WORKSHOP FINAL SUMMARY REPORT

Development of Ecological Tier Testing Schemes for Microbial Biotechnology Applications

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Prepared for

U.S. Environmental Protection Agency
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EXECUTIVE SUMMARY

The U.S. Environmental Protection Agency's (EPA) Office of Pollution Prevention and Toxics (OPPT), Health and Environmental Review Division (HERD), Office of Research and Development (ORD), and Environment Canada, Commercial Chemicals Evaluation Branch jointly sponsored this workshop on the development of ecological tier testing schemes for genetically engineered microorganisms (GEMs). The workshop was held January 11-13, 1994 in Arlington, VA. The workshop included nearly 100 participants representing academia, industry, and government.

The purpose of the workshop was to develop ecological tier testing schemes for naturally-occurring and genetically engineered microorganisms, used in a variety of applications that potentially may be subject to the Toxic Substances Control Act (TSCA) within the U.S. or the Canadian Environmental Protection Act (CEPA). CEPA regulates naturally occurring microorganisms as well as genetically modified ones, whereas TSCA (EPA) only regulates GEMs. Microorganisms used in the following applications were discussed: bioremediation, biomining, mineral leaching, coal transformations, desulfurization of fossil fuels, oil recovery, biomass conversion, fuel production, waste treatment, nitrogen fixation, and closed system fermentation for the production of enzymes or specialty chemicals. Development of tier testing schemes is desired because they provide a logical, organized progression for prioritizing information requirements or testing needs. Workshop participants were divided into various breakout groups according to expertise. On the first day of the workshop, two breakout groups (A and B) discussed the hazard and exposure components for use of microorganisms in the various technologies presented above. Another breakout group (combined C and D) discussed ecological effects and fate endpoints deemed of importance in ecological risk assessment of microorganisms used in these technological applications. A separate breakout group (E) discussed issues specifically dealing with evaluation of microbial pathogenicity or toxicity endpoints. One other group (F) developed a strawman tier testing scheme, using a scheme developed in a previous EPA/Environment Canada workshop (Bioremediation Risk Assessment Workshop, June, 1993, Duluth, MN). This strawman was used during the last two days to develop

final ecological tier testing schemes. On the second and third day of the workshop, participants were split into three groups, depending on expertise, to develop finalized schemes for technologies that were considered to be contained/closed systems (Group I), semi-contained technologies (Group II), and intentional environmental release (Group III). Participants were charged with building onto the strawman scheme given the previous identification of the hazard and exposure components of the various technologies. An issue paper prepared by the organizers with examples of other tier testing schemes, a discussion of ecological effects resulting from the release of microorganisms, and a discussion of the regulation of microorganisms by Environment Canada and EPA was provided to participants several weeks before the workshop. It is important to note that the ecological tier testing schemes developed in the workshop are not new regulatory requirements for industry; instead they are solely for internal guidance within EPA and Environment Canada. Activities of the various groups and subgroups are summarized in the following overview.

Group A: Technology-based Hazard and Exposure Identification

Group A participants focused on the potential hazards resulting from the use of microorganisms in bioremediation, biomining/mineral leaching, coal transformation, desulfurization of petroleum, and oil recovery. They concluded the potential hazards and exposures were site-specific and process-driven for each of the technologies. Ecosystem exposures were categorized as either intentional, or unintentional, and also considered site-specific. The group generally agreed that properly operated systems would have multiple barriers to prevent unintentional release of microorganisms. It was emphasized that strains of organisms for various technologies were quite specialized. Each process was described and, containment, potential releases, exposures, hazards, and level of risk to humans or the environment were discussed.

Bioremediation was defined as a process using microorganisms to degrade organic pollutants contaminating soil, groundwater and sludges. Microorganisms used for bioremediation are usually indigenous organisms present at the site, or ones with altered genetic/biochemical properties adapted to specific processes. Genetic alterations include enhanced enzyme activity, altered regulatory control of enzymes,

modified mobility, suppressed inhibitory traits, modified survival, altered substrate specificity, and the capacity for uncoupling metabolism or modifying the substrate solubility.

The extent of potential exposure can vary from low (closed systems such as bioreactors) to very high (uncontained systems such as treatment of oil spills). Releases from bioremediation applications can occur via surface water, aerosols, ground water, and biological carriers from equipment failure or spills. Although the hazards associated with bioremediation applications were considered low, the release of microbial toxins/enzymes could lead to adverse effects from toxic by-products such as toxic metabolites, halides, hydrogen sulfide, and/or polymeric compounds depending on substrates. In addition, the potential for genetic changes in a genetically engineered microorganism exists if a treated waste contains mutagens.

Biomining was defined as a process using microorganisms to remove unwanted components of ore, whereas mineral leaching uses microorganisms to recover a desired metal by oxidizing metal sulfides and converting insoluble forms to soluble ones for removal from solution. Group participants opined that adhesiveness and the ability to complex with metals are desirable traits for organisms employed for these applications. Both applications are conducted either in closed systems such as bioreactors (up to 10^6 L) or semi-contained systems, such as lined heaps (up to ~400 million tons for copper mining). Releases of microorganisms from these systems can occur via water, aerosols, biological carriers, work activity, and site monitoring. However, survival of organisms used in these processes is limited to highly specialized niches (low pH, high Fe and Mn, etc.); since they survive poorly elsewhere, natural containment is provided within the system. Biomining hazards were considered to be low, and limited primarily to toxic by-products such as sulfuric acid and metal/metalloids.

The use of microorganisms to remove inorganic and organic sulfur from coal through oxidation (i.e., ferrous sulfate, ferric sulfate, elemental sulfur, and sulfuric acid), and liquefication to convert the coal to a more convenient form for transportation, storage, and use was discussed. Although coal transformation is conducted in a semi-contained system, coal demethanation (a potential future application) could be uncontained. Release of organisms from these applications to the environment could

be through aerosols, terrestrial and freshwater media, biological carriers, worker activity, equipment activity, waste disposition, sampling for monitoring, and in the product itself. The hazards associated with coal transformation processes were considered low, and include potential exposure to toxic soluble organics, sulfuric acid, metal/metalloids and biomass.

Group participants suggested that desulfurization of petroleum was still in the experimental stages, and identified two common approaches. One approach uses enzyme preparations or nonviable cells, while the other uses microorganisms developed to metabolize sulfur in oil without metabolizing the carbon. Some experimental systems use bioreactors, but heavier petroleum fractions can be processed in large-scale reactors or semi-contained systems. Thus, releases of the organisms occur either in the product or in process effluents or in both. The hazardous by-products of this application are partially oxidized hydrocarbons, volatile organic compounds, reduced sulfur compounds, spent catalyst, and biomass.

The group discussed addition of nutrients to stimulate growth of the indigenous or added microbial population for production of biomass, gases, polymers, slimes, surfactants or other products as an application involving oil recovery. These substances are microbially produced outside of the reservoir and then injected to plug pores. The bacteria used to produce these plugging agents are inactivated by sterilization before release. The desired characteristics of organisms used in this application include heat tolerance, salinity resistance, reduced oil biodegradation, modified polymer properties, and enhanced production of surfactants or plugging agents. Release of organisms can potentially occur via process fluids, aquifers, equipment failure or defective boreholes, water, aerosols, particulates, biological carriers, worker activity, equipment activity, sampling for monitoring, and in the product. Overall hazards associated with oil recovery were considered low, and attributed only to hazardous by-products such as hydrogen sulfide, biomass, toxic surfactants, polymers, acids, and alcohols.

Group B: Technology-based Hazard and Exposure Identification

This group examined the possible hazards and exposures resulting from nitrogen fixation, waste treatment, fuel production/biomass conversion, and closed system fermentation for enzyme or specialty chemical production. Each process was defined, and potential exposures and hazards associated with each discussed.

Nitrogen fixation was defined as an application designed to increase the nitrogen-fixing capabilities of bacterial symbionts such as *Sinorhizobium*, *Rhizobium,* and *Bradyrhizobium,* used for increasing productivity, particularly of legume crops. Current research is focused on increasing the competitiveness of microorganisms against indigenous symbionts and to improving the efficacy of an introduced strain.

Use of nitrogen fixing rhizobia is uncontained, and may lead to extensive environmental exposure. Although microorganisms are intentionally released at the application site, point source releases also can occur at production facilities. The hazards associated with nitrogen fixation are considered low since there are no hazardous by-products, and because of the benign nature of the microorganisms used. Potential hazards include the microbial contamination of the inoculant, reduction of bioreactivity of the host-symbiont relationship, an increase in the growth of weeds, and the introduction of “competitive” genes whose impact is unknown, but which could cause adverse environmental effects.

Waste treatment was defined as the microbial degradation of municipal and industrial waste material in applications such as sewage treatment, composting, and the removal of hazardous materials from industrial streams. An extensive number of organisms are used in this application and most have not been characterized. Since a typical municipal wastewater facility processes 1.1×10^9 L of wastewater per day, potential exposures from this application could be extensive, and the level of containment varies from contained (sealed) to open systems. Microorganisms are released via plant effluent, aerosols, and the application of processed sludge as fertilizer. Waste treatment poses a low potential ecological hazard, although health hazards will exist due to pathogens naturally present in waste streams.

Fuel production or biomass conversion was defined as the use of microorganisms to degrade plant biomass for the production of valuable products such as ethanol, hydrogen, methane, and other hydrocarbons. Starting material is processed

and fermented in large, enclosed, sterilized vessels, after which this microorganism inoculant is added to the process material in the vessels. The most commonly used microorganisms are *Cellulomonas, Bacillus, Clostridium, Aeromonas, Streptomyces,* and *Phanerochaete*; most of the organisms involved in these applications are not pathogenic and do not produce hazardous by-products. Therefore, hazards were considered low. GEMS are commonly utilized with the goal of increasing the ability of organisms to digest a wider diversity of substrates, and to increase the specificity of the products. Releases of organisms occur from inadvertent leaks or large scale accidents, but the likelihood of release is minimized by using well-defined, process-specific industrial containment measures.

Microorganisms are grown in closed-system fermentors to produce enzymes and specialty chemicals for use in making still other products. The participants expected few or no environmental releases because these applications are performed predominantly in closed systems. Organism releases occur more frequently in urban centers, due mostly to accidental release and aerosols from residuals left on filters or seals. Hazards associated with these processes are considered low because the desired products are usually not toxic. The only concern identified was that introduced genes potentially may be transferred to other microorganisms capable of causing adverse effects.

Groups C & D: Ecological Effects and Exposure/Fate Endpoints

This combined group was organized to identify the ecologically significant endpoints to be included in the tier testing schemes. They considered maintenance of the stability of the ecosystem the most critical endpoint. The participants were in general agreement that assessing the risks of introducing microorganisms into the environment was important because of the potential effect on ecological stability. It was noted that the potential ecological impacts of microorganisms released in the environment have not been well characterized. There are several reasons for this deficit in knowledge. First, it is not yet possible to predict how potential impacts of applied microorganisms will be expressed. Second, the environmental level at which these impacts should be measured has not been clearly defined and, neither the

appropriate techniques for detecting ecological effects nor how the results should be interpreted has been determined. Some participants suggested that ecological endpoints can be determined with microcosms, which the group generally agreed are legitimate approximations or simulations of the natural environment. It was generally agreed that ideally, studies should be performed on “stressed” and “non-stressed” environments.

The impact of an introduced microorganism on primary production was identified as a significant ecological endpoint for both terrestrial and aquatic systems. The participants suggested that the testing scheme should evaluate the effects of the microorganism on the significant primary producers. An adverse effect would be a change in the populations and activities of the primary producers relative to an appropriate control population.

Cycling of limiting nutrients was another identifiable ecological endpoint. Essential tests should determine the effect of introduced microbes on net nitrogen and carbon mineralization as well as the cycling of phosphorous and sulfur for both aquatic and terrestrial ecosystems. Time scale and land use factors should also be considered in the cycling of limiting nutrients in terrestrial systems.

Community structure and diversity was another identifiable endpoint. A broad survey of the species richness and functional groups in the presence of the microorganism should serve as a preliminary test. An initial change in the taxonomic diversity would be evidence of an effect. If changes occur in the number of taxa, the total density, and the density of major groups, or if a species is missing, then additional testing should be performed.

Grazers can be used as indicators of fluctuations in the community structure of environments. A change in the numbers and types of species that graze on primary producers, or on heterotrophic organisms was considered an ecological endpoint.

The effects on sensitive species were also considered significant endpoints. The two types of sensitive species identified were an indigenous organism in the general population that responds rapidly to environmental perturbation and a specific indicator organism that is sensitive to an introduced microorganism and/or its gene product. The

participants agreed that sensitive species should be considered during the survey of taxa used to characterize community structure, rather than a stand-alone endpoint.

Ecologically significant exposure and fate endpoints in both terrestrial and aquatic ecosystems were discussed. A number of issues based on microbial survival and fate were considered within this context. The participants agreed that the population density that constitutes survival of the microorganism is too low to measure due to delayed effects such as population regrowth. Also, the novel genes in an introduced organism might be transferred to indigenous microorganisms. Enumeration of spores, propagules, and biomass are important considerations in determining the fate of microorganisms, although this may not always be possible. It becomes a concern, if the introduced organism displaces its parent, but it is more important to determine if it survives. It is also important to establish quantitative relationships between the number of microorganisms that survive and persist and the potential harm they may do. However, this information usually does not exist. The group generally agreed that 90% of the information about the fate of the GEM could be obtained using less costly microcosms, rather than mesocosms which are extremely expensive.

Group E: Microbial Pathogenicity/Toxicity

This group discussed categorization of frank and opportunistic pathogens, predictive pathogenic traits, and toxin production by microorganisms that may be used for risk assessment for the specified technologies. Identification of microbial pathogens and opportunistic pathogens was considered problematic due to limited research materials. A preliminary list of pathogens for certain genera of particular concern, such as *Pseudomonas* and *Bacillus,* was created. It was generally agreed that the use of genera may be too broad of an approach, and that the use of species-level identification avoids unnecessary testing of non-pathogenic microorganisms within the genera.

The subgroup concluded that there is no one specific phenotypic or genotypic characteristic that is directly associated with, or predictive of, pathogenicity. The development of a list characterizing the ability of an organism to grow would include the ability of the organism to colonize as well as specific environmental requirements for the

organism to sustain itself. Another list of traits that indicate the potential for pathogenicity to plants was created by the subgroup. These traits include the production of cell-degenerative enzymes, toxins, growth factors, or extracellular polysaccharide, ice nucleation ability, and the ability to grow in or on the host. Some of the group participants suggested that bioassay results from oxidase, tobacco hypersensitivity reaction, Gram reaction, and fatty acid analysis tests would be more informative than a list of pathogenic traits. It was also suggested that characteristics of pathogenicity should be broad and should include all environmentally significant species. For testing of toxins/toxin producers, it is useful to employ surrogates, provided that the surrogate organisms are appropriate for the conditions under consideration. After initial tests are performed using standard species, other specialized or more relevant surrogates may need to be used.

Group F: Strawman Tier Testing Scheme

This group consisting of the chairs and co-chairs for the three subgroups (Groups I, II, and III) developed the strawman tier testing scheme that was used to develop the ecological tier testing schemes for closed, semi-contained, and open biotechnology applications. The scheme contains four tiers. Tier 0 contains preliminary information, taxonomic identification, proposed use, and site characterization. Tier 1 contains initial exposure and hazard assessment components. Tier 2 addresses additional questions about exposure and hazard from Tier 1. Tier 3 contains open or limited field tests in the selected environment.

Tier Testing Schemes

Group I developed a tier testing scheme for contained or closed biotechnologies. Rather than defining the level of containment that would qualify as a closed system, the group assumed that incidental or accidental releases of microorganism could occur in any closed system. The only changes the group made to the strawman version of Tier 0 was to fold the application characterization and manufacturing and distribution characterization together, and to add quantitative structure-activity relationship analysis

and the environmental behavior of the GEM to this Tier. This revised tier enables well-characterized products to proceed more rapidly to approval. Tier 1 includes testing for toxin production, pathogenicity, survival, and hazards associated with the DNA product. After satisfying the requirements/concerns of Tier 1, if the organism poses no unreasonable risk to the environment, the organism is approved. If there is a potential risk or uncertainty, then one should proceed to Tier 2. The group decided to combine Tiers 2 and 3 from the strawman scheme since field testing was not deemed necessary for microorganisms used in contained systems. Tier 2 for closed systems addresses concerns about population build-up, toxicity, pathogenicity, the build-up of gene product, unknown host range, and long-term persistence.

Group II created a scheme for semi-contained technologies. They emphasized the difficulty of arriving at a fixed scheme for use across all of the semi-contained applications. They agreed that the scheme needs to be flexible. They also emphasized the importance of evaluating microorganisms as well as their genetic construct and potential by-products. The goal of Tier 0 was characterization of microorganisms through examination of the intended application site, mode of action, intended efficacy, mode of application, and manufacture and distribution. It was agreed that the survival and persistence of the organism and the genetic cassette are of special concern in this tier. If there is no unreasonable risk from the organism, then the submitter may proceed to Tier 3 testing if desired. If there is a concern, the assessment process moves into Tier 1, which includes exposure and hazard assessment addressing issues such as persistence of the microorganism and its genes, fate of the microorganism, pathogenicity, toxicity of by-products, and preliminary ecological effects. Tier 2 involves the further analysis of hazards, predominately more complex ecological effects testing. Tier 3 involves evaluating the use and/or efficacy of microorganisms in selected environments for open or limited field tests. The scheme was evaluated for adequacy using theoretical scenarios in bioremediation, oil recovery, coal transformations, municipal waste treatment, and biomining.

Group III developed a scheme for open or uncontained technologies. Tier 0 involves gathering preliminary Premanufacture Notification (PMN) submission or pre-test information which should include organism characterization, mode of action or

intended efficacy, application characterization, and manufacturing/distribution characterization. Risk (defined as hazard x exposure) is also based on application of the data from Tier 0. Tier 1 involves a short-term screening-level testing of both exposure and hazard endpoints. The four exposure components of concern are the persistence of the organisms and novel DNA, the dispersal of organisms, proliferation, and occurrence of repeated applications. The three hazard components include toxicity, pathogenicity, and other ecological effects. Tier 2 involves longer-term, more comprehensive/complex testing of exposure and ecological effect endpoints. Tier 3 consists of open or limited field tests in selected environments to assess the efficacy of the organism. The scheme for uncontained technologies was evaluated by using it to determine the ecological risks associated with microbially enhanced oil recovery and bioremediation scenarios involving oil spills. The group was in general agreement that the proposed tier testing scheme contained the appropriate screens and questions needed to assess the potential hazards associated with these two scenarios.

INTRODUCTION

Background

The Workshop on the Development of Ecological Tier Testing Schemes for Microbial Biotechnology Applications was jointly sponsored by the U.S. Environmental Protection Agency's (EPA) Health and Environmental Review Division of the Office of Pollution Prevention and Toxics (OPPT), Office of Research and Development, and the Commercial Chemicals Evaluation Branch of Environment Canada. The Workshop was held in Arlington, Virginia, on January 11-13, 1994. The workshop agenda is presented in Appendix A. Appendix B is a list of the various discussion questions for each breakout session. Participants at the Workshop were experts from government, academia, and private industry. A complete list of participants is presented in Appendix C. The workgroup chairs are identified in this list by an asterisk.

Purpose and Goals

Because genetically engineered microorganisms (GEMs) have been used for only a few industrial applications or technologies, there is a lack of familiarity with the risks associated with their use. Therefore, the OPPT Biotechnology Program at EPA currently reviews microorganisms that are subject to the Toxic Substances Control Act (TSCA) on a case-by-case basis. In the interest of standardizing the review process by identifying and ranking the importance of certain testing needs, OPPT is developing ecological tier testing schemes. Tier testing schemes are useful tools because they provide risk assessors with a logical, organized progression for evaluating data and prioritizing testing needs. In addition, the schemes minimize the testing requirements for industry because only baseline tests need to be submitted unless these data trigger a hazard or exposure concern that must be addressed by additional testing. Tier testing schemes are commonly used in other risk assessment applications such as the assessment of microbial pest control agents under Subdivision M of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the assessment of industrial chemicals under TSCA, and the assessment of oil spill bioremediation products. These

tier testing schemes are discussed in detail in the background document developed for this workshop (U.S. EPA 1993).

The participants in this workshop were charged with developing tier testing schemes for assessing the ecological risks associated with the use of microorganisms in various technologies. The Workshop focused on the following technologies/applications: bioremediation, biomining, mineral leaching, coal transformations, desulfurization of petroleum, oil recovery, biomass conversion, fuel production, waste treatment, nitrogen fixation, and closed system fermentation for the production of enzymes or specialty chemicals. The tier testing schemes developed at the Workshop consist of hazards and exposure endpoints of concern with the use of GEMs as well as triggers that initiate further testing if effects are observed at the lower tiers. The ecological tier testing schemes developed during this workshop are solely for the purpose of internal guidance within EPA and Environment Canada. They are not new regulatory requirements for industry.

For the purposes of this workshop, both genetically engineered microorganisms and naturally-occurring microorganisms were considered. It is assumed that the potential exposure and effects scenarios associated with the use of naturally-occurring microorganisms may be similar to, or at least serve as a baseline for, those associated with the use of GEMs. In addition, the Canadian regulations, New Substances Notification Regulations for Biotechnology Products, apply to both genetically engineered and naturally-occurring microorganisms, as well as the products of these organisms (e.g., enzymes).

Workshop Structure

On the first day of the Workshop, participants were organized into five specialized subgroups. Groups A and B identified hazard and exposure concerns resulting from the use of microorganisms in various technologies. Group A focused on bioremediation, biomining/mineral leaching, coal transformations, desulfurization of petroleum, and oil recovery. Group B considered nitrogen fixation, fuel production, biomass conversion, municipal waste treatment, and closed system fermentation. Groups C, D, and E identified endpoints of ecological significance that would apply to all

of the technologies listed above (i.e., not specific to any one technology). Groups C and D were combined to identify both ecological effects and exposure/fate endpoints for terrestrial and aquatic ecosystems. Group E identified pathogenic traits of, or toxin production by, microorganisms that may be used in the technologies considered in this workshop. The Group examined the potential of using screening level tests for assessing the pathogenicity or toxicity of microorganisms. Group F developed a strawman tier testing scheme that later was used by the workshop participants to develop ecological tier testing schemes for closed, semi-contained, and open system technologies.

On the second and third days of the Workshop, participants were re-organized into three break-out groups for the purpose of developing separate tier testing schemes for closed/contained, semi-contained, and open/uncontained technologies. The term "contained" in this context relates to those technologies where microorganisms are released to the environment unintentionally and/or in small quantities. Examples of contained/closed technologies include the use of bioreactors for bioremediation, closed system fermentations for enzyme production, and closed system fermentation for fuel production. Examples of "semi-contained" technologies are prepared bed reactors that are lined to prevent vertical or horizontal movement, or compost piles for biomass transformations of agricultural wastes. Examples of "open/uncontained" technologies include the use of microorganisms on coastal oil spills or the use of nitrogen-fixing microorganisms on a large-scale basis. To develop the tier testing schemes, the break-out groups integrated the hazard and exposure components of the different technologies (as identified by Groups A and B) and the appropriate hazard and exposure endpoints (as identified by Groups C, D, and E) into the strawman scheme developed by Group F. The break-out groups also identified and established triggers at each level that would initiate testing at higher tiers. Group I developed the tier testing scheme for contained/closed system microorganisms. Group II developed the tier testing scheme for semi-contained system microorganisms. Group III developed the tier testing scheme for microorganisms intended for open/uncontained release.

Opening Plenary Session

Greetings and opening remarks were given by Dr. Gwendolyn McClung of the U.S. Environmental Protection Agency, Dr. Terry McIntyre of Environment Canada, and Dr. Charles Hendricks of the U.S. Environmental Protection Agency's Environmental Research Laboratory in Corvallis, Oregon.

After the opening remarks, a number of presentations provided participants with important background information. The first two presentations focused on the regulation of microorganisms by the United States and Canada. Ellie Clark of the U.S. Environmental Protection Agency discussed the regulation of intergeneric genetically engineered microorganisms under TSCA. Dr. Terry McIntyre of Environment Canada described the regulation of microorganisms under the New Substances Notification Regulations provisions of the Canadian Environmental Protection Act. The remaining presentations provided an overview of some existing tier testing schemes. Dr. William Schneider of the U.S. Environmental Protection Agency outlined the scheme used for assessing microbial pest control agents under Subdivision M of FIFRA. Dr. Jerry Smrchek of the U.S. Environmental Protection Agency described the scheme used by OPPT to assess the ecological risks of industrial chemicals under TSCA. Dr. Philip Sayre of the U.S. Environmental Protection Agency discussed the scheme developed for assessing human health and environmental risk for bioremediation agents. The scheme was developed at the Bioremediation Risk Assessment Workshop sponsored by EPA and Environment Canada in 1993. Finally, Dr. Gwendolyn McClung of the U.S. Environmental Protection Agency presented a draft for the OPPT ecological tier testing scheme. Further information on these topics is available in the background document developed for this workshop (U.S. EPA 1993).

SPECIALIZED SUBGROUPS

GROUP A: TECHNOLOGY-BASED HAZARD AND EXPOSURE IDENTIFICATION

Group A examined potential exposure scenarios and discussed factors affecting the potential hazards resulting from the use of microorganisms in bioremediation, biomining/mineral leaching, coal transformation, desulfurization of petroleum, and oil recovery.

The group noted several general themes common to the entire discussion. In characterizing the methodologies used and in assessing the potential hazard and possible exposure, the group consistently noted that these issues are site-specific and process-driven for each of the technologies discussed. The group also found that it could make general statements about many of the specific issues discussed. For example, several of the applications considered use similar genera of organisms; application rates were in the range of 10^6 to 10^{12} organisms per mL or per gram. Open technologies tend to use lower concentrations of cells, whereas closed technologies tend to use more cells. The frequency of microorganism application is also technology driven. Thus, for process technologies or growth-based techniques, the organism is added and allowed to grow. In contrast, multiple applications are used for catalytic processes. Multiple applications may also be necessary if the system is not sufficiently supportive of microbial growth and/or desired metabolic activities.

Potential ecosystem exposure is also site-specific. The applications considered by the group can be located in, or adjacent to, a variety of diverse ecosystems; for example, biomining tends to occur primarily in deserts or in mountainous regions. The group differentiated between intentional ecosystem exposure and unintentional exposure. For example, there is no widespread intentional environmental exposure in biomining, coal transformation, or oil desulfurization, but environmental exposure is possible in the event of an accident. If there was an accidental release from containment, most of the potential means of dissemination are common to all of the technologies considered, as discussed in detail below. However, the group emphasized that a properly operated system has multiple barriers to prevent organism release, as well as systems for containing microorganisms in the event of accidental releases and

preventing their dissemination. The strains of organisms used for the various technologies are quite specialized to highly specific environments, and are not expected to compete well with naturally occurring organisms in other environments. In addition, if a company wants to patent a GEM, it has a vested interest in making certain that the organism is not released to the environment, thus preventing any claim that the organism is naturally occurring. The group also discussed an extensive list of possible exposure routes, although it was emphasized that these were possible exposure scenarios only and that the likelihood of any of them resulting in an actual hazard was very low. Because of the lack of time, the group did not discuss specific hazards, other than toxic byproducts, resulting from the use of microorganisms for these technologies, although it was noted that many of these byproducts occur naturally (e.g. production of H_2SO_4 resulting in low pH of surface waters by thiobacilli in mines).

Bioremediation

Bioremediation involves the use of microorganisms to degrade organic pollutants contaminating soil, groundwater, and sludges. Some current systems promote growth of indigenous microorganisms that degrade the material of interest by incorporating fertilizer (nitrogen and phosphorus) into the contaminated medium. In other systems, naturally occurring microorganisms or, in the future, GEMs that have been selected for their degrading capabilities may be added to the substrate to enhance remediation efficacy in shorter response times.

Organisms/Application

A large variety of different organisms are used in bioremediation applications. These include methanotrophs, pseudomonads, white rot fungi, *Escherichia coli* (especially derivatives of strains K12 W3110), and species of *Rhodococcus*, *Mycobacterium*, *Arthrobacter*, *Cunninghamella*, Cyanobacteria, and *Bacillus*. Cyanide degraders, sulfate reducers, pseudomonads, *Bacillus* species, and *E. coli* are used for remediation of mining wastes. In many field applications, the organisms used are those that are found growing at the waste site. However, for remediations conducted in closed vessels, it is more feasible to use well-characterized organisms. This choice

reflects the two major strategies for choosing the organism to be applied: (1) use an organism that has grown at the site and, so, has demonstrated resistance to the toxic materials found at the site, or (2) use one primary organism with its various biochemical activities or components altered as needed. If the waste being treated contains mutagens, there is a potential for genetic changes within the applied organism. Therefore, even if the microorganism is initially well characterized, it may not be as well-characterized by the end of the treatment period.

A variety of genetic modifications can be, or are being used to develop microorganisms for bioremediation applications. The group did not differentiate between current and future methodologies. Modifications include enhancing enzyme activity, altering regulatory control of enzymes, modifying the mobility of the microorganism, suppressing inhibitory traits, modifying survival, altering substrate specificity, uncoupling metabolism, modifying the solubility of substrate by such methods as surfactant secretion, adding markers, adding or enhancing desirable growth characteristics, increasing shelf life, enhancing the bioavailability of the substrate, adding resistance to toxic materials other than the one(s) targeted by the bioremediation effort, and altering metabolic pathways to produce a directed flow of metabolites that avoids "dead-end" toxic products.

For field applications, concentrations of bioremediation microorganisms of 10^6 to 10^9 colony forming units (cfu)/mL may be applied. Contained bioreactors may use higher concentrations. One or multiple applications may be used, depending on the efficiency of the system. Additionally, staged applications may be used for bioremediation, with one microorganism partially metabolizing the waste to an intermediate product and another microorganism metabolizing the intermediate compound to a final product.

Exposure

The degree of potential environmental exposure depends on the process used. Bioreactors are closed systems, so releases would be expected to be minimal. Bioreactors range in size from a few to more than $9x10^5$ liters. Landfarming at lined sites and some *in situ* groundwater treatment applications, such as treatment of static

aquifers, are semi-contained applications. Semi-contained operations vary in size more than contained operations and range from several hundred liters for biofilters to over 3 X 10^6 liters for lined lagoons. Treatment of oil spills, *in situ* groundwater treatment of active aquifers, and landfarming at unlined sites are considered uncontained technologies, with sizes on the order of square miles. Current bioreactor systems either use a chemical disinfectant or do not inactivate the microorganisms before disposal. There are no current commercially viable technologies that are effective against spores; autoclaving and gamma-irradiation are the only effective technologies, but autoclaving is not cost-effective on a large scale, and gamma-irradiation is not practical. In the absence of an accident, exposure of environmental media from a bioreactor would be minimal compared to the semi- or uncontained technologies where all environmental media could potentially be exposed, depending on the location of the site to be remediated and the surrounding ecosystems.

Microorganisms can be routinely released from bioreactors in waste streams and products, including exhaust air, and in the process effluent. In addition to these routes, releases from semi-contained systems can occur via surface water, aerosols, groundwater, and biological carriers. Releases could also occur from contained and semi-contained systems as a result of equipment failure or spills. Once out of containment, a variety of dissemination methods are possible. Although these are hypothetical scenarios, incorporating all possible modes of dissemination in a worst-case scenario is necessary. Dissemination could occur by surface and subsurface (saturated and unsaturated zones) water, aerosols, particulates, biological carriers, worker activity, equipment activity, waste disposition, and sampling conducted to monitor the site. The media affected would also be site-specific.

Hazards

The hazards associated with the use of GEMs to remediate toxic wastes are considered to be very low. The potential for production of toxic by-products depends entirely on the material(s) being remediated and the microorganisms used. Depending on the substrate, toxic metabolites, halides, hydrogen sulfide, and/or polymeric compounds could be byproducts. Metals or metalloids could become more accessible

or be concentrated via biosorption. The microorganism could also release microbial toxins or certain enzymes that may have adverse effects.

Biomining/Mineral Leaching

Biomining applications use microorganisms to remove unwanted components of ore, while mineral leaching uses microorganisms to recover the desired metal, usually by oxidizing the metal sulfides and converting insoluble forms to soluble ones that can be recovered from solution.

Organisms/Application

The species used include *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans*, and *Leptospirillum ferrooxidans*. Archaeobacteria that are moderate or extreme thermophilic iron oxidizers are also used. Organisms used as surface property modifiers include *Pseudomonas* sp. and fungi. The traits of interest include the ones discussed under bioremediation, as well as resistance to metals, thiocyanate, and chloride. Adhesiveness and the ability to complex metals are also desirable. However, the potential use of microorganisms and the potential for useful genetic manipulations is limited by the nature of the ore and the generally poor bioavailability of the substrate.

Biomining or leaching operations typically take place on a very large scale. Closed systems range from 2×10^5 to 9×10^6 liters, and the heaps used in semi-contained systems typically are 6 to 8 million tons of soil and rock for gold mining and about 400 million tons for copper mining. Microorganisms are generally applied at approximately 10^6/g solid material. The processes can use either a one-time inoculation or multiple applications, depending on the specific process used.

Exposure

Biomining and bioleaching can be conducted in either the closed system of a bioreactor or in semi-contained lined heaps; therefore, there is no intentional environmental exposure. Because the microorganisms are recycled to re-inoculate the ore, inactivating the microorganisms would be counterproductive. Potentially affected

media would be defined by the site; however, these operations often take place in mountainous or desert environments. The organisms involved survive in these highly specialized niches (low pH, high Fe and Mn, etc.), and survive poorly outside those environments, thus, providing for some degree of natural "containment". A potential for fresh water exposure via aerosols exists if lakes or rivers are near the operation, although exposure of these media is less likely. Microorganisms can be released from contained systems in waste streams or process effluent, in addition to equipment failure or spills. With semi-contained systems, aerosols, and biological carriers, such as animals or birds, can also result in transport of the microorganism. Once released from containment, the microorganism could be transported by water, aerosols, biological carriers, worker activity, and site monitoring.

Hazard

Toxic by-products of these processes are sulfuric acid and metals or metalloids. However, these by-products are not specific to biomining and would also occur with traditional mining methods. The overall hazard of using GEMs for biomining was considered to be low.

Coal Transformations

Coal desulfurization involves the use of microorganisms to remove inorganic (pyrite and other metal sulfides) and organic sulfur from coal by oxidizing them into easily removed products such as ferrous sulfate, ferric sulfate, elemental sulfur, and sulfuric acid. Coal liquefaction is useful for converting coal into a form that is more convenient for transportation, storage, and use.

Organisms/Applications

A variety of genera are used for removing organic sulfur from coal. These organisms include *Pseudomonas*, *Rhodococcus*, *Acinetobacter*, *Desulfovibrio* and other sulfur reducing bacteria, *Brevibacterium*, and *Corynebacterium*. Inorganic sulfur can be removed by the same microorganisms that are used for biomining and mineral leaching, i.e., *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans*, *Leptospirillum ferrooxidans*, and

archaeobacteria. Pseudomonads, fungi, and white rot fungi are used for coal liquefaction. Microorganisms used for these applications may be genetically modified as described for bioremediation to alter the microorganism's metabolism, identifiability, and ability to survive and grow. In addition, enhanced adhesiveness is a trait of specific interest for coal transformation. The number of microorganisms applied, approximately 10^{10} to 10^{11}/g of solid, is higher than that used for biomining. Inasmuch as coal transformation is a process technology, it is conducted as a continuous reaction, and microorganisms are added once per volume processed.

Exposure

Coal transformation is currently carried out as a semi-contained process in slurry reactors. A future possible application, demethanation of coal, would be conducted as an uncontained process. Currently, there are no full-scale coal transformation operations using microorganisms. Based on engineering concerns from needed coal throughput, a site would be estimated to be approximately 4 to 8 hectares. Exposure of environmental media could occur via aerosols, with terrestrial and freshwater environments potentially affected. Since the process would be conducted as a semi-contained procedure, no ecosystem would be intentionally exposed. Potentially affected ecosystems would depend entirely on the site and could include deserts, mountains, or other ecosystems. In the event of a release from the site, microorganisms could be disseminated in water, aerosols, via biological carriers, worker activity, equipment activity, waste disposition, sampling for monitoring, and in the product.

Hazards

Hazardous byproducts of coal transformation include toxic soluble organics, sulfuric acid, metals and metalloids, and biomass. Except for biomass, all by-products would be produced by the corresponding conventional technologies. The overall hazard of using GEMs for coal transformation was considered to be low.

Desulfurization of Petroleum

The use of microorganisms for petroleum desulfurization is currently in the experimental stage. Of two experimental approaches, the first one uses enzyme preparations or nonviable cells. If the enzyme product were used for petroleum desulfurization, the cells would be grown separately and the enzyme purified before addition to the oil. Otherwise, the cells are grown and then inactivated before they are introduced into the oil. For the second approach, microorganisms are also being developed that can metabolize the sulfur in the oil without metabolizing the carbon. If viable cells were added directly to the oil, a minimal number would probably be used. Because petroleum desulfurization is a process technology, cells would be added once per volume processed. It is difficult to estimate the eventual size of microbial petroleum desulfurization operations, as this process has not yet been commercialized; however, the size of closed and semi-contained systems are both estimated at1.5×10^5 to 7.5×10^6 liters.

Organisms/Applications

Petroleum desulfurization is conducted using the same microorganisms that are used for coal desulfurization. Traits of interest for this application include solvent resistance, heat and pH tolerance, and altered substrate specificity. Because this is an experimental process, the size of the vessel and associated engineering aspects that would be used commercially are not known.

Exposure

Some of the current experimental systems employ closed systems (bioreactors). The use of lower levels of containment is limited by the volatility of some petroleum components, but heavier fractions could be processed in semi-contained large-scale reactors. As the current systems are closed, environmental media would not likely be exposed and ecosystems would not be intentionally affected. The ecosystems that would be affected in the case of an accidental release would depend on the environment surrounding the bioreactor. Potential avenues of microbial release from containment are in the product or in process effluents. The amounts released by these

pathways would be expected to be small. Equipment failure could also lead to release of microorganisms. In the unlikely event of release, microorganisms could be disseminated from the site by the same pathways as were discussed for coal transformation.

Hazard

Hazardous byproducts that could be formed by microbial desulfurization of petroleum are partially oxidized hydrocarbons, volatile organic compounds, reduced sulfur compounds (e.g., disulfides), spent catalyst, and biomass. Only the last byproduct is unique to the involvement of microorganisms in this process.

Oil Recovery

Microbially enhanced oil recovery involves the addition of nutrients to stimulate either the indigenous or added microbial population in the production of biomass (cells), gases (CO_2, H_2, N_2, and possibly CH_4), polymers, slimes, surfactants, or other products within the reservoir. These substances selectively plug pores in the subsurface reservoir and diminish the available volume in which the oil can flow. Oil that was previously inaccessible may then be displaced by injecting water or brine, which now follows along different flow paths.

Organisms/Application

Bacteria used for oil recovery include *Bacillus*, *Clostridium*, *Leuconostoc*, *Klebsiella*, *Acinetobacter*, *Rhodococcus*, and *Pseudomonas*. Alternatively, plugging agents such as xanthan gum and scleroglucans can be microbially produced outside of the reservoir and then injected to plug pores. In this case, the bacteria used for xanthan gum production are inactivated by sterilization before release. The general traits described under bioremediation may also be of interest for this application. Additional desired traits are reduced plant pathogenicity, heat tolerance, salinity resistance, reduced oil biodegradation, modified polymer properties, and enhanced production of surfactants and plugging agents. Under good conditions, only one application of microorganisms is necessary, but in practice, additional applications may be necessary

to improve efficiency. Current experimental systems range in size from 12,000 cubic meters (one well) to a 6.2 x 10^6-cubic meter pilot project. Application areas could be potentially become much larger as the technology develops.

Exposure

Surfactants and polymers may be produced in a closed bioreactor and then injected into the reservoir. Injection of the microorganisms directly into the oil reservoir is considered semi-contained; only the subsurface environment would be exposed. However, other media could be exposed in the event of an accident, with the affected media being determined by the site. Release from containment could occur in process fluids, in aquifers, or as a result of equipment failure or a defective borehole. In the event of a release, the microorganisms could be disseminated via water, aerosols, particulates, biological carriers, worker activity, equipment activity, sampling for monitoring, and in the product.

Hazards

Hazardous byproducts of the technology include hydrogen sulfide, biomass, and possibly toxic surfactants, polymers, acids, and alcohols. Except for the production of hydrogen sulfide, all of these byproducts are unique to the use of microorganisms for this process. However, the byproducts would be contained either within a bioreactor or within the reservoir of oil, itself a toxic material, and the overall hazard of the use of genetically engineered organisms (GEMs) for oil recovery is considered low.

GROUP B: TECHNOLOGY-BASED HAZARD AND EXPOSURE IDENTIFICATION

Nitrogen fixation, waste treatment, fuel production/biomass conversion, and closed system fermentation for enzyme or specialty chemical production were examined by this subgroup. The group attempted to characterize the sources of the hazards and exposures associated with these technologies.

Nitrogen Fixation

The symbiotic association of nitrogen-fixing microorganisms with leguminous plants (e.g., soybean, alfalfa) has been used in agriculture for centuries to increase the nitrogen-fixing capabilities of crop plants. With the expansion of genetic technologies, nitrogen-fixing microorganisms can be modified to increase the efficiency of nitrogen fixation and to stimulate the competitive abilities of these organisms to form nodules on the host plant. There are many potential benefits of introducing GEMs into this symbiotic system. The use of some GEMs is still in the testing stages; however, a limited commercialization of a genetically modified *Sinorhizobium meliloti* (formerly known as *Rhizobium meliloti*) has been approved.

Organisms/Application

The primary microorganisms used for nitrogen fixation with legume crops are from the genera *Sinorhizobium*, *Rhizobium* and *Bradyrhizobium*. In addition to the use with legume crops, the nitrogen-fixation application is used for fern and algae symbiosis in rice paddies and on woody crops (e.g., alder). Specific modifications presently being tested include the insertion of additional *nif* genes, promoters, and *dct* genes for C-4 dicarboxylic acid transport. Also, work is being done on increasing the competitiveness of the microorganisms against indigenous symbionts. Specifically, microbiologists are focusing on *Sinorhizobium* strains RCR2011 and RMBPC-2. The two primary goals (target traits) for genetically engineered technologies are to improve the efficiency of nitrogen fixation and to improve the efficacy of the introduced strain in reaching the target plant.

The application of the microorganisms to the host plant typically occurs at the stage of seed preparation. The seeds may be coated on-site or at a manufacturing facility. The number of organisms applied to each seed is in the range of 100 to 10,000 depending on the size of the seed. Field application can involve 2.5×10^5 to 2.5×10^6 seeds/hectare, resulting in 2.5×10^7 to 2.5×10^{10} organisms/hectare. However, this number of microorganisms is small compared to the 10^7 to 10^8 cells/g soil of saprophytic

bacteria typically found in soils. The organism-coated seeds are applied annually, or every three to five years depending on the type of crop.

Exposure

The use of nitrogen-fixing microorganisms is a uncontained process; therefore, environmental exposures associated with this technology are extensive. Not only are the organisms intentionally released at the application site, but point-source releases can occur at the production facilities. Because nitrogen fixation is important in agricultural applications, typical areas of use are very large. In Wisconsin, for example, over 1.2 million hectares of alfalfa have been inoculated with wild-type rhizobia. Approximately 2.6 million hectares of soybeans are inoculated each year with *B. japonicum*, which is approximately 10% of the total U.S. soybean acreage. Presently, the organisms are utilized only on croplands. The literature indicates that *Rhizobium* are not highly mobile in soil. However, runoff from flooding could be a potential route of transport and the organisms may be capable of surviving at low numbers for extended periods.

Hazards

The hazards associated with the release of genetically modified, nitrogen-fixing microorganisms are generally considered to be low. With the possible exception of gene transfer of antibiotic resistance genes from the inoculant strains, there are no materials that would be characterized as potentially hazardous by-products of this technology. Instead, the specific hazards identified for this technology are based mainly on the uncertainties of the effects of new genetic material and modified organisms on the environment. For example, the lack of knowledge regarding the insertion of genes into cryptic sites (those in which there is no known function associated with the DNA) could be a potential hazard. Although few adverse effects are predicted, the outcomes of these insertions have not been clearly documented. In addition, because of the nature of the inoculant production process, there is the potential for microbial contamination of the inoculant strain, resulting in the introduction of unknown microorganisms to the environment. Another hazard associated with this technology

concerns a potential reduction in biodiversity of the host-symbiont relationship, a concern that extends to the use of restricted host plant genotypes as well. Concerns have also been expressed regarding whether the use of nitrogen-fixing microorganisms will unintentionally increase the growth of weeds; however, this effect is expected to be minimal because the microorganisms that are released are specific to only certain plant species. Finally, there is a potential hazard associated with the introduction of "competitiveness" genes. The impact of these genes is unknown, but the modification of genes that increase the ability of a microorganism to out compete other microorganisms could have unforeseen effects. It is important to ensure that modified microorganisms are more competitive only under conditions where a high inoculum dosage is artificially provided, and are not more competitive in terms of long range persistence in natural environments.

Waste Treatment

The microbial degradation of waste material is a natural process with which scientists have extensive experience. This technology is used in a variety of applications such as sewage treatment and composting (i.e., the conversion of organic wastes). It also is an effective method for removing hazardous materials from industrial waste streams. This subgroup considered waste treatment in terms of the use of microbial organisms for the degradation of municipal and industrial wastes. In the case of municipal waste treatment, the use of GEMs is not expected to contribute significantly to the treatment process. In contrast, the use of GEMs for treating industrial wastes may be advantageous.

Organisms/Applications

There are extensive, uncharacterized microorganisms involved in the municipal waste treatment process. It would be nearly impossible to document the variety of organisms in a typical sewage treatment facility or to track the specific traits exhibited by each of these organisms. As a result of the diverse nature of the waste treatment consortia and the inherent efficiency of its degradation ability, the introduction of GEMs is not expected to improve the municipal waste treatment process. In fact,

microorganisms engineered for the degradation of specific contaminants (e.g., detergents) may be out-competed by members of the natural consortia that degrade a wide range of substrates. The diversity of substrates available to these microorganisms in most municipal waste streams would lead to the elimination of any specific organism that is unable to adapt to different amounts and compositions of waste. This subgroup also examined industrial waste treatment, a situation in which the actual waste composition often can be well characterized. In this case, the use of waste-specific engineered microorganisms might, in fact, make the degradation process more efficient, such as in constructed wetlands.

A rough estimate of the concentration of microorganisms in a typical municipal wastewater stream is 10^9 to 10^{11} microorganisms/mL. A typical municipal wastewater facility processes 1.1 X 10^9 L of wastewater per day. In this application, the microorganisms would have to be applied to the waste stream continuously, to replenish those lost in the release of treated wastewater.

Exposure

Environmental exposures associated with the municipal waste treatment process are generally considered to be extensive. The level of containment for the wastewater consortia varies; large municipalities may have sealed tanks, whereas small, rural facilities may be completely open. Inactivation procedures, such as chlorination, ozonation, and ultraviolet (UV) radiation treatment, are fairly effective for preventing large-scale release of microorganisms. The use of UV irradiation is increasing due to the resistance of some organisms such as *Cryptosporidium* to chlorination, raising concerns about the creation of mutant strains. However, the level of UV radiation employed is usually high enough to kill the organisms. The means by which the organisms are released from a sewage treatment plant include release in the effluent, release from open tanks as aerosols, and release in sewage sludge that may be used as agricultural fertilizer. A typical municipal wastewater facility could release approximately 10^{21} to 10^{23} microorganisms per day in the effluent. The plant effluent is subject to extensive transport such that both freshwater and marine coastal ecosystems could be exposed to the microorganisms. Aerosol release and use of the sludge as

fertilizer could result in exposure of terrestrial ecosystems. Although there are numerous routes for the release and transport of the microorganisms, the stability/persistence of the microorganisms and their genetic makeup (for GEMs) has not been examined. In situations where GEMs are used, it is possible that the inserted DNA sequences may be released as is also the case with fragments of DNA from natural microbial consortia. The tracking process is hindered by the size and diversity of the naturally occurring microbial populations. In the case of industrial waste treatment, the small size of the facilities and the desire to contain the specialized microorganisms for proprietary measures is expected to minimize these types of releases.

Hazards

The extensive experience with the naturally occurring microorganisms associated with municipal wastewater treatment applications indicates that these populations present a low ecological hazard (health hazards will always exist because pathogens are present naturally in waste streams, but this aspect is not relevant to the current discussion). The use of GEMs in municipal wastewater treatment is unlikely because GEMs will be quickly outcompeted by naturally occurring populations. In the case of GEMs developed for specific industrial uses, there may be potential hazards. The GEMs or selected naturally occurring consortia would be developed and used for a specific trait or purpose. Despite increased containment measures, accidental releases could occur. If the GEM is released into an environment in which it will face competition from naturally occurring populations, then the hazard is expected to be minimal; however, the GEM may not be outcompeted in all environments. In the case of accidental release of GEMs, the transfer of the introduced DNA to other microorganisms could present a potential hazard.

Fuel Production/Biomass Conversion

This technology uses the ability of microorganisms to degrade plant biomass for the production of valuable products, including ethanol, hydrogen, methane, and other hydrocarbons. For this process, the starting material is processed and fermented in

large, enclosed, sterilized vessels, and then the inoculant containing the specially selected microorganism or GEM is added to the material in the vessels. As a result of the high specialization required to degrade many of the complex plant materials, GEMs are expected to have a significant role in this technology.

Organisms/Applications

Organisms that display the necessary genomes for the degradation of cellulose, hemicellulose, or lignin include species of *Cellulomonas*, *Bacillus*, *Clostridium*, *Aeromonas*, *Streptomyces*, and *Phanerochaete*. *E. coli* is often used to clone and express the relevant genes found in these organisms. In addition, *Saccharomyces cerevisiae* has been used extensively to produce ethanol from corn starch. This subgroup based its discussion on the extensive experience with the use of *S. cerevisiae* in the corn-ethanol industry. The product yield in this application is determined largely by the efficiency with which the microorganism uses the available substrate. The goals of genetic engineering are to increase the ability of organisms to digest a wider diversity of substrates and to increase the specificity of the product.

The concentration of microorganisms used for this technology is estimated to be 10^{18} to 10^{20} organisms for an ethanol plant with a capacity of 150 million liters per year. In some facilities, microbial stocks in the process tanks are replenished intermittently by addition from a seed tank; other production methods rely on the natural reproduction of the population within the vessel.

Exposure

The biomass conversion and fuel production applications are conducted in contained vessels, and the process is designed to maximize the recovery of all volatile products. Releases resulting from inadvertent leaks or large-scale accidents could result in microorganisms entering the natural environment on a large scale; however, the likelihood of release can be reduced by the use of certain industrial containment measures. Aerosol release from the system is a potentially large point-source exposure, but it can be mitigated through the use of scrubbing technologies. As a result of the large numbers of organisms used, the large amount of substrate required, and

the high cost of extensive containment, the industry relies mainly on primary containment in outdoor vessels. Secondary containment in the form of diking could be used to lessen the threat of accidental release.

Hazards

The hazards associated with the use of GEMs in this application are expected to be low. Many of the organisms utilized are non-pathogenic and do not produce hazardous by-products (e.g., *Saccharomyces cerevisiae*). Organisms that are plant pathogens are useful for converting plant debris; however, this application is not considered to be a problem as long as the pathogen is significantly disarmed or the desired genes from the pathogen are placed in an organism that is considered safe. For example, *Xanthomonas campestris* is a plant pathogen that has been used in industry for many years to produce xantham gum with no apparent problems. The main concern is the creation of specialty microorganisms that are able to degrade many different types of plant materials and that have competitive benefits not found in nature because it is possible that these GEMs will attack native sources if released accidentally to the environment. In most cases, however, GEMs are at an inherent disadvantage outside of the environment for which they were constructed. Ideally, the GEMs will devote a large amount of energy to the production of the desired end product (e.g., ethanol, methane) and then will be unable to compete successfully with the natural populations of microorganisms. In addition, organisms in dedicated fermentation vessels need a specialized, sterile environment; they are not adapted to natural environments with many stressors (e.g., starvation conditions). Therefore, the genetic modifications to the microorganisms are expected to increase their susceptibility in natural environments, effectively minimizing the hazard associated with accidental release.

Closed System Fermentation for Enzyme or Specialty Chemical Production

The use of bacteria to produce enzymes and other specialty products involves growing the specific microorganisms in fermenters and collecting the desired product (e.g., proteases, amylases). This technology is an area in which GEMs are expected to

have a significant impact. Currently, a variety of bacteria have been modified to produce various enzymes or specialty chemicals. The traits that are of interest for genetic engineering are based on the specific organism and the desired end product.

Organisms/Application

There are a number of different organisms used in this application. The organisms are selected and/or genetically engineered based on the desired end product. Some typical organisms used include strains of *Bacillus*, *Aspergillus*, *Trichoderma*, *Hemicula*, *Streptomyces*, *Clostridium*, *Xanthomonas*, and *E. coli*. The genetics of the organisms used are fairly well known and most contain stable genetic systems. The stability of these systems is maintained even with the introduction of modifications. Some Canadian producers rely on an OECD-based guidance criteria called the Good Industrial Large Scale Practice (GILSP) Criteria. Under this standard, the genetic elements of the organism must be well characterized, the host and the modified organism must be non-pathogenic, the organism must be used in a safe environment, the organism must have a limited survival in the environment, there must be a benefit/endpoint for the modification, the vector must be well characterized, and the introduced genetic material must be limited in size and lack mobility.

This application involves large numbers of organisms with figures in the range of 10^8 to 10^{12} organisms/mL in fermentors that are approximately 10,000 to 200,000 liters in size. Therefore, total populations in a single fermentor can reach 10^{15} to 10^{18} cells. The frequency of the application is batch-dependent. Typical turnover rates are every 2 to 3 days or 8 to 10 days.

Exposure

Little or no environmental release of the microorganisms used in this application is expected. The processes occur in closed systems, and inactivation procedures are usually employed at every point in the operation. Containment of GEMs is of great interest to the producers because it would be disadvantageous to allow anyone else to have cells of the organism. Economically, it is beneficial to maintain exclusive knowledge concerning the genetic nature of the engineered organism, and the primary

insurance against the loss of secrecy is the containment of the organisms. In addition, release within the manufacturing plant will be low because of other regulations (e.g., workers exposure must be controlled in response to regulations for occupational safety). Moderate environmental exposure could occur as a result of accidental release, but containment procedures reduce the risk of this exposure route. Containment is nearly complete with a closed system that consists of sealed fermentors that are housed in specially designed buildings. To inactivate wastewaters and other materials that may be contaminated with the organism, companies use "kill" tanks, special drainage systems, scrubbers, and barriers to the release of the organisms. Any inadvertent releases are expected to be in the form of aerosols from residuals left on filters and seals or from accidental release. Exposure would be to the areas surrounding the plant, many of which are close to urban centers.

Hazards

The hazards associated with the use of microorganisms in closed system fermentors are low as a result of the regulation and specificity of the microorganisms. As a result of industry standards, the microorganisms used often are approved under the Good Industrial Large Scale Practice (GILSP) Criteria. As discussed earlier, organisms that meet the GILSP criteria are expected to present only a minimal hazard. In addition, the organisms are not expected to compete and survive outside the controlled environment because the high degree of specialization of these organisms renders them poor competitors in other ecosystems. Also, some of the genetically engineered varieties are specifically modified to be disabled in natural environments. There may be some hazard associated with the potential mobility of the introduced genes; however, organisms with this tendency would not meet the GILSP Criteria. It is also doubtful that the organisms have the ability to persist long enough to transfer the genes. As mentioned previously, specialization limits their survival in natural environments and, therefore, reduces the opportunity to transfer genetic information. The only other potential hazard involves the products of these organisms. The desired products are not usually toxic; however, there is the possibility that the genetically

manipulated organisms may produce unknown by-products that were not originally anticipated, although these are probably checked for regularly in this industry.

GROUP C & D: ECOLOGICAL EFFECTS AND EXPOSURE/FATE ENDPOINTS

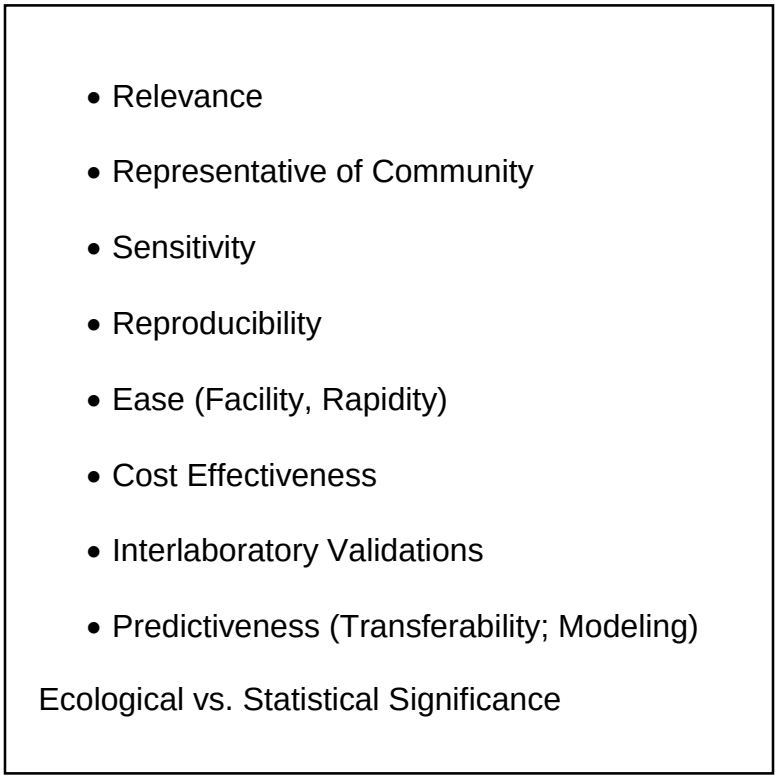
Ecological Effects

This group identified ecologically significant endpoints that should be included in the tier testing scheme. The overriding concept in determining the endpoints of ecological effects discussed below is to maintain the sustainability of the ecosystem. A defensible series of tests should be designed to identify demonstrable and significant effects on the ecosystem. The group suggested developing a set of generic endpoints and a set of specific, case-by-case endpoints for sites into which GEMs may be introduced. Dr. Guenther Stotzky generated a list of desirable characteristics of methods for assessing ecological effects of introduced microorganisms (**Figure 1**).

There are several reasons why potential ecological impacts of inocula have not been well characterized. It is not yet possible to predict how potential impacts will be expressed. Moreover, the environmental level at which these impacts should be measured (e.g., micro- vs. macroecology), the appropriate techniques to use to detect ecological effects, and how the results should be interpreted (e.g., statistical vs. ecological significance) are not clearly defined.

Many of the endpoints discussed below can be determined with microcosms. The microcosms should represent the recipient environment as well as environments through which the organism may pass. It must be a legitimate approximation or simulation of the natural environment, and not just an environmental sample. Inasmuch as any potential effects may require a long time to manifest themselves, the absence of a short-term effect does not necessarily eliminate risk, and this should be considered when designing a study. Mesocosms may provide extra information, but it must be clearly determined whether their use justifies the extra cost and time.

Figure 1. Desirable Characteristics of Methods for Assessing Ecological Effects of GEMs.



Source: Stotzky, G, Broder, MW, Doyle, JD, and Jones, RA. 1993. Selected methods for the detection and assessment of ecological effects resulting from the release of genetically engineered microorganisms to the terrestrial environment. Adv. Appl. Microbiol. 38:1-98.

Another issue is that the studies should be performed on "stressed" and non-stressed environments. "Stressed" environments would include the presence of substrates upon which the enzymes encoded by the novel DNA function, or antimicrobials to which the novel DNA confers resistance. The addition of these materials might also help to identify any selection and competitive advantages.

Primary Production (CO_2 fixation)

Any impact of an introduced microorganism on primary production is a significant ecological effect endpoint for both terrestrial and aquatic systems. The testing scheme should include an evaluation of the effects of the microorganism on the significant primary producers in the environment of concern (e.g., forest, crop land, prairie, desert).

The endpoint to be identified is a change, positive or negative, in the populations and activities of the primary producers compared to controls (e.g., no introduction of a GEM). System recovery and delayed effects must be considered when choosing the duration of the study (i.e., acute vs. chronic). The types of controls must also be carefully determined, e.g., the controls may include soil before bioremediation, soil after bioremediation, and soil of comparable type that is not inoculated. Relevant soils, determined on a case-by-case basis, should be used.

In environments where primary production is not the main source of carbon such as subsurface soils, groundwater, or publicly owned treatment works (POTW) facilities, the effects of the introduced microorganism on catabolic functions should be assessed. A change in the degradation and transformation of organics or metals, for example, may impact water purity. Dissolved organic carbon, total organic carbon, and tests with model compounds may be used to measure an effect on this ecosystem function.

Mineralization and Losses of Limiting Nutrients

The cycling of limiting nutrients is another significant ecological endpoint. In many terrestrial habitats, the limiting nutrients are nitrogen, phosphorus, sulfur, and, in some cases, carbon. The test should determine the effect of the introduced microorganism on net nitrogen mineralization (e.g., NO_3^-, NO_2^-, NH_4^+). When it is important in the subject environment, the effects on nitrogen fixation should also be studied. The effects of the introduced microorganism on the cycling of phosphorus and sulfur is measured by determining the availability of phosphorus and sulfur to the primary producers in the relevant ecosystem. The effect of the organism on mineralization of carbon should be studied when the introduced organism has the ability to depolymerize complex organics. Time scale and land-use factors must also be considered in the cycling of limiting nutrients.

In aquatic systems, the significant limiting nutrients are usually nitrogen and phosphorus. The effects of the introduced microorganism on net mineralization of nitrogen should be assessed. If the microorganism is expected to be released to an environment where nitrogen fixation is important, the effects of the organism on this function should also be studied. The availability of soluble orthophosphate (PO_4^{3-}) in

the water column and sediment in the relevant ecosystem should be measured. Both of these parameters are linked to primary production.

Community Structure (Diversity)

In both terrestrial and aquatic environments, the community structure is an indication of the health, sustainability, and resiliency of the ecosystem. A preliminary test could involve making a broad survey of the species richness and functional groups (e.g., major algal groups, benthic fauna) in the presence of the introduced microorganism, although this may be difficult to do in a microcosm. If the introduced microorganism is affecting the community structure, it is expected that the taxonomic diversity will change first. Changes in the number of taxa or changes in the community structure would trigger additional testing. If there is a significant change in total density or in the density of major groups, or if a species is missing, then additional testing should be performed. If feasible, the lowest trophic level should be studied.

Grazers

Predators or grazers may be used as indicators of fluctuations in the community structure of environments, as they are a link to both higher and lower organisms and an important point for transfer of trophic energy in the ecosystem. A change in the numbers and types of species that graze on primary producers, or on heterotrophic organisms, positive or negative, is an important ecological endpoint. In terrestrial systems, the density and grazing rate of nematodes and protozoa that feed on bacteria and fungi should be assessed. In aquatic systems, total protozoa, including both vegetative and encysted forms, should be measured. Effects on other aquatic groups (e.g., algae, insects and other invertebrates) may also need to be studied. This type of effect may be important at higher levels of tier testing, i.e., changes in community structure should trigger testing to identify the effect of an introduced microorganism on grazers in the ecosystem.

Sensitive Species

The group identified two types of sensitive species. A sensitive species may be an indigenous organism in the general population that responds rapidly to environmental perturbation, or it may be a specific indicator organism that is sensitive to the introduced microorganism and/or its gene product. Effects on sensitive species were considered as possible significant endpoints; however, the consensus was that sensitive species should not be used as stand-alone endpoints. Rather, they should be considered during the survey of taxa used to characterize community structure.

Exposure/Fate

Due to time constraints, this group was unable to discuss fully the ecologically significant exposure and fate endpoints. Techniques to measure exposure (except for dispersal) are available, and it is possible to predict the fate of microorganisms in the environment; however, such predictions are not very accurate or precise. The size of microbial populations depends on the environmental conditions, and these conditions are often unpredictable. Instead of a thorough discussion, brief answers to a number of questions on microbial survival and fate are presented below.

Survival and Persistence

The fate and transport of the microorganism and its novel DNA must be determined. Periodic tests should be conducted even after the novel genes or introduced organisms can no longer be detected because the organisms may regrow and the novel genes may reappear in other organisms. Persistent gene products may also pose a risk.

Competition

The determination of the competitiveness of an introduced microorganism is less important than determining if the microorganism survives. Some participants believed that competition between introduced microorganisms and indigenous populations could not be measured accurately *in vivo* or *in situ*. Consequently, the group decided that it is not necessary to study competition separately.

Responses to Exposure/Fate Questions

1. At what population density and for what length of time does the persistence of an introduced microorganism constitute survival of the microorganism?

   The consensus was that the population density that constitutes survival of the microorganism is too low to measure. There may be delayed effects if the population regrows to higher levels. Moreover, the novel genes in an introduced organism may be transferred to indigenous microorganisms.

2. Is it appropriate to consider the number of individual propagules, the total biomass, or both, of an introduced microorganism, especially with respect to fungi?

   Enumeration of spores, propagules, and biomass may not be possible in many cases. However, when possible, it should be done.

3. What constitutes a valid test of microbial competitiveness? Is there a concern with the competitive ability between a GEM and its parent, between a GEM and the indigenous populations, or both?

   If the organism displaces its parent, it is a concern. However, it is more important to determine if the introduced organism survives.

4. Is it important for risk assessment to establish quantitative relationships between the numbers or biomass of introduced GEMs that survive and the harm that they may do? Does such information exist?

   It is important for risk assessment to establish quantitative relationships between the number of microorganisms that survive and persist and the

harm that they may do. However, this information does not exist in most cases.

5. If a microcosm is not appropriate for evaluating the survival, proliferation, dispersal for great distances, and effects for particular environments or applications of GEMs, are there suitable mesocosms for these purposes?

   Mesocosms are extremely expensive, and their use must be well justified. The consensus was that 90% of the information about the fate of the GEM could be obtained using less costly microcosms.

GROUP E: MICROBIAL PATHOGENICITY/TOXICITY

This subgroup discussed the categorization of frank and opportunistic pathogens, predictive pathogenic traits, and toxin production by microorganisms that may be used for risk assessment for the technologies under discussion in this workshop.

The subgroup determined that the issues of microbial pathogenicity/toxicity outlined in the discussion questions were not amenable to across-the-board solutions but, rather, should be addressed in a system-specific manner. ("System" refers to the subject organism, the potentially affected organisms, the receiving environment, and the conditions of use.) The development of each system-specific analysis should rely on consultation with expert panels. Using such consultations, system-specific databases and catalogs could be instituted that, ultimately, would form the core of an "expert system." This expert system would be an invaluable tool in the regulatory review of biotechnology applications.

Information Sources

Although there are several incomplete lists of microorganisms known to be frank pathogens of animals, plants, or aquatic organisms, these lists are not well-organized. It would most likely be necessary to consult textbooks and to search the primary scientific literature for such information. However, the appearance of an organism on one of these lists would certainly lead regulators to require further testing to ensure that

the product is free of pathogenicity (i.e., the lists would trigger "red flags"). The identification of opportunistic pathogens is even more problematic; still, the few incomplete lists, when available, would provide triggers for further testing.

Species of Particular Concern

For certain genera frequently used in the technologies under consideration (e.g., *Pseudomonas, Bacillus*), a preliminary list of pathogens was drawn up by this workshop subgroup. This list can be expanded by members of the subgroup (using available information sources in their respective offices and institutions), but other experts beyond the subgroup members should also be consulted during the construction of a pathogen list. It was noted that the use of genera may be too broad an approach for such an exercise, especially for the large genus of *Pseudomonas*. For many genera, the species level is necessary to avoid unnecessary testing of nonpathogenic microorganisms.

Predictors of Pathogenicity or Toxicity

The subgroup concluded that there are no across-the-board specific phenotypic or genotypic characteristics that are directly associated with, or predictive of, pathogenicity. The development of a list characterizing the ability of an organism to grow in the host would include information such as the ability to colonize, and specific environmental requirements (e.g., temperature, pH, oxygen conditions), and the list would need to be tailored to each specific system. As a start, the subgroup created a list of traits that indicate the potential for pathogenicity to plants. This list included the production of cell-degradative enzymes, toxins, growth factors, or extracellular polysaccharides; ice nucleation ability; and the ability to grow in or on the host.

The subgroup agreed that bioassay results will be much more informative than lists of pathogenic traits. Four examples of tests that, when properly combined, could assist in categorizing bacteria as plant pathogenic were suggested: oxidase, tobacco hypersensitive reaction, Gram reaction, and fatty acid analysis.

Research needs concerning predictive characteristics include: the extension of databases from their current limited focus on a few organisms of public health or agronomic importance to a broader inclusion of environmentally significant species. This exercise should include consultation with experts.

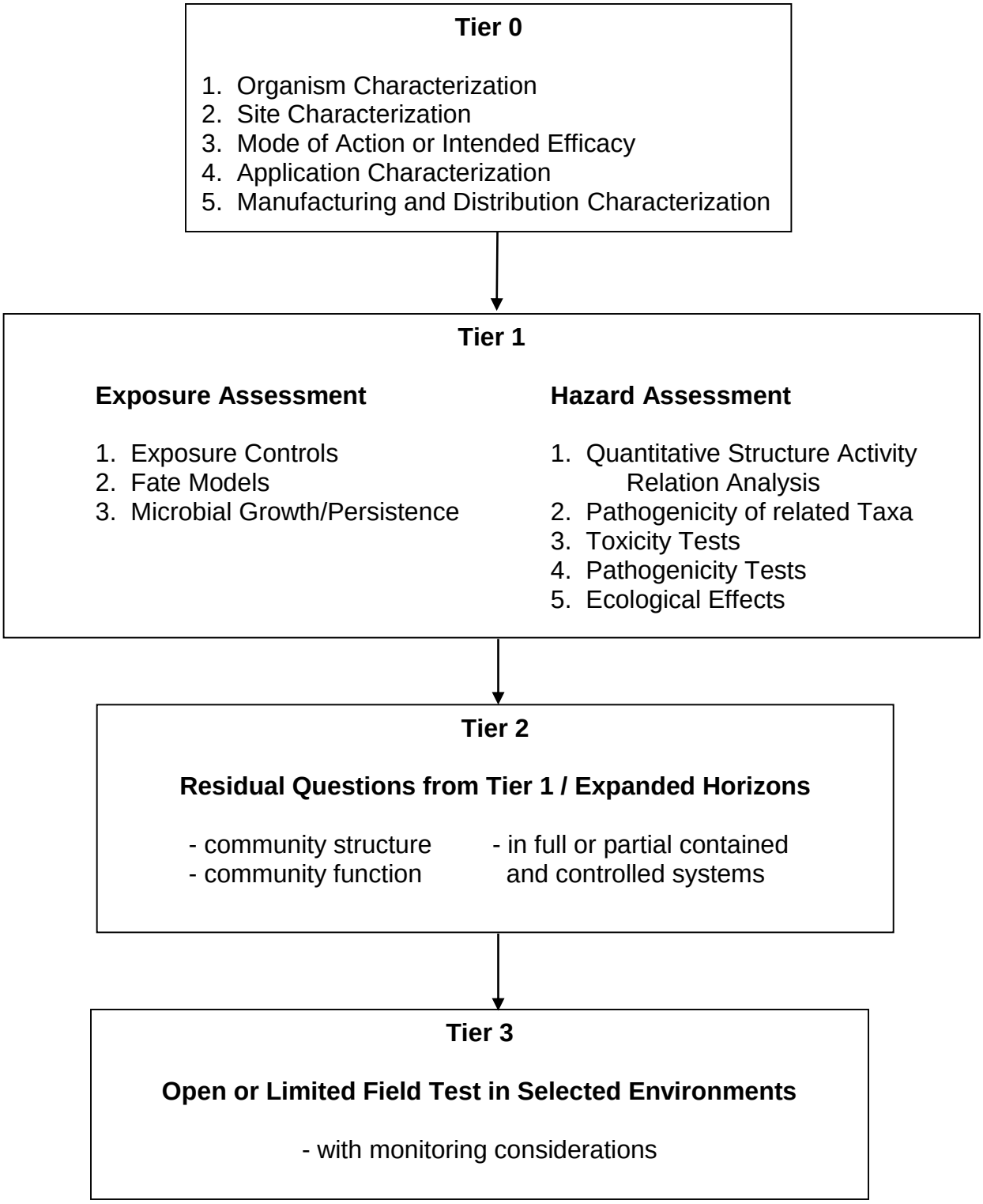
Mycotoxins and Other Toxins of Concern

Mycotoxins are produced in the environment and their expression is under environmental control, but few examples are understood. Many toxins other than mycotoxins are produced, but again, analysis is system dependent. In testing for toxins/toxin producers, it would be useful to employ surrogates, as currently used for chemical toxicity testing, provided the surrogate organisms are appropriate for the conditions under consideration. After initial tests using standard species, other specialized or more relevant surrogates may need to be used.

GROUP F: STRAWMAN TIER TESTING SCHEME

This subgroup developed the strawman tier testing scheme that was used by the breakout groups on Day 2 and Day 3 to develop the ecological tier testing schemes for closed, semi-contained, and open biotechnology applications. The scheme (**Figure 2**) contains four tiers. Tier 0 includes preliminary (i.e., pre-test) information, including taxonomic identification, proposed use, and site characterization. Tier 1 contains the initial exposure and hazard assessment components. Tier 2 would address any residual questions about exposure and hazard from Tier 1. Tier 3 is open or limited field tests in selected environments.

Figure 2. Strawman Tier Testing Scheme



TIER TESTING SCHEMES

GROUP I: SCHEME FOR CONTAINED/CLOSED TECHNOLOGIES

This group reviewed the strawman tier testing scheme, shown in **Figure 2**, developed the day before by Group F, and used it to develop a tier testing scheme for contained biotechnologies. The group did not define the level of containment that would qualify as a closed system but, rather, assumed that incidental or accidental releases of microorganism could occur in any closed system. The system must qualify as a closed system through an exposure characterization. Because the group participants ranged from individuals with considerable knowledge of and experience with their products to people working with novel products that are not well characterized, the tier testing scheme was modified to allow approval based on how much information is available regarding the ecological risks associated with the microorganism. The low levels of exposure commonly associated with contained systems allowed for a greater flexibility in developing the tier testing scheme.

Tier 0

The only changes the group made to the original strawman tier testing scheme was to fold the application characterization and manufacturing and distribution characterization together and add quantitative structure-activity relationship (QSAR, a correlation between toxicity and physical/chemical properties) analysis and environmental behavior of the GEM to Tier 0. This tier enables well characterized products for which a substantial amount of information exists about their safety to gain approval if no unreasonable ecological risks are identified. These "early-out" organisms are those that would meet the GILSP (Good Industrial Large-scale Practices) criteria (see **Table 1**). Conversely, if specific concerns are not resolved, further testing will be required to address these concerns in Tier 1.

Table 1. Suggested Criteria for recombinant DNA (rDNA) Good Industrial Large Scale Practice (GILSP) Microorganisms

Host Organism	rDNA Engineered Organism	Vector/Insert
Non-pathogenic;	Non-pathogenic;	Well characterized and free from known harmful sequences;
No adventitious agents;	As safe in industrial setting as host organism but with limited survival without adverse consequences in the environment.	Limited in size as much as possible to the DNA required to perform the intended function; should not increase the stability of the construct in the environment (unless that is a requirement of the intended function);
Extended history of safe industrial use; OR		Should be poorly mobilizable;
Built-in environmental limitations permitting optimal growth in industrial setting but limited survival without adverse consequences in environment.		Should not transfer any resistance markers to microorganism not known to acquire them naturally (if such acquisition could compromise use of drug to control disease agents).

Five fundamental concerns were identified and are listed in the Tier 1 box in **Figure 3**. For example, if persistence (survival) data are lacking at Tier 0, then Tier 1 tests to determine the persistence of the organism will be required.

Tier 1

Tier 1 involves testing for specific concerns that were raised in the Tier 0 analysis (**Figure 3**) and, for the most part, conforms to the strawman tier testing scheme. If a concern exists because there is a lack of information regarding the organism's persistence, the submitter must further characterize the hazard and fate issues. Persistence and survival studies incorporating site-specific parameters may be required. For a GEM, the suggested trigger for further testing is if the GEM persists better than the host organism or exceeds the background levels of indigenous microorganisms. The regrowth of the GEM would be a concern that would also trigger further testing from Tier 1 to Tier 2. Concerns about the hazards associated with the DNA product may be addressed in Tier 1 by studying the persistence of the genetic material. If the DNA product persists, then determine if the DNA can be transferred to indigenous bacteria. If the microorganism is not well characterized in terms of its identification, Tier 1 tests for determining higher level taxonomy and taxonomical grouping may provide information that will resolve other concerns. Negative identification (determining which species a organism does not belong to) was also suggested as a possible method for eliminating some hazard concerns. If there is concern resulting from uncertainty about the host range or the pathogenicity of the introduced microorganism, testing on surrogate plant and animal species should be performed. Similarly, Tier 1 includes testing to determine if the introduced microorganism produces a toxin that may adversely affect the environment. If concerns about toxicity exist, Tier 1 calls for testing using appropriate indicator or surrogate species. Because this testing scheme is for contained systems, the toxicity concern may be resolved with a change in the process to alleviate potential releases of the microorganism, or a change in the type of microorganism used. If at the end of the Tier 1 testing, it is determined that the organism poses no unreasonable risk

Figure 3. Contained Systems Tier Testing Scheme

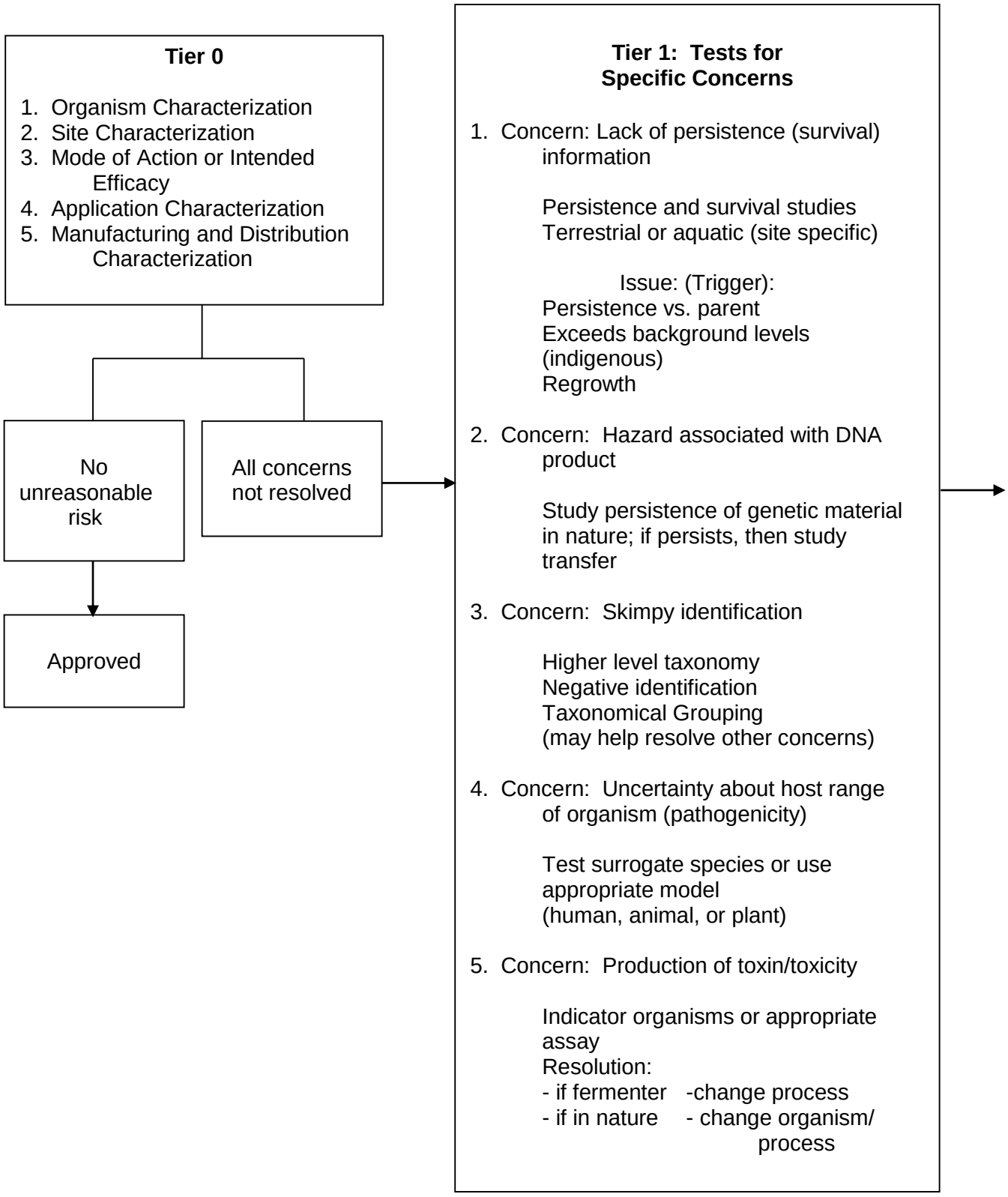
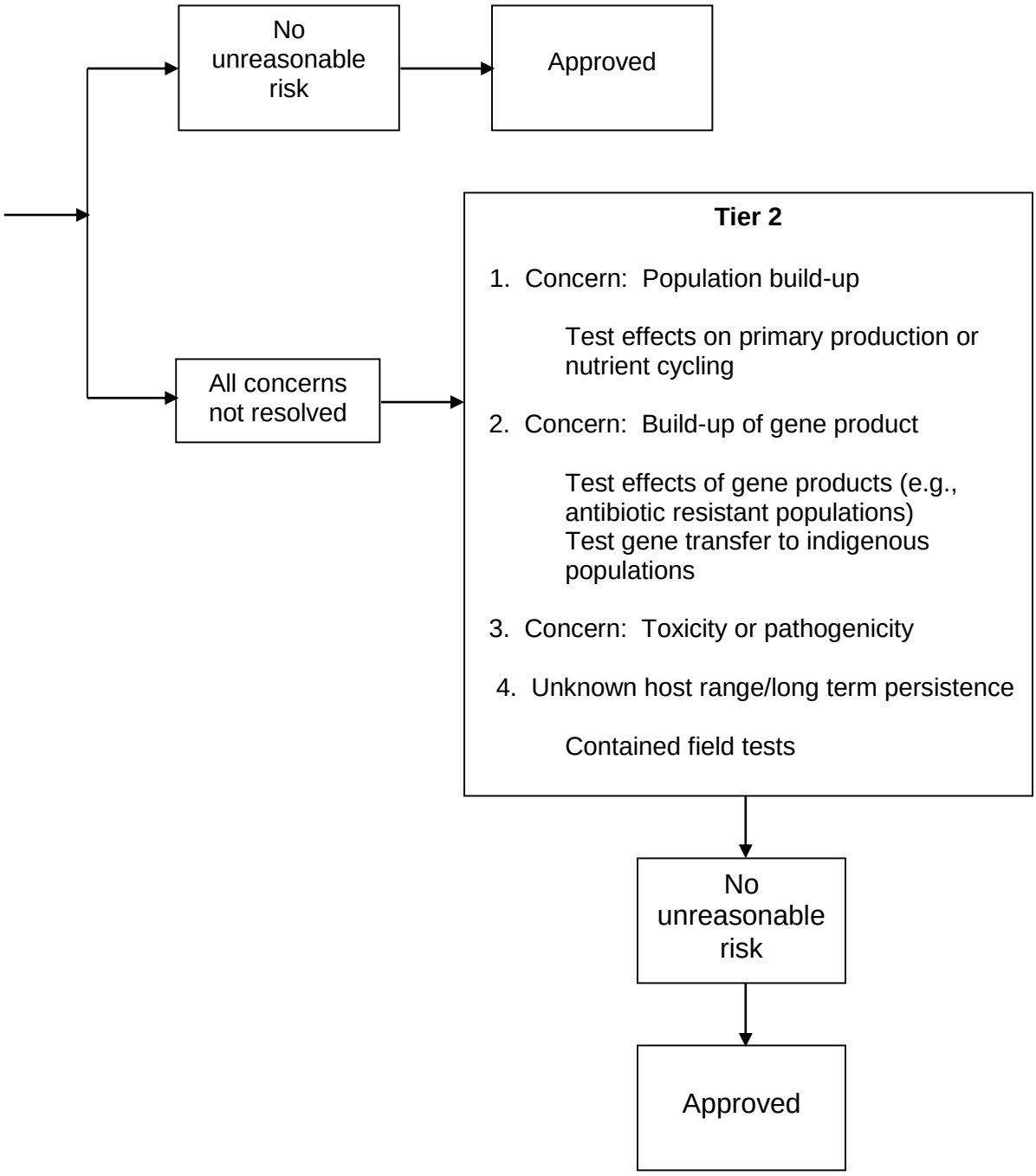


Figure 3 (continued)



to the environment, the microorganism is approved. If all concerns are not resolved, further testing is required in Tier 2.

Tier 2

Tier 2 and Tier 3 of the strawman scheme (Figure 2) were combined into Tier 2. Similar to Tier 1, Tier 2 designates tests that address specific concerns. The fundamental concerns identified in this Tier include population build-up, toxicity, pathogenicity, build-up of gene product, unknown host range, and long-term persistence. If population build-up is a concern, then Tier 2 calls for tests to determine the effects of the introduced microorganism on primary productivity and nutrient cycling. If the gene product is expected to persist and possibly transfer, tests to determine the impact of the gene product and expression in recipient organisms should be conducted. For example, tests may determine if antibiotic resistant populations may increase when the organism is released. If there is a concern about the toxicity or pathogenicity of the introduced microorganism, more precise testing to identify the threshold levels, e.g., LD_{50} or $LC_{50,}$ is required. "Contained" field tests (those in which there is an attempt to mitigate dispersal) may also be employed in Tier 2 to characterize the host range and long-term persistence of the introduced microorganism.

GROUP II: SCHEME FOR SEMI-CONTAINED TECHNOLOGIES

This group examined the general strawman tier testing scheme (**Figure 2**) to determine what modifications would be necessary to develop the tier testing scheme for semi-contained technologies (i.e., bioremediation, oil recovery, coal transformations, municipal waste treatment, and biomining). The discussion group emphasized that arriving at a fixed scheme for use across all of the semi-contained applications is a difficult process. The most important characteristic of a workable, efficient, and simple tier testing scheme is flexibility. A rigorously structured tier testing scheme will not suffice given the variety of current and future technologies. Flexibility is especially important within tiers in terms of the endpoints selected. The specific tests that will be used within each tier should depend on the characteristics

of the organism that have not been sufficiently described in previous tiers. Throughout the modification process, care was given to create a scheme with this underlying flexibility. The tier testing scheme developed for semi-contained applications is presented in **Figure 4**. This scheme maintains simplicity in its design while providing a functional framework for the examination of naturally occurring and genetically engineered microorganisms. It also is flexible, both as a whole scheme and within each Tier, as was shown in theoretical scenarios with some of the technologies. Another key element of this system is that the submitters should not only evaluate the microorganisms, but also the genetic material and the potential byproducts.

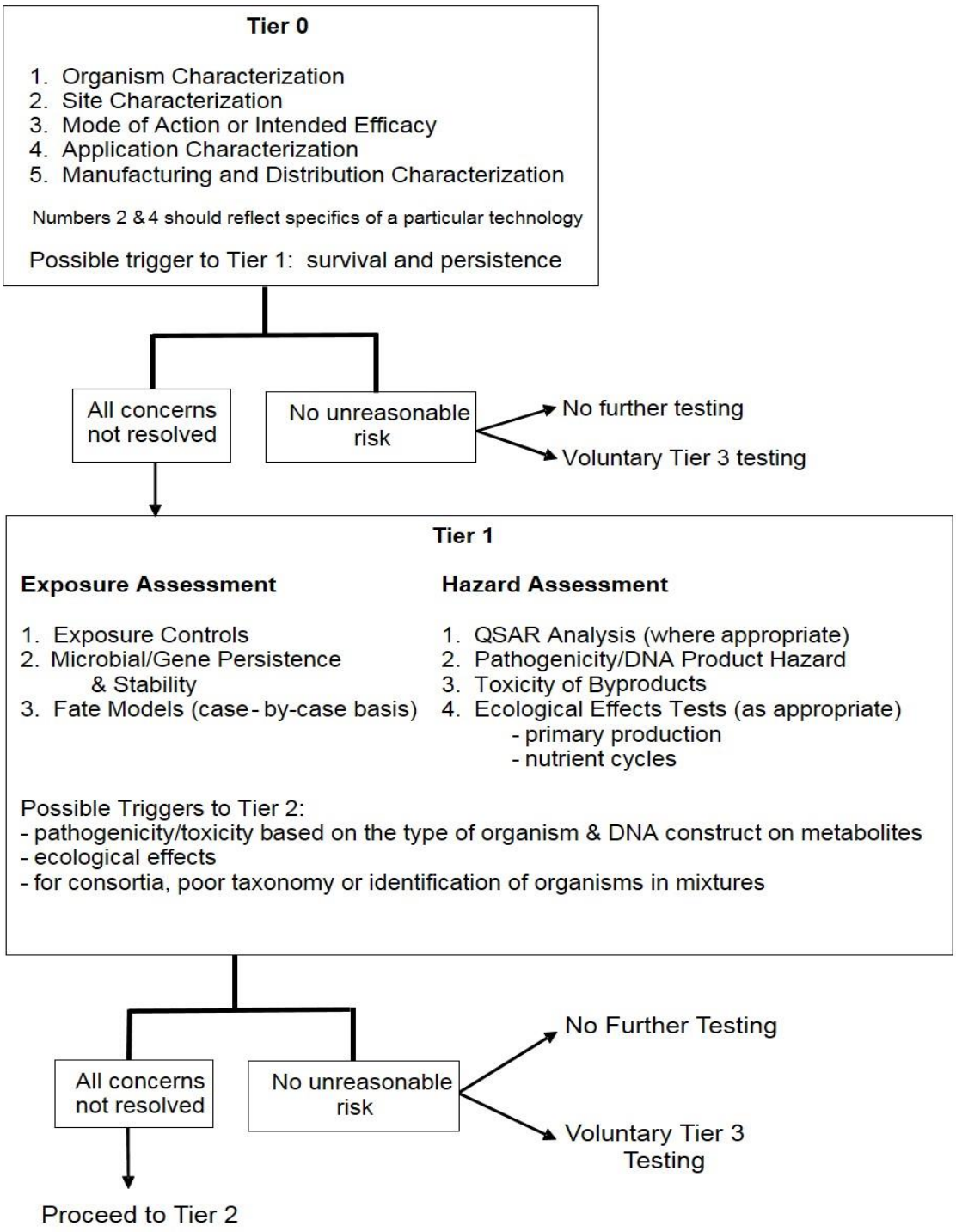
Tier 0

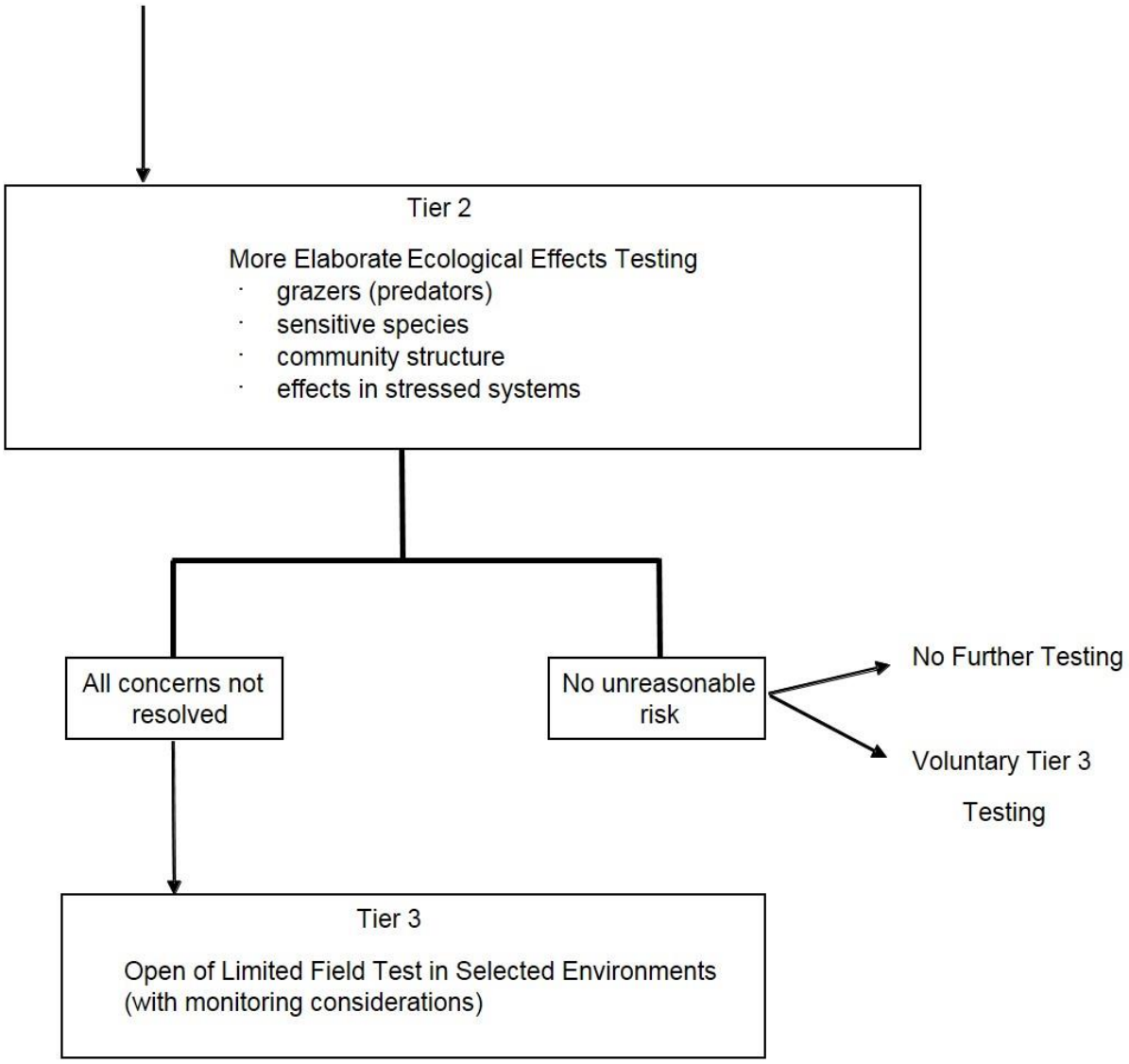
The goal of this tier is to characterize the microorganism based on the submitter's description of the organism and the intended production and use of the microorganism. Specifically, it examines the organism (including the genetic material), the intended application site, the mode of action, the intended efficacy, the mode of application, and the manufacture and distribution of the microorganism. In general, the information required for assessment in this tier is similar to the data provided in Pre-manufacturing Notifications (PMN) submitted by industry to the U.S. EPA before the manufacture or importation of a new product. It is anticipated that the submitter could provide this type of information without laboratory testing, especially if the microorganism is already well characterized in the published literature. A special concern in this tier is the issue of the survival and persistence of the organism and the genetic cassette.

Once the background data have been reviewed, a decision will be made as to whether use of the organism presents an unreasonable risk to the environment. This decision is based on the resolution of concerns about issues such as genetic persistence. If there is no unreasonable risk, the submitter may exit the scheme or may move directly to Tier 3. Tier 3 would not be a requirement in this case; however, manufacturers often perform field

studies to examine the efficacy of their product regardless of EPA requirements. If all concerns have not been resolved in Tier 0, then the assessment process moves into Tier 1.

Figure 4. Semi-contained Tier Testing Scheme




Tier 2
More Elaborate Ecological Effects Testing
· grazers (predators)
· sensitive species
· community structure
· effects in stressed systems
All concerns not resolved
No unreasonable risk
No Further Testing
Voluntary Tier 3 Testing
Tier 3
Open of Limited Field Test in Selected Environments (with monitoring considerations)

Tier 1

This tier includes exposure and hazard assessment, although both assessments may not be necessary. In certain cases, the unresolved concerns from Tier 0 may only apply to problems with exposure, in which case, only the exposure assessment would be conducted; other cases may require only hazard assessment based on the concerns identified in Tier 0.

The exposure assessment includes an examination of methods to control the release of the organism/genetic material and a review of studies of the persistence and stability of the microorganism and novel genes. Fate models may also be examined in some cases, but it is important to recognize that they are very complex and may not always be available or useful.

The hazard assessment includes four components. First, quantitative structure-activity relationship analyses of the by-products of the application may be evaluated, if appropriate for the given technology. Second, data from pathogenicity tests on the microorganism, the DNA product, and the transfer of the genetic material should be reviewed. Third, toxicity tests on the by-products using an indicator organism or appropriate assay should be considered. Finally, tests that examine the effect of the organism on environmental processes, such as primary production and nutrient cycling, should be evaluated. The pathogenicity, toxicity, and ecological effects data may be adequately described in the literature or in a previous PMN submission, or the submitter may have to perform the tests. In all cases, the tests used should be appropriate to each specific technology.

After the necessary data have been collected, the exposure assessment and/or hazard assessment will be conducted. If the concerns identified in Tier 1 have been resolved, then the submitter may exit the tier scheme, or, as in Tier 0, the submitter may voluntarily initiate Tier 3 studies. Possible triggers to the next tier include significant pathogenicity and/or toxicity of the organism, the DNA, or metabolites, notable ecological effects, persistence of the organism or genetic material, or lack of taxonomic identification in the case of microbial consortia.

In this tier, any additional hazard and exposure issues from Tier 1 will be examined. Some of the issues evaluated in this tier may include the build-up of

population or gene products, the long-term persistence and transfer of gene products, and pathogenicity/toxicity concerns. To determine whether these issues are indicative of possible environmental risk, more elaborate ecosystem-level tests must be evaluated. These tests may examine the effects of the microorganism on grazers (predators) and sensitive species, changes in community structure, and/or effects of the microorganism on stressed systems. If any hazard or exposure issues are still unresolved, Tier 3 testing may be necessary. If the organism does not pose unreasonable risk, no further assessment would be required.

Tier 3

Tier 3 can, upon approval, be entered voluntarily from any of the previous tiers based on the monitoring needs of the applicant (i.e., the manufacturer needs these tests to evaluate the use and/or efficacy of the microorganism). This tier also can be entered from Tier 2 if there is a regulatory need for further assessment. Tier 3 consists of open or limited field tests undertaken in selected environments. It is important to consider monitoring issues when conducting these types of tests. After Tier 3 evaluation has been completed, the submitter may receive permission to manufacture the product. In some cases, however, it may be necessary to restrict the use of the product as the result of residual hazard or exposure concerns associated with the organisms.

Evaluating The Scheme

To evaluate the adequacy of the tier testing scheme developed for semi-contained technologies, the group used theoretical scenarios for these applications (i.e., bioremediation, oil recovery, coal transformations, municipal waste treatment, and biomining).

Biomining was considered as an example of a more contained technology, based on current practices that provide a kind of biological containment. Specifically, bioleaching is conducted on an impervious lined base, and biooxidation is conducted in reactor tanks. Following Tier 0 evaluation, it is expected that biomining products would need to be further evaluated based on concerns related to exposure controls and persistence. In this case, the information obtained in the data-gathering process of Tier

1 would be used to address the exposure questions generated in Tier 0. Growth and persistence of the organism and the persistence of genetic inserts are the most important characteristics that need to be assessed; fate models would not be relevant for biomining. Biomining also provides an example of an application that would not require a hazard assessment. The selected or genetically modified organism would be extremely substrate-specific and, therefore, very sensitive to ambient pH levels; this characteristic would minimize the risk of extensive environmental exposure. Biomining products are expected to exit the scheme after Tier 1; however, manufacturers may wish to conduct Tier 3 tests for their own purposes.

Industrial waste treatment was considered as an example of a less contained application. It is anticipated that environmental exposure will be associated with this technology; therefore, Tier 1 assessment will most likely be necessary. Depending on the concerns identified in Tier 0, both hazard and exposure assessments may be necessary. In the exposure assessment, fate modeling is appropriate. Models already exist for tracing effluent plumes and the compartmentalization of streams. Under the hazard assessment, gene transfer should be examined based on concerns about the persistence of the novel DNA. Tests with the microorganisms may be difficult to perform as a result of the complexity of the consortia; however, it may be possible to trace genetic inserts in ecological effects and pathogenicity testing. Depending on the characteristics of the organism, persistence tests may need to be performed. Chronic persistence would be a trigger for Tier 2, and perhaps Tier 3, testing.

Bioremediation (bed reactors, *in situ* groundwater treatment, and composting) also was considered as an example of a less contained technology. Similar to industrial waste treatment, it may involve complex consortia. The difficulty associated with characterizing the consortia would most likely necessitate Tier 1 assessment. In this case, concerns that trigger further testing might be exposure-based (e.g., the persistence of the introduced genetic material) or might be based on an inability to adequately characterize the organisms of the consortia. These concerns are expected to lead to Tier 2 and Tier 3 testing.

GROUP III: SCHEME FOR OPEN/UNCONTAINED TECHNOLOGIES

This group reviewed the strawman tier testing scheme (**Figure 2**) and developed an appropriate tier testing scheme for open biotechnology applications (i.e., bioremediation, oil recovery, coal transformations, waste treatment, or nitrogen fixation applications). This scheme, which resembles a decision tree more than the traditional tier testing scheme, is presented in **Figure 5**. The tiered system was considered to be a series of concerns or questions rather than a set of testing requirements. The tier testing scheme for open microbial applications features a generic set of concerns that need to be addressed for every product; however, only the relevant or appropriate issues would trigger further testing. Each tier has a decision box after evaluation of the available data. There are three options for actions at each of the decision points in this tiered approach system: 1) based upon the information available, the concerns are low and the product may proceed to Tier 3 field testing; 2) there is not enough information, so certain tests must be performed to determine risk; or, 3) the product is rejected because the risks are unacceptable, and further testing is not productive for the applicant.

Tier 0

Tier 0 involves gathering the preliminary PMN submission or pre-test information. The information should include organism characterization, site characterization, mode of action or intended efficacy, application characterization, and manufacturing/distribution characterization. The organism characterization should include an identification of the recipient organism and the novel DNA. The possibility of release during transport of the organism from the laboratory to the field should be covered in the manufacturing/distribution characterization. The Tier 0 data should provide enough background information to enable the assessor to formulate questions about hazard and exposure concerns. Evaluation and confirmation of the quality of the data used in Tier 0 is imperative, as these data are fundamental to the risk assessment. Once the information in Tier 0 has been reviewed, the analysis moves to the first Decision Box.

Figure 5. Open Systems Tier Testing Scheme

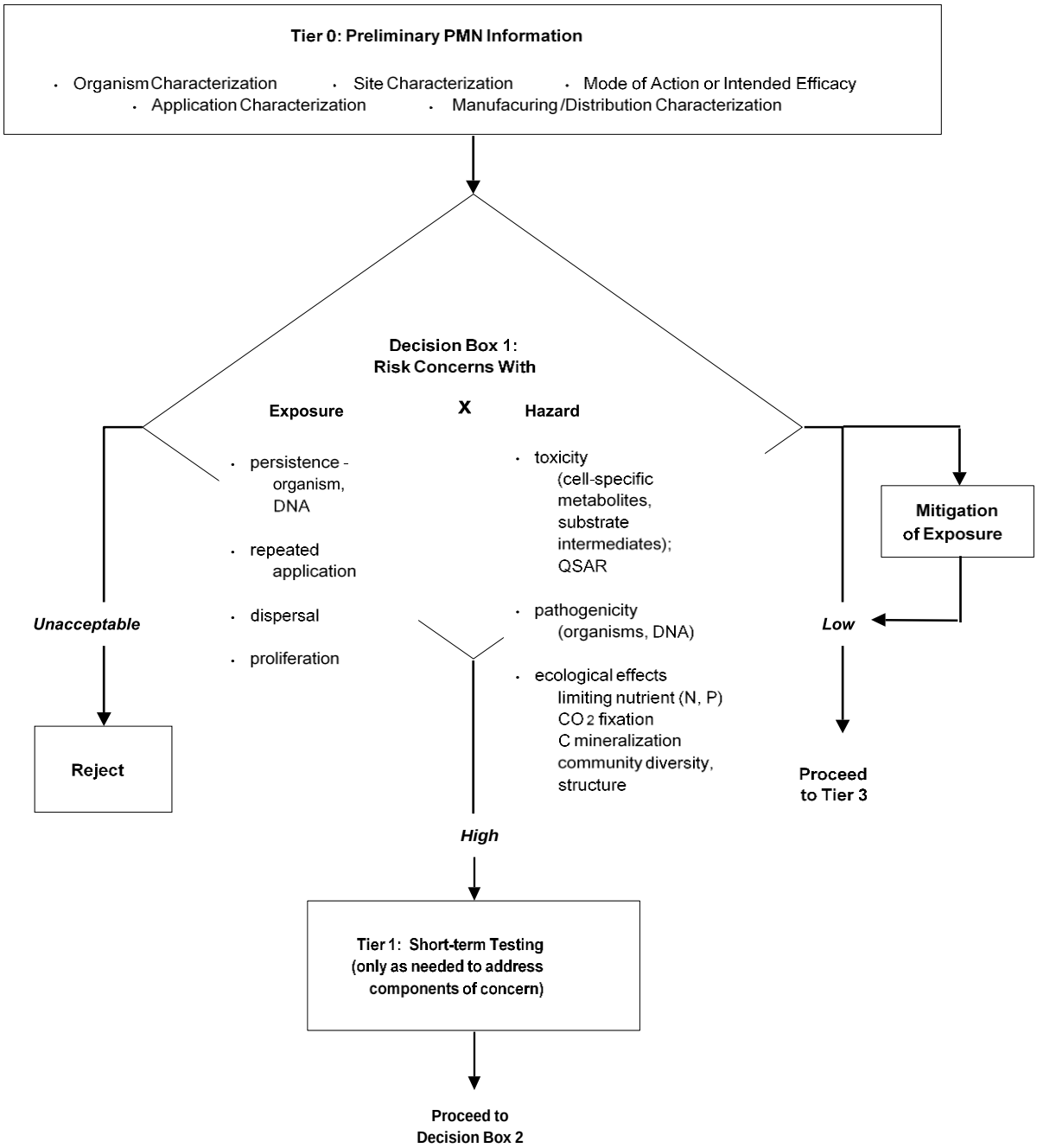
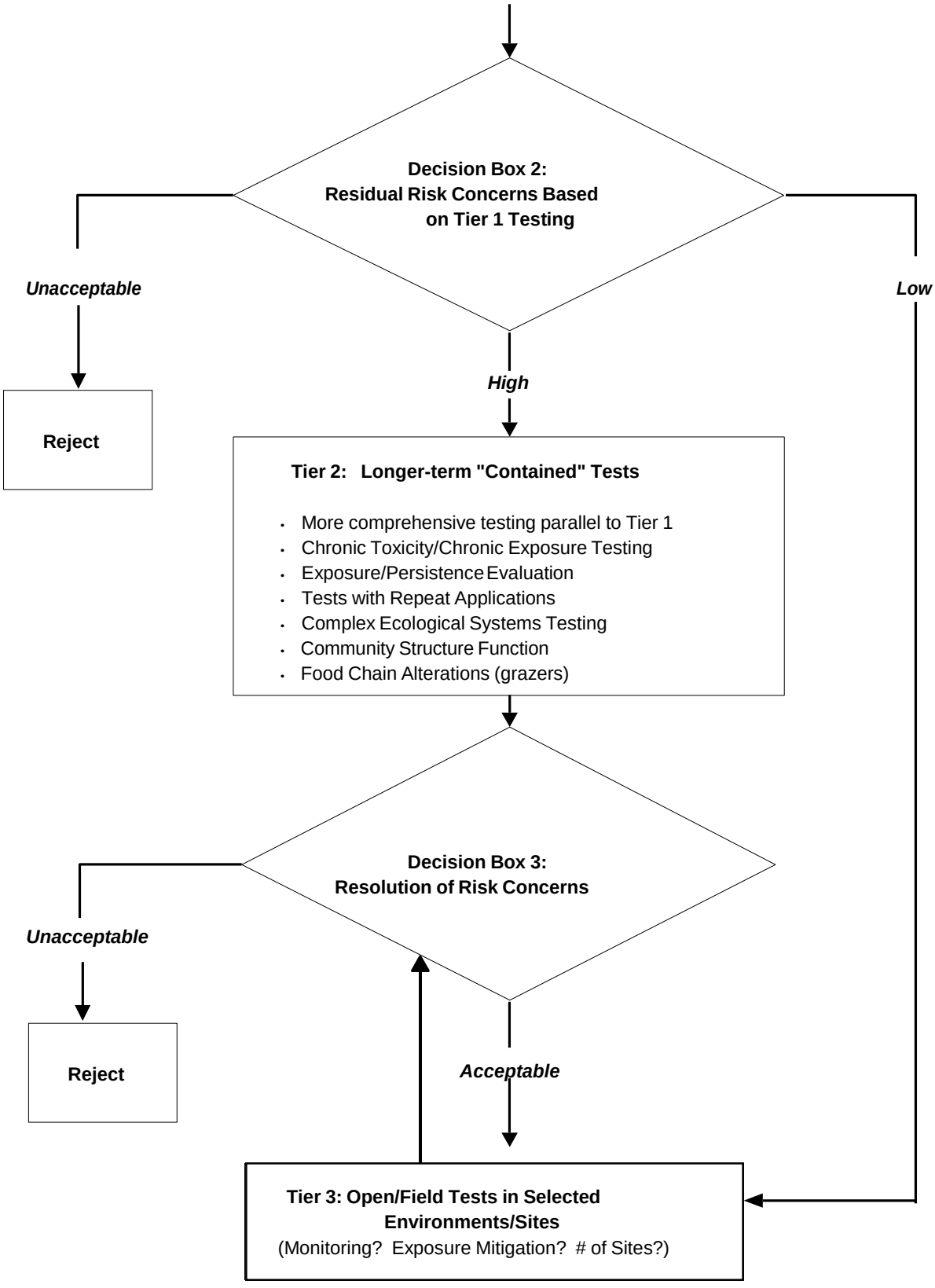


Figure 5 (continued)



Decision Box 1

Decision Box 1 features the exposure and hazard concerns that should be generally addressed when characterizing the risk of a microorganism used in open applications that may be subject to the Toxic Substances Control Act. At this point, the relevant risk concerns must be determined based on the data provided in Tier 0. Both exposure and hazard components are evaluated, and the risk concerns are determined according to the risk formula: Risk = Hazard X Exposure. Either the exposure must be of low concern or the hazard must be of low concern for a product to proceed to Tier 3 field testing. If any one of the exposure or hazard components is of high concern, the product must undergo appropriate testing of these components in Tier 2. Some members of the group believed that it cannot be assumed that microbial exposure is ever zero or low enough to be of low concern, because the organism may drop to below detection limits and then may or may not grow back at a later time. It was also suggested that the assessor may want to consider whether the risk formula and probability assessment are appropriate for determining the ecological risk associated with uncontained applications of microorganisms.

There are four exposure components in Decision Box 1. They include persistence of the organism and the novel DNA, dispersal of the organism, proliferation, and whether repeated applications occur. Persistence may be derived from growth curves of the organism, most likely in microcosms simulating the recipient environment, and the trigger for concern may be when the population maintains a stable equilibrium instead of dying off. If the inserted DNA encodes for some characteristic of concern, the risk of transfer occurring should be explored by evaluating the persistence of the novel DNA as well.

There are three hazard components in Decision Box 1: toxicity, pathogenicity, and other ecological effects. The toxicity of substrate intermediates and other organism-specific metabolites are considered. The toxicity of these chemicals may be estimated with an analysis of quantitative structure activity relationship (QSAR) if data do not exist at this stage. Other ecological effects are also evaluated at this point, including limitation of nutrient availability (nitrogen and phosphorus), effects on primary productivity, carbon mineralization, and community diversity.

If there are no concerns, the product may proceed directly to Tier 3 field testing. The assessment must consider each type of environment to which the organism may be released. If concerns are raised about the risk resulting from the exposure and hazard assessment endpoints/information listed in the Decision Box 1 in **Figure 5**, testing is required to address these concerns. Alternatively, the submitter can provide information regarding the mitigation of exposure. New information regarding hazard and exposure that is generated from testing or provided by the submitter is evaluated in Tier 1. The hazard components may remain a gray area, however, and should be carefully considered on a case-by-case basis.

Tier 1

Tier 1 involves direct short-term screening-level testing to address only the concerns about hazard or exposure raised in Tier 0. It was suggested by some group members that the testing performed in this tier be screening-level tests (i.e., maximum hazard dose), with the assumption that in open systems, exposure is always high; however, other group members argued that exposure is not always high, e.g., with the release of *Rhizobium*, and exposure endpoints should still be considered and tested at this Tier, as the results may mitigate risk. If there are residual concerns, Tier 2 testing is required.

Decision Box 2

If residual risk concerns still exist based upon the results of the Tier 1 testing, Tier 2 testing is triggered. There may also be issues of duration inherent to the open uses of microorganisms that may trigger chronic or more complex testing in Tier 2. The residual Tier 1 concerns may also be low enough to allow the product to undergo Tier 3 field tests, or the risks may be unacceptable, and the product may be rejected at this point.

Tier 2

Tier 2 involves longer-term, comprehensive and/or complex testing that parallels the testing performed in Tier 1. The studies that may be performed at this stage include

tests to determine chronic toxicity, complex ecological systems testing, and evaluations of persistence. The more complex ecological testing should determine more specifically the effects on community structure and function, e.g., food chain alterations or effects on grazers. Testing may be performed in stressed as well as in unstressed environments, as suggested by Group C/D. The tests performed in Tier 2 should feature more realistic exposure or dose regimens as opposed to the screening level or maximum hazard type of dosing used in Tier 1 tests.

Decision Box 3

A final resolution of risk concerns occurs in Decision Box 3, and the product is either rejected, if the concerns are considered unacceptable, or approved for Tier 3 testing if the risks are considered acceptable.

Tier 3

Tier 3 tests consist of open or limited field tests in selected environments. These tests are often performed by the applicant to assess the efficacy of the microorganism. The tests may or may not include monitoring and/or mitigation of exposure, depending on the resolution of risk concerns identified in Decision Box 3.

Evaluating the Scheme

The group evaluated the scheme developed for uncontained technologies by applying the scheme to determine the ecological risks associated with microbially enhanced oil recovery (MEOR) technology. Only naturally occurring organisms have been used in MEOR field applications, and although the possibility has been explored, GEMs are not expected to be used for this application in the short-term. The commercial products are biosurfactant-producing bacteria that are used for treating wax fouling (i.e., for dewaxing of the well bore). Often this application is considered to be semi-contained because a pressure gradient develops with the removal of oil that forces the flow of water from adjacent aquifers into the well. Exposure is also mitigated by biocide controls and corrosion protection. However, environmental exposure still depends on site-specific characteristics. Hazards that may be associated with the

organism include surfactant production, gas production, acid production, and possible mobilization of toxic substances in the environment. The consensus of the group was that the proposed tier testing approach contains the screens and questions needed to assess the potential hazards of microbial use in this scenario.

The group also tested the scheme with a bioremediation scenario involving an oil spill. Commercially-available, naturally-occurring microorganisms were used to remediate the Alaska shoreline and open coastal environment which had been a healthy ecosystem before the "acute" contamination from the Exxon "Valdez" oil spill. Because of the high exposure to aquatic and terrestrial wildlife, pathogenicity and toxicity of byproducts were a concern. Nutrients were also added during the application, and the impact of each of these added nutrients would also need to be evaluated in the scheme. Microcosm studies on community structure were performed. Again, the proposed tier testing scheme appeared to ask the appropriate questions for determining the potential hazards associated with this scenario. It was noted that problems may arise in cases where a question or step asked for in the tier testing scheme may not be able to be reasonably answered.

CONCLUSIONS

From the workshop group efforts, conclusions could be drawn from discussions on technology-based hazard and exposure identification, ecological effects and endpoints for exposure and fate, microbial pathogenicity and toxicity, and for tier testing schemes developed for contained/closed technologies, semi-contained technologies, and open or uncontained technologies.

Hazard and exposure identification are both technology-specific and dependent upon the type(s) of containment (i.e., contained/closed, semi-contained, or uncontained) for each application. Concerns are centered on the type and concentrations of microorganisms and chemical products/by-products that might potentially be released. In general, the uncontained processes are expected to have the greatest potential for causing adverse effects to the environment, due to widespread dispersal of the introduced organism and/or the potential transfer of introduced genes, notably from

GEMs, to indigenous microorganisms. However, hazards associated with these technological processes are considered to be low. In some instances, the only potential hazards from these technological applications are from by-products (e.g., oil recovery) not necessarily unique to the use of a GEM. For other processes (e.g., nitrogen fixation or biomining) the impact of the microorganism itself is the major concern. In general, the hazard potential for all technologies are expected to be low provided the applications are properly conducted. Industrial processes are expected to occur predominantly in closed systems which use well-defined, process-specific measures to ensure protection against accidental release. Although potential exposures from closed and semi-contained systems are either rare and/or low, ecological exposures from the uncontained systems can be extensive (e.g., *Rhizobium*-coated seeds). Hence, concern with uncontained systems centers on survival and persistence of the introduced microorganism(s) if any hazards of the microorganism exist.

Ecological effects and the endpoints for exposure and fate must focus on ecological stability. Although it is important to assess the potential risk/impact of an introduced microbe on ecological stability, there is a deficit of knowledge on how the impact might be expressed. Effects on primary productivity, nutrient cycling, and community structure and diversity are deemed of importance. Survival of the introduced organism is also a major concern, especially if the introduced organism displaces its indigenous parent.

On the subject of microbial pathogenicity and toxicity, it was concluded that lists of potential pathogens in genera of concern would be useful, but are not available. Accurate identification, to the species level was considered critical in assessing potential pathogenicity of toxicity of the microorganism. It was also generally concluded that no one specific phenotypic or genotypic characteristic can be used to predict pathogenicity, but the ability to colonize and sustain a presence would be important. Plant pathogenicity traits identified were production of cell-degenerative enzymes, toxins, growth factors, extracellular polysaccharide, ice nucleation ability, and ability to grow in or on the host.

Finally, ecological tier testing schemes were developed for closed, semi-contained, and open biotechnology applications. The three schemes developed, during

the workshop, (closed, semi-contained, or uncontained), have common elements. Tier 0 is a preliminary screen, containing pre-test information on taxonomic identification of the organism(s), proposed use, and site characterization. Tier 1 contains initial exposure and hazard assessment components. Tier 2 addresses additional questions about exposure and hazards from Tier 1 open system if necessary and, Tier 3 contains open or limited field tests in the selected environment. The scheme was evaluated by application to uncontained systems such as a microbial-enhanced oil recovery (MEOR) technology scenario and a bioremediation scenario involving an oil spill. It was concluded that the proposed tier testing schemes developed in this workshop contain the screens and questions needed to assess the potential hazards and exposures associated with these technological uses of microorganisms, with the caveat that problems can arise where a question or step in the scheme cannot be reasonably answered given our current limitations in methodology and knowledge of microbial ecology.

REFERENCES

U.S. EPA. 1993. Issue Paper: Development of Ecological Tier Testing Schemes for Microbial Biotechnology Applications. United States Environmental Protection Agency, Office of Pollution Prevention and Toxics, Washington, D.C.

APPENDIX A: WORKSHOP AGENDA

DEVELOPMENT OF ECOLOGICAL TIER TESTING SCHEMES FOR MICROBIAL BIOTECHNOLOGY APPLICATIONS

January 11-13, 1994 at the Rosslyn Westpark Hotel in Arlington, VA
Sponsored by U.S. Environmental Protection Agency and Environment Canada

Tuesday, January 11, 1994 - ROSSLYN BALLROOM

8:00 - 8:15 am Registration
8:15 - 8:30 am Welcome and Introductory Remarks
8:30 - 10:15 am Plenary Session:

- TSCA Coverage of GEMs & Use Areas - Ellie Clark
- CEPA Coverage - Terry McIntrye
- Overview of Tier Testing:
  - Microbial Pest Control Agents - Subdivision M - Bill Schneider
  - OPPT Chemical Tier Testing Scheme - Jerry Smrchek
  - Bioremediation Workshop Testing Scheme - Phil Sayre
  - OPPT Draft Ecological Testing Scheme - Gwen McClung
- Charge for the Day

10:15 - 10:30 am Break

10:30 - 12:00 noon Specialized Subgroups:
(A) Hazard & Exposure Identification
(B) Hazard & Exposure Identification
(C&D) Ecological Effects Endpoints and Exposure/Fate Endpoints
(E) Microbial Pathogenicity/Toxicity
(F) Strawman Tier Testing Scheme

12:00 - 1:15 pm Lunch

1:15 - 3:00 pm Specialized Subgroups (continued)

3:00 - 3:15 pm Break

3:15 - 5:00 pm Specialized Subgroups (continued)

5:00 pm Adjourn

Wednesday, January 12, 1994 - SHENANDOAH SUITES A & B

8:00 - 10:15 am — Plenary Session:
- Presentation of Strawman Scheme (Group F)
- Presentation of Hazards and Exposure (Group A)
- Presentation of Hazards and Exposure (Group B)
- Presentation of Effects Endpoints and Exposure/Fate Endpoints (Groups C&D)
- Presentation of Pathogenicity/Toxicity Testing (Group E)
- Charge for the Day

10:15 - 10:30 am — Break

10:30 - 12:00 noon — Breakout Sessions: Integration of Hazard and Fate Components into Strawman Scheme
(I) Contained Systems
(II) Semi-contained Uses
(III) Open Releases

12:00 - 1:15 pm — Lunch

1:15 - 3:00 pm — Breakout Sessions (Continued)

3:00 - 3:15 pm — Break

3:15 - 5:00 pm — Breakout Session (Continued)

5:00 — Adjourn

Thursday, January 13, 1994 - SHENANDOAH SUITES A & B

8:00 - 9:30 am Plenary Session: Review of Tier Testing Schemes Developed on Day 2

- Presentation of Tier Testing Schemes:
  (I) Contained Systems
  (II) Semi-contained Uses
  (III) Open Releases
- Charge for the Day

9:30 - 9:45 am Break

9:45 - 11:45 noon Breakout Sessions:

(I) Contained Systems
(II) Semi-contained Uses
(III) Open Releases

11:45 - 1:00 pm Lunch

1:00 - 2:45 pm Breakout Sessions (Continued)

2:45 - 3:00 pm Break

3:00 - 3:30 pm Plenary Session: Summary/Follow-up

3:30 Adjourn

APPENDIX B: DISCUSSION GROUP QUESTIONS

TUESDAY JANUARY 11th - SPECIALIZED SUBGROUPS

Group A - Technology-based Hazard and Exposure Identification

Application Areas to be Discussed:

Bioremediation
Biomining/Mineral Leaching
Coal Transformations
Desulfurization of Petroleum
Oil Recovery

For all of the above application areas, and for each technology or process falling within that category (e.g. for bioremediation - bioreactors, land farming, composting, in situ groundwater treatment, oil spills, etc.) **identify all unique potential hazard and exposure scenarios associated with that technology/process.** In order to accomplish that objective, questions such as the following should be addressed.

1. What microorganisms (both naturally-occurring and genetically engineered) are currently used or may be used in the future with these technologies?

2. What traits resulting from genetic manipulations are of interest with that technology?

3. What numbers (or biomass) of microorganisms are applied at any specific time?

4. With what frequency are the microorganisms applied?

5. What levels of containment are associated with the technology?

6. What inactivation procedures, if any, are used before disposal or emptying of the vessels or tanks in closed system technologies? Are these inactivation procedures effective against bacterial endospores or fungal spores?

7. How large are the sites used in these technologies (e.g., acreage involved, aquifer, vessel size, etc.)?

8. What environmental media would be exposed (e.g., terrestrial, freshwater, marine, or combinations of the above)?

9. What environments/ecosystems are involved with these technologies (e.g., cropland, forests, deserts, subsurface soils, sediments, freshwater, marine)?

10. By what means are microorganisms released from the site of application or from the closed vessels?

11. By what means is there a potential for dissemination of the microorganisms away from the site of use or release (e.g., aerosols, vertical or horizontal movement of the microorganisms in soil or sediment, etc.)?

12. What are the by-products of the technologies/processes (e.g., metals, sulfuric acid, cyanide, etc.)?

13. What are the by-products of the technologies/processes formed specifically through the use of microorganisms (e.g., toxic metabolites with bioremediation, acid or surfactant production with microbially enhanced oil recovery, etc.)?

14. For each of the technologies under consideration, are there unique potential hazards associated with the use of either naturally-occurring or genetically modified microorganisms (e.g., carbon cycle disruption with ligninases)?

15. Are there any effects of the microorganisms on nontarget organisms?

TUESDAY, JANUARY 11th

Group B - Technology-based Hazard and Exposure Identification

Application Areas to be Discussed:

Nitrogen Fixation
Municipal Waste Treatment
Fuel Production
Biomass Conversion
Closed System Fermentation for enzyme or
specialty chemical production

For all of the above application areas, and for each technology or process falling within that category (e.g. for biomass conversion - ethanol production, acetone/butanol production, methane production, oil production, composting of agricultural wastes) **identify all unique potential hazard and exposure scenarios associated with that technology/process.** In order to accomplish that objective, questions such as the following should be addressed.

1. What microorganisms (both naturally-occurring and genetically engineered) are currently used or may be used in the future with these technologies?

2. What traits resulting from genetic manipulations are of interest with that technology?

3. What numbers (or biomass) of microorganisms are applied at any specific time?

4. With what frequency are the microorganisms applied?

5. What levels of containment are associated with the technology?

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13. What are the by-products of the technologies/processes formed specifically through the use of microorganisms (e.g., toxic metabolites with bioremediation, acids or surfactants production with microbially enhanced oil recovery, etc.)?

14. For each of the technologies under consideration, are there unique potential hazards associated with the use of either naturally-occurring or genetically modified microorganisms (e.g., carbon cycle disruption with ligninases)?

15. Are there any effects of the microorganisms on nontarget organisms (e.g., nodulation of legumes within or outside the cross-inoculation group)?

TUESDAY, JANUARY 11th

Group C & D - Ecological Effects and Exposure/Fate Endpoints

These groups are to identify endpoints of ecological significance, regardless of which technology would lead to an examination of that endpoint.

The technologies under consideration include:

bioremediation
biomining/mineral leaching
coal transformations
desulfurization of petroleum
oil recovery
nitrogen fixation
municipal waste treatment
fuel production
biomass conversion
closed system fermentation for specialty chemicals

Ecological Effects

Participants will identify **ecologically significant** endpoints in both terrestrial and aquatic ecosystems that should be included in the tier testing schemes to be developed on day 2 and 3 of the workshop. These endpoints for effects should not include pathogenicity or toxicity of the microorganisms. There is a separate specialized breakout group, Group E, which is addressing these effect endpoints. To accomplish identification of ecological effects testing needs, the following questions may be useful.

1. What are the important ecological effects endpoints for terrestrial, freshwater, and marine environments with the introduction of microorganisms used in these various technologies under discussion in this workshop? Examples of effects endpoints that may warrant consideration include:

   - microbial community structure
   - disruption of nutrient cycling
   - soil respiration
   - algal photosynthesis
   - soil enzyme activities
   - alteration in species diversity of plant or animal communities
   - predator/prey interactions
   - trophic interactions
   - energy flow

(Many of the ecosystem structure and function endpoints listed above are general terms. Please elaborate and be as specific as possible.)

2. Which of these ecological effects endpoints should have the highest priority for testing of adverse impacts resulting from the introduction of microorganisms to the environment?

3. Using experience from natural observation or microcosm testing, what readily observable **phenotypic traits** are predictive or indicative of the likely behavior of microorganisms in soils, sediments, surface waters, aquifers, or in or on plants and animals?

3.a. Which of these can be linked to specific environmental effects that can be associated with general environmental effects (e.g. acidification of environmental media, reduction of available oxygen, etc.)?

4. What chemical and physical information on properties of the **environment** receiving the microorganism is *really* useful (and what is not) for predicting effects of bacteria and fungi?

Exposure/Fate Endpoints - This group will identify ecologically significant exposure and fate endpoints in both terrestrial and aquatic ecosystems that may be appropriate for any of the technologies to be discussed in this workshop (e.g. survival, proliferation, competition, gene transfer, etc.). Also, the use of models for significant endpoints will be discussed. For some of the technologies under consideration in this workshop, the need for testing both on-site and off-site should be specified.

1. At what population density and for what length of time does the persistence of an introduced microorganism constitute survival of the microorganism?

2. Is it appropriate to consider the number of individual propagules, biomass or both, especially in regard to fungi?

3. Should viability be defined as capacity to proliferate in culture media, or should it be defined as number of metabolically active propagules or size of metabolically active biomass?

4. From knowledge of their behavior in nature or in microcosms, what phenotypic traits or characteristics of bacteria or fungi are known to be important for survival, multiplication, dispersal, and gene transfer of bacteria and fungi in soils, sediments, surface waters, aquifers, and in or on plants or animals?

5. Is it possible to predict which microorganisms will proliferate and to what extent in soils, sediments, surface waters, aquifers, and in or on plants and animals? If the answer is yes, what are the appropriate predictors? If the answer is no, what approach is needed to establish those predictors?

6. What chemical and physical information on properties of the environment receiving the microorganism is *really* useful (and what is not) for predicting survival, multiplication, and gene transfer of bacteria and fungi?

7. What constitutes a valid test of microbial competitiveness? Is there a concern with the competitive ability between a GEM and its parent, or between a GEM and the indigenous populations, or both?

8. Is it important for risk assessment to establish quantitative relationships between the number or biomass of GEMs and the harm they may do? Does such information exist? Assuming that data on such a relationship do not exist and that a simple quantitative relationship cannot be established, how can exposure and hazard be related in a risk analysis?

9. Are there useful models for proliferation or survival of bacteria and fungi in natural environments? What approaches or parameters are needed to develop such models?

10. Can the existing epidemiological models and models for dispersal of microorganisms be used for application to transport of GEMs? What modifications or additional parameters need to be included, such as rate of loss of viability? What information is needed to develop models for vector or water dispersal of microorganisms?

11. Are there suitable microcosms for evaluating survival, proliferation, dispersal, and effects for the likely uses of GEM in the potentially impacted environments? If suitable microcosms do not exist, what factors need to be considered in designing microcosms suitable for these evaluations?

12. If a microcosm is not appropriate for evaluating survival, proliferation, dispersal for great distances, and effects for particular environments or uses of GEMs, are there suitable mesocosms for these purposes? If suitable mesocosms do not exist, what factors should be considered in designing mesocosms suitable for these evaluations?

13. How should the validity of microcosms or mesocosms as simulants of natural environments be established with specific reference to GEMs?

14. What types of evaluations of survival, proliferation, or dispersal can only be conducted in the field? What restrictions or constraints should be placed on field trials to minimize the likelihood of inadvertent transport of GEMs to sites where they may be a hazard?

TUESDAY, JANUARY 11th

Group E - Microbial Pathogenicity/Toxicity

1. Are there readily available information sources (literature or databases) which list microorganisms known to be frank pathogens to animals (vertebrates or invertebrates), plants, and aquatic organisms? Is the documentation behind these lists extensive or merely anecdotal?

1.a. Are these lists regularly updated?

1.b. Are there similar sources for summaries of microorganisms found to behave as opportunistic pathogens of animals, plant, or aquatic microorganisms?

2. Are the lists of frank pathogens sufficiently inclusive to warrant concluding that microorganisms absent from those lists are not a concern with pathogenicity when released into the environment?

3. Are there lists or databases of microorganisms (bacteria and fungi) that are capable of producing toxins (i.e., endotoxins, exotoxins, and mycotoxins) which affect terrestrial animals (vertebrates and invertebrates), aquatic organisms, or plants?

4. Which microorganisms that may be used for the technologies under consideration in this workshop (such as those listed in the brief technology descriptions in section I.E. of the issue paper) pose potential concerns because of potential pathogenicity or toxicity?

5. Since *Pseudomonas*, *Bacillus*, *Thiobacillus*, and certain other genera are often used in the technologies under consideration in this workshop, are there certain species within these genera which are pathogenic or are there certain species which produce toxins of concern?

6. Are there specific phenotypic and genotypic traits or characteristics of bacteria or fungi that are directly associated with or predictive of pathogenicity to plants, terrestrial animals, and aquatic organisms?

7. Are there other indicators such as certain enzymes, chemicals, or gene sequences produced by microorganisms used in these technologies that may indicate potential pathogenicity or toxicity?

8. What *in vitro* tests can be used to predict pathogenicity to plants, invertebrates, and vertebrates (e.g. cutinases, pectic enzymes, hyaluronidase, collagenase, elastase, gene probes or effects on tissue or cell culture)?

9. What types of research should be conducted to establish additional traits of the sorts mentioned in questions 6 - 8?
10. Under what conditions are mycotoxins produced by fungi used in these technologies of concern after release to the environment? Are mycotoxins typically produced by fungi in the environment, or are mycotoxins produced only under certain controlled conditions?

11. What other microbial toxins are of concern with the use of microorganisms for closed system or environmental releases with these technologies?

12. How commonly are extracellular toxins of microorganisms found to be the mechanisms of disease or injury to plants and animals? Do these toxins affect a broad or a narrow range of plant or animal species? Can a testing scheme be developed based on use of only a few indicator (surrogate) species to predict possible effects of such toxins on a wide range of plants or animals?

13. Assuming that a microorganism acts on a narrow range of hosts or susceptible populations, what basis should be used to establish the individual plant or animal species that are of sufficient economic, aesthetic or ecological importance to be considered in a tier-testing scheme or for the designation of suitable surrogates?

14. Is there any concern in regards to microbial community structure or function with the use of microorganisms that produce bacteriocins, antifungal compounds, or other antimicrobials against other members of the same genus or other genera of microorganisms?

TUESDAY, JANUARY 11th

Group F - Strawman Tier Testing Scheme

- This group is to develop strawman tier testing schemes such as those presented in Section II of the issue paper (pp. 26-28; 40-41; 51-52; 58-61). The draft schemes presented on p.58 and 51 more closely represent the type of strawman scheme needed for the breakout groups to build on the next two days.

- This group must decide how many strawman schemes are necessary for the three breakout sessions based on containment (1) closed/contained systems, (2) semi-contained technologies, and (3) open releases to the environment.

    1. Should there be different schemes for closed systems vs. environmental release since the exposure is quite different?

    2. Is there any utility in having different schemes for semi-contained technologies (those in which there are some provisions to limit dissemination of the microorganism or the processes are on a localized, smaller scale) vs. intentional, large-scale, open environmental release?
    Or is it more reasonable to assume that if the microorganism is introduced in the environment and is capable of disseminating, then there is really no difference between semi-contained and open, intentional releases?

- These strawman schemes should contain the following basic units:

    - preliminary (pre-test) information (e.g., taxonomic identity, construct/genetic modification, laboratory or greenhouse data such as growth properties, etc., site characteristics)

    - exposure

    - hazard

    Designate at which tier(s) exposure or hazard testing should be conducted - whether exposure or hazard should come first, or whether there are various components of each that need to be addressed within each tier

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