Draft Algae Guidance for the Preparation of TSCA Biotechnology Submissions

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I. INTRODUCTION

A. STATUTORY AUTHORITY

The U.S. Environmental Protection Agency (EPA) Office of Pollution Prevention and Toxics (OPPT) implements microorganism regulations under Section 5 of the Toxic Substances Control Act (TSCA) to regulate certain new genetically engineered microorganisms (GEMs). "New" microorganisms are defined as microorganisms formed by the deliberate combination of genetic material that was originally isolated from organisms of different taxonomic genera (also called "intergeneric"). TSCA regulates the manufacture, import, processing, distribution in commerce, use, and disposal of new microorganisms used for commercial purposes. Manufacturers are required to report certain information to EPA 90 days before commencing the manufacture of intergeneric microorganisms that are not listed in the TSCA Inventory of Chemical Substances.

The Frank R. Lautenberg Chemical Safety for the 21st Century Act (an amendment to TSCA, signed on June 22, 2016) did not specifically affect the microorganism regulations under that law (40 CFR 725). However, EPA is now required to make an affirmative finding that addresses whether or not a new substance (including a new microorganism) may present an unreasonable risk of injury to health or the environment, including the risk to a potentially exposed or susceptible subpopulation under the conditions of use of the microorganism. This determination must be made without consideration of costs or other nonrisk factors. Within the applicable review period, EPA will determine the following:

- The microorganism [significant new use] presents an unreasonable risk;
- The information available is insufficient to permit a reasoned evaluation of the health and environmental effects of the microorganism;
- The microorganism [significant new use] may present an unreasonable risk;
- The microorganism [significant new use] may have substantial or significant environmental release or human exposure; or
- The microorganism [significant new use] is not likely to present an unreasonable risk

The regulatory determinations for microorganisms are published at https://www.epa.gov/reviewing-new-chemicals-under-toxic-substances-control-act-tsca/epa-pre-manufacture-notice-review.

EPA reviews the information submitted in a notification, conducts a risk assessment, and determines if the new microorganism may present an unreasonable risk of injury to human health and the environment. Production can begin once EPA makes a determination that the microorganism "is not likely to present an unreasonable risk". Once a notice of commencement of manufacture or import is received, the microorganism will be listed on the TSCA Inventory. For all other determinations, EPA may issue an order or rule to the extent necessary to protect against risks identified.

B. PURPOSE OF THE POINTS TO CONSIDER GUIDANCE DOCUMENT

GEMs subject to EPA oversight are used in a wide variety of applications, including, but not limited to, fuel production, biomass conversion, waste treatment, biofertilizers, bioremediation, biomining, biosensors, and production of enzymes or chemicals. EPA uses the data and information pertaining to a new GEM, its manufacturing process, and its intended use to conduct a risk assessment. To assist companies in providing the necessary information, a guidance document entitled, "*Points to Consider in the Preparation of TSCA Biotechnology Submissions for Microorganisms*," (hereafter referred to as the Points to Consider document) was developed in the 1980s and made available to the public. The Points to Consider was last revised in 1997 with the promulgation of the EPA's Biotechnology Rule, Microbial Products of Biotechnology; Final Regulation Under the Toxic Substances Control Act (https://www.gpo.gov/fdsys/pkg/FR-1997-04-11/pdf/97-8669.pdf).

The 1997 Points to Consider is a guidance document designed to ensure adequate data and information accompany the submission of a required notification to EPA. The required notification is fulfilled by the submission of either 1) a Microbial Commercial Activity Notice (MCAN), or 2) a TSCA Experimental Release Application (TERA), as appropriate. Thorough MCAN/TERA submissions are essential so EPA may perform an efficient and effective review of the new microorganism. TSCA requires, for notifications under Section 5, that submitters provide "…*any test data in the possession or control of the person giving such notice which are related to the effect of any manufacture, processing, distribution in commerce, use, or disposal of such substance … on health or the environment, and a description of any other data concerning the environmental and health effects of such substance*," which are known or reasonably ascertainable. This is a broad mandate and can be difficult for a potential submitter to interpret, especially as it relates to microorganisms subject to regulations under TSCA. Thus, EPA has developed guidance explicitly designed to assist those who must submit notices to EPA for biotechnology applications (MCANs/TERAs).

This guidance has been issued in the form of a set of data and information needs relevant to biotechnological risk assessment. Suggested methods for acquisition and presentation of these data and information are included so both the submitter and EPA personnel may view them from the same perspective. The list of specific information needs is not intended to be applicable to all types of biotechnology products and uses. Rather, it can be seen as a menu from which potential submitters may select those points that are relevant to their GEM. Many submitters who have used this guidance have also found that the order of information presented in the guidance helps them organize their data and information for their notices.

C. UPDATING THE POINTS TO CONSIDER DOCUMENT

The initial Points to Consider guidance document was issued approximately a decade prior to the promulgation of formal rules governing receipt of notifications for biotechnology microorganisms and products under TSCA. This was a time during which EPA's policy under the Coordinated Framework for Regulation of Biotechnology (1986) was in effect, but formal rules were still under development. Voluntary submission of notices under the existing regulations for chemical substances was encouraged for biotechnology applications, and many such Pre-Manufacturing Notices (PMNs) were received. When the final TSCA Biotechnology Rule was issued in 1997, a revised Points to Consider document was issued at that time that explained the new submission types for microorganisms and the data and information needs associated with each. The many changes in biotechnology products and production technologies in recent years is the impetus to update the existing guidance. This specific guidance for genetically engineered (GE) microalgae and cyanobacteria, hereafter referred to as algae, serves as a supplement to the existing Points to Consider document to accommodate the needs of producers from a rapidly developing algae industry sector.

D. RATIONAL FOR FOCUS ON ALGAE

Within the last decade, there has been significant progress in development of GE algae that would be subject to TSCA oversight. Several such cases have been the subject of Section 5 notifications and reviews by EPA. Potential submitters have interacted with EPA via a pre-notice communication system set up for developers of products subject to TSCA. Many of these submitters have requested that EPA's guidance be updated to reflect the nuances of their algal-related technologies. EPA has listened to these and other similar requests and agreed that inclusion of algae production-specific information be included in a revision of its Points to Consider. However, updates to other subjects in the Points to Consider document are also pending and a complete revision may take some time. Therefore, given the pressing need currently expressed by the regulated community, EPA has chosen to develop this "Draft Algae Guidance for the Preparation of TSCA Biotechnology Submissions", hereafter referred to as "Draft Algae Guidance" separate from a wholly updated Points to Consider document.

E. 2015 EPA WORKSHOP FOR PUBLIC INPUT ON CONSIDERATIONS FOR RISK ASSESSMENT OF GENETICALLY ENGINEERED ALGAE

Algae producers are not the only stakeholders with a desire for understanding EPA's need for data and information to enable robust reviews of biotechnology submissions. The public also needs to be confident that EPA is using appropriate data and information resources during the course of risk assessments.

By issuing this "Draft Algae Guidance", EPA will be describing the essence of its review process. This transparency demonstrates EPA's risk assessments are based on sound science.

To increase transparency, on September 30, 2015, EPA's OPPT hosted a public workshop entitled, "Workshop for Public Comment on Considerations for Risk Assessment of Genetically Engineered Algae" (https://projects.erg.com/conferences/oppt/workshophome.htm). At this meeting EPA solicited input from the regulated community and the public regarding the data and information needs EPA thought applicable for algal biotechnology submissions. Comments received during the workshop and in the associated docket, as well as input from other scientific and stakeholder sources, have been incorporated into the Considerations for Risk Assessment of Genetically Engineered Algae to create this updated "Draft Algae Guidance" document.

F. ORGANIZATION OF THIS DRAFT ALGAE GUIDANCE

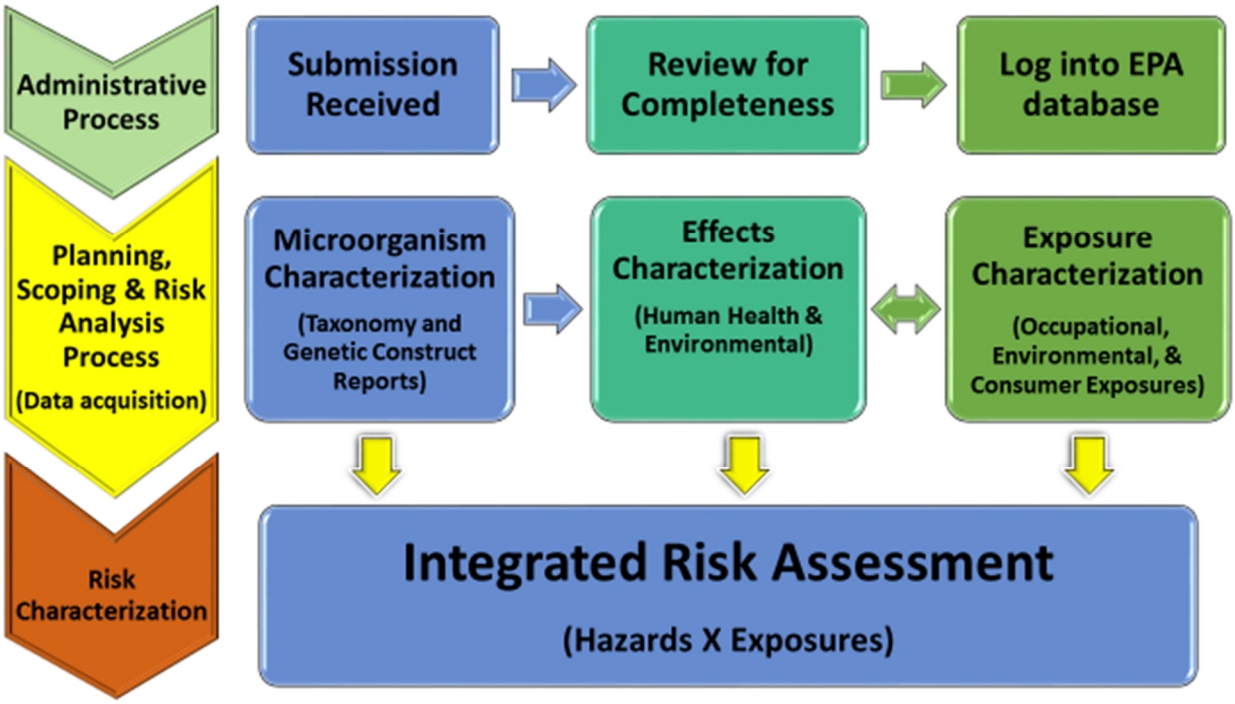
Section II of this document discusses EPA's risk assessment process for GEMs. Section III presents the data and information needed from submitters for EPA to conduct a risk assessment. Appendix A gives an overview of various manufacturing processes for algal biofuels or bioproducts.

II. EPA RISK ASSESSMENT PROCESS

EPA's internal processing of a microbial submission and its risk assessment process are depicted in the following diagram (Figure 1). The first step of the risk assessment process involves the administrative processing of the submission as shown in the first row. The second step as shown in the second row is the characterization of the microorganism, characterization of human health and ecological hazards, and characterization of exposures. The third step as shown in the third row is the merging of hazards and exposures into an integrated risk assessment.

EPA conducts a risk assessment on the TSCA subject microorganism under the paradigm that Risk = Hazard × Exposure. There are various, separate reports that contribute to the risk assessment including: (1) a Microbial Identification Report that verifies of the taxonomy of the TSCA subject microorganism, (2) a Genetic Construction Report that analyzes the construction of the TSCA subject microorganism, (3) a Construct Hazard Analysis that evaluates any potential hazards associated with the genetic modifications and the potential for horizontal gene transfer, (4) a Human Health Hazard Assessment that evaluates the potential pathogenicity, toxicity, and immunological effects of the TSCA subject microorganism, including the effects on a potentially exposed or susceptible subpopulation under the conditions of use, (5) an Ecological Hazard Assessment that evaluates the potential pathogenicity/toxicity of the TSCA subject microorganism to terrestrial and aquatic animal species and to plants and its potential interactions in

Figure 1. Overview of EPA's Risk Assessment Process for MCANs.



the environment, (6) an Engineering Report that assesses conditions of use, worker exposure, and microbial releases to the environment through manufacturing or during field applications, and (7) an Exposure Assessment that evaluates the potential for survival and dissemination of the microorganism in the environment and exposures to the general human population and consumers, including susceptible subpopulations.

The proposed conditions of use of the microorganism is first considered. However, of key importance in the amended TSCA is the need to identify all foreseen future uses of the microorganism. Thus, consideration will also be given to the future use of the microorganism in a different manner than that proposed in the submission.

Under TSCA, there is no specified list of information and/or data elements for a microorganism. Rather submitters must provide to EPA all relevant data and information in their possession or reasonably ascertainable. These data must be sufficient to enable EPA to conduct a robust risk assessment. The Points to Consider document provides guidance to the submitter as to the type of data and information needed by EPA to conduct a scientifically credible risk assessment. The key element to the risk assessment is the amount of data and information that EPA is supplied with or can obtain concerning the TSCA subject microorganism.

III. INFORMATION NEEDS FOR RISK ASSESSMENT

A. RECIPIENT MICROORGANISM CHARACTERIZATION

Determining the identification of a microorganism is usually the first step of a regulatory risk assessment. Microbial identity is essential to the basic characterization of the microorganism. Many of the traits of the recipient microorganism will be considered in assessing the potential for the GE algae to cause adverse effects in humans and/or the environment.

1. Taxonomy

a. General

1) Usually the bulk of the genetic information in any TSCA subject microorganism is derived from the recipient microorganism. It is therefore likely that any added features from a donor organism will be insufficient to warrant a different species name for the TSCA subject microorganism than that of the recipient microorganism.

2) Modern classification schemes for microorganisms rely on nucleic acid analyses (e.g., 16S/18S rRNA genes) to a great extent and, for many taxa, phylogenetic analysis is the primary means of identification. Combining gene sequencing technology with traditional phenotypic methods generally provides a better approach to unique identification than using single methods in isolation.

3) Information substantiating the taxonomy of the recipient microorganism:
The submission should explain the taxonomic approaches chosen for identification. There are two means of supplying substantiating information: (1) a letter from a standard culture collection establishing an organism's identification, or (2) data/analyses used by the submitter or its agent in establishing the identity of the recipient microorganism.

b. Specific Issues

1). Cyanobacteria:
The international prokaryotic systematics community has adopted a naming convention based on the existence of bacterial names on the “Approved List” (1980), or on subsequent lists of validly published names as found in current issues of the International Journal of Systematic and Evolutionary Microbiology. A list of current valid names, including preferred synonyms, may be found at http://www.dsmz.de/bacterial-diversity/prokaryotic-nomenclature-up-to-date.html or at http://www.bacterio.net.

This system does not, however, adequately cover cyanobacteria, where only a few genera appear on the approved list. For now, cyanobacterial systematists

often yield to Botanical Code (Melbourne Code or later) names for these microorganisms. Some may use a taxonomic system found in the National Library of Medicine's NCBI Taxonomy Browser (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?name=cyanobacteria). EPA recommends that the submitter contact the Agency for the most current guidance on naming of those genera/species of cyanobacteria that do not appear on the Approved List.

2). Algae:
Naming conventions have not been codified for other microorganisms using approved names like they have for bacteria and viruses. The Botanical Code (Melbourne Code or later) may be cited for algae (http://www.iapt-taxon.org/nomen/main.php. EPA recommends that the submitter contact the Agency for the most current guidance on accepted taxonomy for algae.

2. General Description and Characterization

A description of the source from which the recipient microorganism was originally isolated (e.g., freshwater lake, estuarine bay, ocean, soil) is important in understanding the nature and ecology of the recipient microorganism. Provide details from which the recipient microorganism was obtained, if applicable, and the following:

c. Gram reaction
d. Specific growth rate (under various temperatures and light conditions)
e. Natural growth forms or patterns (e.g., unicellular, colonies/coenobia, chains, filamentous, mats)
f. Photosynthetic ability
g. Pigments
h. Nitrogen fixation ability
i. pH range and optimum
j. Temperature range and optimum for growth
k. Illumination conditions optimal for growth (intensity, photoperiod)
l. Salinity tolerance (e.g., marine water, freshwater, brackish water, euryhaline)
m. Habitat (e.g., soils, fresh or marine waters or sediment, wastewater, desert soils)
n. Position in water body or water column (e.g., planktonic, benthic, periphytic)
o. Prevalence/distribution in the environment
p. Dormancy structures/strategies (e.g., spores, cysts, viable but nonculturable [VBNC] state)
q. Reproductive methods
r. Importance in aquatic food web/trophic interactions
s. Alga composition (protein, carbohydrate, oil content, lipid yields, % ash-free dry weight (AFDW), specific fatty acids produced)
t. Strain selection considerations

B. GE ALGA CHARACTERIZATION

This section is intended to provide information to EPA that is useful in identifying the taxonomy of the TSCA subject microorganism and the taxonomy of the donor organisms. Although EPA focuses its risk assessment on the intergeneric nucleic acids, all introduced nucleic acid sequences (both intra- and intergeneric sequences) need to be identified. Other manipulations such as deletions, mutagenesis, directed evolution, etc. should also be described. This allows EPA to fully compare the TSCA subject microorganism to the recipient microorganism in order to assess any altered characteristics of the TSCA subject microorganism.

1. Taxonomy of the TSCA Subject Microorganism

If genetic manipulation is so extensive that a TSCA subject microorganism might be more appropriately assigned to a different taxon than the recipient microorganism, the submitter should provide support for the designation of the TSCA subject microorganism equivalent to that which was provided for the designation of the recipient microorganism (Section III.A.I.a.).

Likewise, if the TSCA subject microorganism is wholly, or significantly synthetic, it may not sufficiently resemble any existing species such that a 'recipient microorganism' can be determined. In those cases, identification of all contributors to the genome of the TCSA subject microorganism should be described to the extent possible, along with full descriptions of synthetic sequences that may be relevant to taxonomic assignment.

2. Taxonomy of the Donor Organisms/Synthetic Sequences

Taxonomic characterization of donor microorganisms which contribute intergeneric nucleic acids to the TSCA subject microorganism, or provide intrageneric nucleic acids that may affect the expression or stability/transfer of the intergeneric sequences is needed. Characterization could include identification of a genus, species, and strain designation for each donor organism.

C. GENETIC MODIFICATIONS

1. Construction of the TSCA Subject Microorganism

This section is where the methods and source organisms used to produce the TSCA subject microorganism are best described. A preferred approach consists of a flow diagram(s) with explanatory text. The diagrams and text should describe the names, functions, and sources of donor organisms, recipient strain, and vector nucleic acids which have been manipulated to produce the TSCA subject microorganism.

a. A brief summary of the construction strategy or genome editing techniques should be presented. The summary should indicate why the genetic manipulations were done and their effect(s) relative to the recipient microorganism.

b. Final recipient strain characterization; examples of useful information:

1) Prior modifications (deletions, additions)
2) Presence of plasmids and their ability to promote mobility/transfer, or affect the expression, of the introduced genetic material
3) Gene sequences and whole genomes where known
4) Stability of gene integration
5) Location of endogenous gene(s) homologous to the introduced nucleic acid sequences that could promote mobility/transfer of the introduced genetic material
6) Characterization of the insertion site for the introduced genetic material
7) Use of antibiotic resistance marker genes
8) Characterization of gene silencing/RNAi technology employed and description of gene(s) that are downregulated
9) Stability of gene silencing
10) Potential for transfer of RNAi to non-target organisms

c. As many circular plasmid/vector maps of intermediate constructs as necessary to clearly show genetic manipulations and gene modification. A linear portion of a plasmid representing only the changes is adequate for plasmids which have been illustrated in their entirety earlier in the diagram. These intermediate construct illustrations should be sufficiently detailed to trace and verify the origins of intergeneric genetic material shown in the final construct illustration.

d. Sizes of important gene fragments retained and lost, sequences altered, and addition and/or deletion of restriction sites.

e. Methods for isolating and identifying the nucleic acid sequences used to modify the recipient microorganism.

f. Catalog references for commercial systems used such as special recipient strains, plasmids, cosmids, etc.

g. If sequences are chemically synthesized, give the accession number for the sequence on which it is based. State whether or not the sequence was codon optimized for the recipient microorganism.

2. Final Genetic Construct

It is recommended to provide an illustration of the final genetic construct which is in the TSCA subject microorganism. The legend which accompanies the final genetic construct illustration should focus on the intergeneric genetic material, and

intrageneric nucleic acid sequences that could affect expression or genetic transfer of the intergeneric genetic material. Introduced intrageneric structural genes, promoters, leaders, repressors, antibiotic resistance markers, transposons or transposon fragments, other gene fragments, and cloning sites (which affect the expression, stability, or mobility of the intergeneric genetic material) should be identified.

D. POTENTIAL HUMAN HEALTH EFFECTS OF THE GE ALGA

Potential health effects of the GE alga on workers conducting fermentations, harvesting, and applying or manipulating the microorganisms in the field, on the general population, and on consumers are assessed by EPA. This section identifies information which is helpful in assessing both the potential for the TSCA subject microorganism to cause pathogenicity, toxicity, or allergenicity to humans, including the effects to a potentially exposed or susceptible subpopulations under the conditions of use, as well as other aspects of the manufacturing/production processes that may result in adverse human health effects.

1. Pathogenicity to Humans

a. Infectivity (e.g., chlorellosis, protothecosis)
b. Production of, or presence of genes encoding virulence factors (e.g., adhesins, invasins, exotoxins, capsules)

2. Toxin Production

a. Major cyanotoxins or phycotoxins (e.g., microcystins, nodularins, anatoxin-a, anatoxin-a(S), cylindrospermopsins, saxitoxins, lyngbyatoxin-a, brevetoxins, ciguatoxins, azaspiracids, yessotoxins, palytoxins, etc.) produced and the presence in the genome of any genes known to encode these toxins
b. Lesser cyanotoxins or phycotoxins (e.g., aplysiatoxins, endotoxin A/lipopolysaccharides, domoic acid) produced and presence in the genome of genes known to encode these toxins
c. Secondary metabolites (e.g., β-methylamino-L-alanine (BMAA), 2,4-diaminobutyric acid (DAB)) produced
d. Route of exposure of toxins or secondary metabolites (e.g., dermal absorption, inhalation, ingestion)
e. Pathway(s) for toxin formation (e.g., polyketide synthetase, non-ribosomal peptide synthetase)
f. Genetic regulation of toxin production

3. Immunological Effects of the GE Alga or its Products

a. Allergy (IgE-related hypersensitivity)
b. Changes in cell wall or outer membrane proteins
c. Asthma (occupational) (e.g., hypersensitivity to *Chlorella* powder)
d. Skin irritation/rashes
e. Eye irritation

4. Harmful Volatile Compounds

a. Methane derivatives
b. Volatile fatty acids

5. Presence/Prevention of Microbial Pathogens (Contaminants) in Ponds

E. POTENTIAL ECOLOGICAL EFFECTS OF THE GE ALGA

Potential effects of the GE alga on organisms other than humans in the environment either through releases from fermentation facilities or photobioreactors, or from use in the environment such as in open ponds need to be assessed by EPA. In order to examine these potential effects, it is helpful to provide a review of the ability of the TSCA subject microorganism (or the recipient) to cause diseases, or be associated with disease in organisms other than humans. Also important are the interactions of the TSCA subject microorganism with other microorganisms, its role in biogeochemical cycles, and potential effects on aquatic food webs if the TSCA subject microorganism survives in the environment.

1. Toxicity to Animals

a. Cyanotoxins, phycotoxins, secondary metabolites produced and the presence of genes encoding toxin production (see Human Health section III.D.2.)
b. Toxicity of triglycerides/lipids or bioproducts produced
c. Route of exposure (ingestion, dermal absorption, inhalation)
d. Host range (e.g., toxicity to aquatic species, terrestrial animals, etc.)
e. Environmental triggers of toxin production

2. Pathogenicity to Animals

a. Chlorellosis, protothecosis of mammals
b. Other adverse effects on aquatic species (e.g., invasion & erosion of carapace of American horseshoe crab by green algae)

3. Pathogenicity to Plants

(e.g., *Cephaleuros* red rust, a green alga pathogenic to red algae)

4. Propensity for Bloom Formation

a. Flotation (e.g., presence of genes encoding gas vesicles [*gvp* genes])
b. Increased buoyancy resulting from increased lipid content
c. Mat formation (tendency to aggregate)

5. Potential Effects on Primary Productivity

a. Changes in photosynthesis rate (carbon fixation/Calvin cycle, oxygen generation)
b. Changes in antenna complexes/photosynthetic pigments/light capture, especially if changes endow the ability to live at different depth

6. Potential Effects on Other Biogeochemical Cycles

a. Nitrogen cycle – changes in nitrogen fixation of cyanobacteria
b. Phosphorus cycle
c. Sulfur cycle (e.g., generation of sulfhydryl compounds during mat decay)

7. Potential Effects on Microbial Food Web/Trophic Level Changes

a. Changes in palatability
b. Trophic transfer of fatty acids
c. Effects of varied fatty acids diets on zooplankton growth and reproduction
d. Secreted triglycerides/lipids vs. intracellularly retained

8. Potential Effects on Other Ecologically Important Relationships

a. Lichens – both cyanobacteria and green algae
b. Desert soil crusts
c. Specific food source for specific organisms

9. Potential Effects on the Surrounding Environment

a. Increased pH of water due to bicarbonate uptake by cyanobacteria or green algae
b. Metal availability – affected by redox
c. Phosphorus availability – precipitation as iron phosphates at low pH
d. Suspended solids

10. Bioaccumulation of Metals in the Microorganism, in Liquid and Solid Wastes, and in the Final Product from Flue Gas or Other Sources

F. FATE OF THE GE ALGA

The potential for exposure of the TSCA subject microorganisms to other organisms in the environment needs to be assessed. The transfer or transport mechanisms that may result in exposures need to be understood. This section identifies information which is helpful in assessing both the potential for the TSCA subject organism to survive, persist, and disseminate from the site of release or use, and out-compete indigenous microorganisms.

1. Survival in Potential Aquatic and Terrestrial Receiving Environments

a. Survival relative to recipient microorganism
b. Ability to overwinter
c. Desiccation tolerance features
d. Known pathogens or grazers

2. Competition with Indigenous Species

a. Ability to out-compete/displace indigenous species
b. Selective advantage(s) imparted to the TSCA subject microorganism
c. Effects on microbial community structure

G. INFORMATION APPLICABLE TO SMALL-SCALE FIELD TESTS

This section provides guidance for information needed regarding small-scale field tests or small-scale environmental introductions.

1. Objectives of the Tests
2. Nature of the Site (e.g., size, elevation, slope, proximity to water bodies, prevailing winds)
3. Field Test Design
4. Application Methods
5. Monitoring Endpoints and Procedures for Isolating/Detecting the TSCA Subject Microorganism
6. Sampling Procedures
7. Measurement Methodologies and Quality Assurance/Quality Control
8. On-Site Containment Practices
9. Termination and Mitigation Procedures
10. Record Keeping & Reporting Test Results

H. MANUFACTURING PROCESS DESCRIPTIONS & PRODUCTION VOLUMES

This section provides guidance on submitting information on the use, the production volume of microorganisms, and the manufacturing process descriptions for various algal production platforms. It also identifies by-products from microbial production.

1. Heterotrophic Fermentation

a. Use and annual production/processing volume for each of the first 3 years
b. Number and location of sites
c. Process descriptions
d. Number of batches or operating days per site per year
e. Fermentor volume (for batch processes) or daily colony-forming units (CFUs) for continuous processes
f. Inactivation methods
g. Concentration (CFUs/ml) in each process stream
h. Cleaning of fermentors
i. Disposal of spent biomass/use of spent biomass
j. By-products

2. Photobioreactors (PBRs)

a. Number/volume of PBRs
b. PBR design and arrangement of PBRs at the site
c. Size/volume/cell density - and whether batch or continuous culture
d. Number of harvests per year and time between harvests (batches)
e. Amount of microorganisms harvested - production alga and contaminants/pathogens
f. Harvesting technologies
g. PBR material (thickness, mil, tensile strength, etc.)

h. Integrity/weatherability of materials used in PBRs
i. Longevity/replacement time of PBRs
j. Junctions of inlet and outlet tubing and potential for leaks
k. Biofuel or bioproduct produced (may need Pre-Manufacture Notice)
l. Amount and source of CO_2 and potential contaminants (e.g., metals in flue gases)
m. Amount and sources of supplied nutrients
n. Water source (e.g., freshwater, salt water, wastewater, recycled water)
o. Water characteristics (e.g., N and P concentrations, presence of heavy metals, arsenic, other contaminants)
p. Characteristics of algogenic organic material (AOM)
q. Distance to surface and underground water sources
r. Use of antimicrobials or pesticides in media
s. Inactivation methods
t. Releases of wastewater
u. Disposal of spent biomass/use of spent biomass
v. Cleaning of PBRs for re-use or disposal of PBRs
w. By-products

3. Open/Raceway Pond Construction and Design

a. Number/volume of ponds
b. Pond size/dimensions/surface area
c. Size/volume/cell density - and whether batch or continuous culture
d. Number of harvests per year and time between harvests (batches)
e. Amount of microorganisms harvested - production alga and contaminants/pathogens
f. Harvesting technologies
g. Pond construction materials
h. Use of liners
i. Use of berms
j. Circulation system and rate and potential for bioaerosols
k. Biofuel or bioproduct produced (may need Pre-Manufacture Notice)
l. Amount and source of CO_2 and potential contaminants
m. Amount and sources of supplied nutrients
n. Water source (e.g., fresh water, salt water, wastewater, recycled water)
o. Water characteristics (e.g., N and P concentrations, presence of heavy metals, arsenic, other contaminants
p. Characteristics of algogenic organic material (AOM)
q. Distance to surface and underground water sources
r. Use of antimicrobials or pesticides in media
s. Inactivation methods
t. Releases of wastewater
u. Disposal of spent biomass/use of spent biomass
v. Disinfection of ponds between batches
w. By-products

4. Additional Siting Information for Commercial-Scale PBRs and Open Ponds

a. Location
b. Climate
c. Precipitation - annual total and seasonal distribution
d. Prevailing wind direction(s) and seasonal changes if applicable
e. Frequency and severity of storms
f. Potential for catastrophic weather events (e.g., hurricanes, tornados, location in flood zone)

I. EXPOSURES OF THE GE ALGA

The potential for exposure of the microorganism to workers, the general population (including susceptible individuals), the environment, and consumers needs to be assessed. This section provides guidance on submission of information for occupational, environmental, and likely consumer exposures to the TSCA subject microorganism or its products.

1. Occupational Exposure

a. Processes that Influence Potential Workers Exposure
   1) Worker activity (e.g., sampling, cleaning)
   2) Number of workers involved per shift per activity
   3) Number of shifts per day
   4) Exposure days per year
   5) Exposure duration
   6) Personal protective equipment (PPE) used
b. Inhalation Exposure (CFUs)
c. Dermal Exposure (CFUs)

2. Environmental and General Population Exposures

a. Environmental Releases from Commercial Facilities to Various Media
   1) Releases to Air
   2) Releases to Water
   3) Releases to Land
b. Inactivation Methods and Pollution Control Technologies
   1) Efficiency of inactivation methods (supported by experimental data)
   2) Clean-in-place procedures
   3) Limit of detection for waste stream sampling
c. Environmental Exposures
   1) Proximity to surface water bodies
   2) Proximity to sensitive ecosystems (e.g., coral reefs, estuaries, mangroves)
   3) Proximity to migratory bird routes
d. General Population Exposures (including susceptible populations)
   1) Inhalation Exposure
   2) Drinking Water Exposure
   3) Proximity to the general human population, urban centers, schools, etc.
   4) Proximity to aquaculture farms, agricultural crops/poultry/livestock

3. Consumer Exposures

(including effects on a potentially exposed or susceptible subpopulation under the conditions of use)

J. MONITORING OF THE GE ALGA

1. Monitoring Endpoints and Procedures

a. Endpoints that will be evaluated in samples that are collected
b. Techniques used to detect the microorganism in test samples
    1) use of watermarks/biocodes/sequences or other strategies that can be used for monitoring
    2) use of negative and positive controls
c. Sensitivity and reliability of the method and the actual limit of detection
d. Efficiency of recovery for each of the sampling techniques if applicable
e. Frequency and type of observations to be made

2. Sampling Procedures

a. How, where and when samples will be taken for each monitoring endpoint
b. Standard procedures for preserving, processing, and analyzing samples
c. Methods of measurement, equipment, precision bias, accuracy and repeatability of the methods
d. Methods for the statistical analysis of field data

K. TERMINATION AND EMERGENCY CONTAINMENT PROCEDURES

a. Type of unexpected effects that would necessitate the emergency termination of a field test or environmental use
b. Emergency termination procedures to be followed if adverse environmental
a. effects are observed
c. Handling of spills or leaks

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00049C; FRL–5577–2] RIN 2070-AB61. Available at: https://www.federalregister.gov/documents/1997/04/11/97-8669/microbial-products-of-biotechnology-final-regulation-under-the-toxic-substances-control-act

APPENDIX A: ALGAE MANUFACTURING PROCESSES

Commercial production of algae for biofuels or bioproducts subject to TSCA oversight is typically accomplished by use of one of the following manufacturing platforms: (1) heterotrophic closed-system fermentation, (2) outdoor photobioreactors (PBRs), or (3) autotrophic open/raceway ponds.

I. Closed-System Fermentors

While microalgae are typically grown autotrophically, a process that requires sunlight or artificial light, some researchers and companies are pursuing an alternative "heterotrophic" fermentation approach. In this approach algae convert sugars into oil and biomass in the dark (ABO, 2015).

A. Process Description

The industrial fermentation process has three main steps: laboratory propagation, fermentation, and recovery.

Laboratory propagation consists of preparing a liquid medium that contains a suspension of the algae and nutrients that are required for growth. An initial culture is prepared by aseptically transferring the microorganisms from vials that have been stored in liquid nitrogen or lyophilized (freeze-dried) to small shake flasks containing sterile growth medium. This transfer typically occurs under a laminar flow hood to prevent culture contamination.

The shake flasks are incubated until the cell density increases to the desired concentration. Then the culture is transferred aseptically to larger flasks, and the cell concentration is again increased. Finally, the culture is transferred to a seed fermentor, which has a typical volume ranging between 1 and 20 percent of the main production fermentor (U.S. EPA, 1997). After growth to the desired cell concentration in the seed fermentor, the fermentation broth is transferred aseptically to the main production fermentor. Production fermentors are typically submerged, deep tank fermentors that have a variety of sealed ports for: sampling, addition of fresh medium, sterile air or oxygen sparging (for aerobic processes), addition of antifoam agents, fermentor off-gas vents (with filters to prevent contamination, as well as potential release, of the GEM), and impellers to facilitate thorough mixing and aeration (U.S. EPA, 1997).

Once the main fermentation is completed, the algae is inactivated or sterilized (killed) for bioproduct recovery. Inactivation processes are very case-specific and may include a combination of the following techniques (U.S. EPA, 1997):

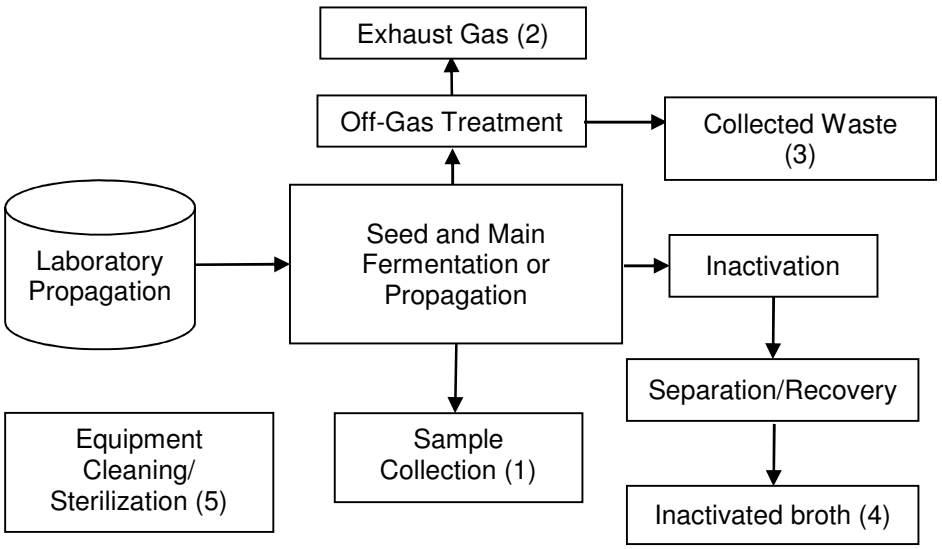
- addition of a germicide or bactericide (e.g., hypochlorite);
- addition of strong acids or bases to achieve an extreme pH;
- cessation of aeration and agitation (to cause oxygen depletion in aerobic processes);

- extreme agitation (to create an extreme shear stress that lyses the cell); and/or
- heat treatment.

A number of techniques are available for harvesting the microalgae. These techniques include, but are not limited to: flocculation centrifugation, filtration, and ultrafiltration (U.S. EPA, 1997, U.S.EPA 2010).

B. Flow Diagram and Potential Release Points

Figure 1 presents a general process flow diagram for industrial fermentation that includes potential release points. Figure 2 presents an example of a closed fermentor.



Environmental Release Points:
1. Sample waste
2. Exhaust gas
3. Collected off-gas system waste
4. Inactivated fermentation broth
5. Equipment cleaning/sterilization waste

Figure 1. Closed Fermentation Process Flow Diagram

Figure 2. Example of Closed-System Fermentors[1]

II. Photobioreactors

Photobioreactors use the same growth mechanism as open/raceway ponds, but differ in that the algae are enclosed in a transparent vessel, which are generally tubular, bag-type or panel designs, and may come in many configurations (ABO, 2011).

A. Process Description

PBRs generally follow the following process (Oilgae, 2010):

1. Water, algae, CO_2 and nutrients are added to a feeding vessel.
2. From the feeding vessel, the flow progresses to the diaphragm pump, which moderates the flow of the algae and CO_2 into the PBR.
3. The PBR promotes biological growth by controlling environmental parameters including light; the PBRs are designed to have light and dark intervals to enhance the growth rate.

[1] https:www.cstindustries.com/uploadedImages/Home/Products/CST_Storage/Vitrium/Aquastore-Ethanol-Fermenter-Tanks-1024.jpg. Used with permission from CST Industries, Inc.

4. After the algae have completed the flow through the PBR, it passes back to the feeding vessel. As it progresses through the hoses, oxygen sensors determine how much oxygen has built up and this oxygen is released in the feeding vessel itself. Optical cell density sensors also determine the harvesting rate.
5. When the algae are ready for harvesting, they pass through a connected filtering system, which collects algae for processing (discussed below), while the remaining algae passes back to the feeding vessel for recirculation.

The extraction process is a two-step process involving the mechanical pressing of the algae. The press bursts the cell walls, thus releasing the algal oil and separating the solids (biomass) from the liquids (oil and water). The second step is the separation of the oil and water via centrifugation. Water recovered from this stage will not contain live algae. However any water suitable for reintroduction into the system will be processed for return to the inoculation tanks (Algae Production Systems, 2009). Figure 3 provides a schematic of the PBR process.

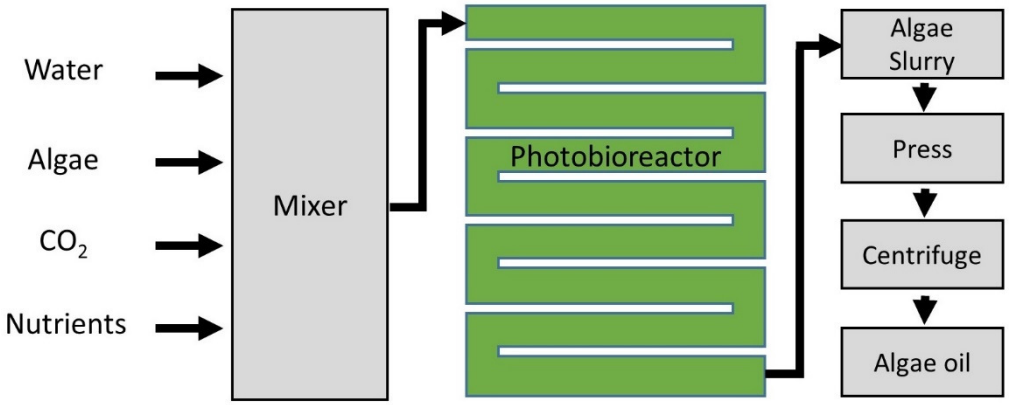


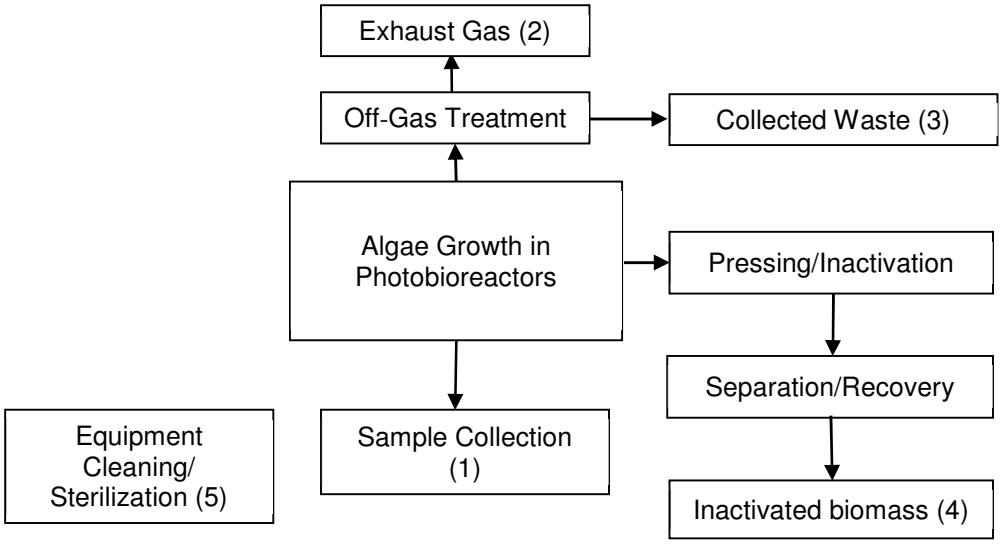
Figure 3. Schematic of Photobioreactor System

PBRs can come in many different setups, including, but not limited to (Oilgae, 2010):

- Tubular Reactors
  - Horizontal
  - Vertical
- Flat panel reactors
- Vertical column reactors
- Bubble column reactors
- Air lift reactors
- Stirred tank photobioreactors
- Immobilized bioreactors

B. Flow Diagram and Potential Release Points

Figure 4 outlines the potential release points from a PBR system, and Figure 5 provides an example of a PBR system.



Environmental Releases:
1. Sample waste
2. Exhaust gas
3. Collected off-gas system waste
4. Inactivated biomass
5. Equipment cleaning/sterilization waste

Figure 4. Potential Release Points from Photobioreactor Systems

Figure 5. Flat Plate Photobioreactors[2]

[2] http://www.nanovoltaics.com/portfolio/photobioreactors. Used with permission from NanoVoltaics, Inc.

III. Open/Raceway Ponds

Open pond systems are the most common system of autotrophic algae cultivation. In the United States, they are used commercially to produce nutritional bioproducts and treat wastewater. Open pond systems use shallow ponds (typically one-foot deep) that range in size from one to several acres. When exposed to sunlight, algae in the ponds use photosynthesis to convert carbon dioxide (CO_2, supplied and atmospheric) into biomass and bio-oils. Raceway ponds resemble a racetrack and often use paddle wheels or other mechanical aeration devices to keep the algae circulating (ABO, 2011).

A. Process Description

In a typical open/raceway pond system, algae are added to the pond with constant mixing and circulation to maintain microalgae growth productivity. Mixing and circulation are provided via paddlewheel systems. Carbon dioxide is bubbled at the bottom of the open ponds via diffuser systems, while nitrogen and phosphorus are added using commercially-produced nitrates and phosphates (OCJ, 2012).

The harvesting method is often a two-stage procedure based on the particular properties of the algae and process requirements. A fraction of the pond culture is harvested daily. Then water is removed to concentrate the algal biomass.

Subsequently, the biomass is processed further, using solvent or mechanical methods to extract lipids/algal oil. Then the lipids/oil is converted into biodiesel, jet fuel, or other oil-based products. The residues can be used for other bioproducts (ABO, 2011; ABO, 2015). Figure 6 shows a schematic of a raceway pond.

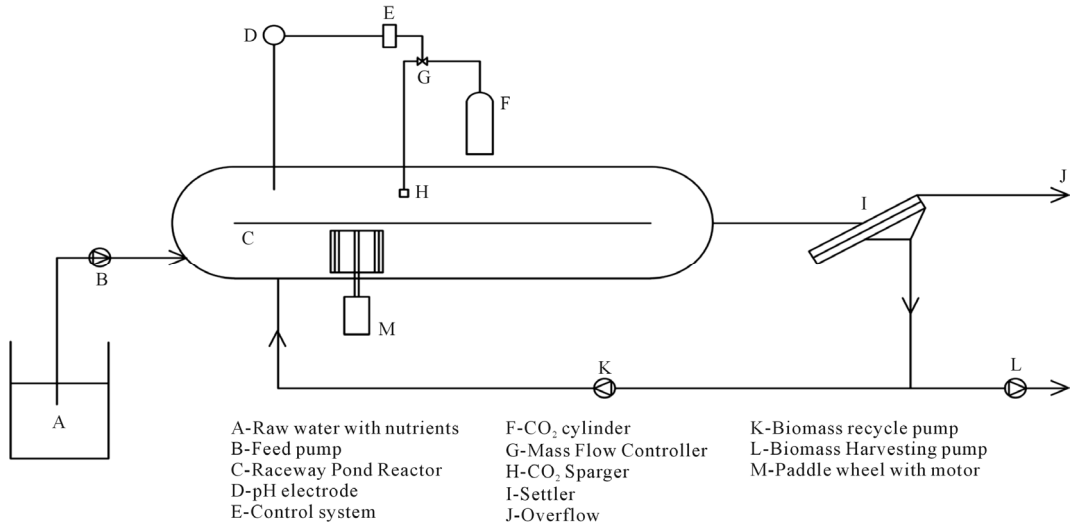
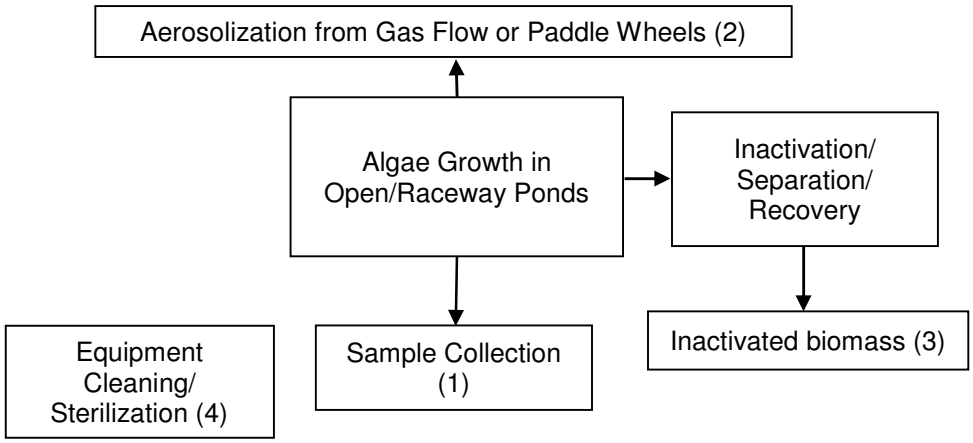


Figure 6. Raceway Pond Schematic[3]

[3] http://file.scirp.org/pdf/AJPS_2015093014342112.pdf. Used with permission.

B. Flow Diagram and Potential Release Points

Figure 7 outlines the flow diagram and potential release points from open/raceway ponds. Figure 8 is an example of a raceway pond.



Environmental Releases:
1. Sample waste
2. Aerosolization from gas flow or paddle wheels
3. Inactivated biomass
4. Equipment cleaning/sterilization waste

Figure 7. Potential Release Points for Open/Raceway Ponds

Figure 8. Raceway Pond (AZCATI, 2014)

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