Algae Supplement to the Guidance Document "Points to Consider in the Preparation of TSCA Biotechnology Submissions for Microorganisms"

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I. INTRODUCTION

A. PURPOSE OF THIS SUPPLEMENT

The initial "Points to Consider in the Preparation of TSCA Biotechnology Submissions for Microorganisms" guidance document, hereafter referred to as the Points to Consider, was issued in the 1980s, approximately a decade prior to the promulgation of formal rules governing Section 5 reporting requirements for biotechnology submissions of intergeneric[1] microorganisms under TSCA. This was a time during which EPA's policy under the 1986 Coordinated Framework for Regulation of Biotechnology[2] 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-Manufacture Notices (PMNs) were received. When the final TSCA Biotechnology Rule[3] was issued in 1997, the Points to Consider document was revised to explain the new submission types for microorganisms and the recommended information and data associated with each. These new submission types include the Microbial Commercial Activity Notice (MCAN) for those microorganisms ready for commercialization, and the TSCA Experimental Release Application (TERA) for those microorganisms still at the research and development stage but intended for environmental introduction.

In recent years new technologies have emerged, both in terms of novel microorganisms not previously thought to be used in TSCA applications and the dramatically different design and manufacturing systems for these microorganisms. In addition, there have been extensive advances in genetic engineering and genome editing techniques. Acknowledging the need to update the Points to Consider for the emerging technologies and recent biotechnological developments, EPA decided to first address the production of genetically engineered (GE) algae for biofuels and bioproducts. The specific recommendations for information that EPA finds useful for GE eukaryotic microalgae and cyanobacteria, hereafter referred to collectively as algae, is provided in this "Algae Supplement" to the existing Points to Consider document.

B. RATIONALE FOR FOCUS ON ALGAE

Within the last decade, there has been significant advancement in the development of GE algae that are, or would be, subject to TSCA oversight. Several such cases have been the subject of Section 5 notifications and reviews by EPA. Since some algae production systems greatly differ from traditional fermentation systems (e.g., outdoor open ponds), EPA thought that algae production-specific information should be included in a revision of its Points to Consider. Given the emerging algal industry for biofuels and bioproducts, EPA developed this "Algae Supplement" to the Points to Consider document to address the use of novel

[1] Intergeneric microorganism means a microorganism that is formed by the deliberate combination of genetic material originally isolated from organisms of different taxonomic genera.
[2] Executive Office of the President. Office of Science and Technology Policy. Coordinated Framework for Regulation of Biotechnology, 51 FR 23302, at 23302-23303 (June 26, 1986) (1986 Coordinated Framework). Available online at: http://www.aphis.usda.gov/brs/fedregister/coordinated_framework.pdf.
[3] 40 C.F.R. Parts 700, 720, 721, 723, and 725 - Microbial Products of Biotechnology; Final Regulation Under the Toxic Substances Control Act; Final Rule. Federal Register / Vol. 62, No. 70 / Friday, April 11, 1997, 17910.

microorganisms using different production systems.

C. DEVELOPMENT OF THIS "ALGAE SUPPLEMENT"

Acknowledging the need to update the Points to Consider for the emerging technologies and recent biotechnological developments, EPA decided to first address the production of genetically engineered (GE) algae for biofuels and bioproducts since in recent years there has been significant advancement in the development of GE algae subject to TSCA oversight.

On September 30, 2015 in Washington, DC, EPA's Office of Pollution Prevention and Toxics (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/2015meeting.htm). At this meeting, EPA solicited input from the regulated community and the public regarding information and data described in the "Considerations for Risk Assessment of Genetically Engineered Algae" (https://projects.erg.com/conferences/oppt/docs/Biotech_Workshop_Report_Final_2015-12-21.pdf, Appendix F) that EPA thought applicable for algal biotechnology submissions. Comments received during the workshop and in the associated docket (EPA-HQ-OPPT-2015-0508), as well as input from other scientific and stakeholder sources, were then incorporated into a second document, "Draft Algae Guidance for the Preparation of TSCA Biotechnology Submissions".

A second public meeting was held on October 27, 2016 in Tempe, AZ to further solicit input from stakeholders and the public (https://projects.erg.com/conferences/oppt/workshophome.htm). Comments received during this meeting, "Public Meeting and Opportunity for Public Comment on EPA's Draft Algae Guidance for the Preparation of TSCA Biotechnology Submissions" (https://projects.erg.com/conferences/oppt/docs/Draft_Algae_Guidance_October2016.pdf), and those received in the associated docket (EPA-HQ-OPPT-2015-0508), were incorporated into this final "Algae Supplement" to the Points to Consider. A companion document, Agency Response to Public Comments on EPA's 'Draft Algae Guidance for the Preparation of TSCA Biotechnology Submissions' that addresses comments received in the docket and at the second public meeting, is also being released.

D. ORGANIZATION OF THIS "ALGAE SUPPLEMENT"

Section II of this "Algae Supplement" presents the data and information from submitters that EPA finds useful for conducting risk assessments of GE algae. EPA/OPPT only has oversight over microorganisms, thus, this document applies to cyanobacteria and eukaryotic microalgae, not to all algae. This Algae Supplement was written broadly to cover both cyanobacteria and eukaryotic microalgae. Not all information elements in the Algae Supplement will apply to all algal strains. Thus, submitters should examine the individual elements and determine which are relevant to their specific algal strain and production platform. Appendix A gives an overview of various manufacturing processes for algal biofuels or bioproducts.

II. INFORMATION USEFUL FOR RISK ASSESSMENT OF A GENETICALLY ENGINEERED ALGA

A. RECIPIENT MICROORGANISM CHARACTERIZATION

The recipient microorganism[4] is the strain into which the intergeneric genetic material is introduced. Determining the identity of the recipient microorganism is usually the first step of a TSCA risk assessment. Microbial identity information [as required at 40 C.F.R. § 725.155(d)] is essential to the basic characterization of the microorganism. Many of the traits of the recipient microorganism will be considered in assessing the potential human health and ecological effects of the subject strain (i.e., the GE alga).

1. Taxonomy

a. General

1) Usually the bulk of the genetic information in any 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 subject microorganism than that of the recipient microorganism.

2) Information substantiating the taxonomy of the recipient microorganism [as required at 40 C.F.R. § 725.155(d)(1)(i)]:
An explanation of the taxonomic approaches chosen for identification is useful for ensuring an accurate identification. There are two means of supplying substantiating information: (1) a letter from a standard culture collection establishing the recipient microorganism's identification, or (2) data/analyses used by the submitter or its agent in establishing the identity of the recipient microorganism.

3) 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 method of identification. Combining gene sequencing technology with traditional phenotypic methods generally provides a better approach to unique identification than using a single method in isolation. A polyphasic approach to taxonomic identification generally enables a more accurate species designation.

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,

[4] Recipient microorganism is the strain into which the intergeneric DNA is introduced and which generally determines the taxonomic designation of the subject microorganism.

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. However, only a few genera of cyanobacteria appear on the approved list since historically cyanobacteria were known as blue-green algae, not prokaryotes. Systematists often yield to the International Code of Nomenclature for Algae, Fungi, and Plants (https://www.iapt-taxon.org/nomen/main.php), (Shenzhen Code, 2018, or a more current version if available) for names of cyanobacteria. 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 prokaryote Approved List.

2) Eukaryotic Algae:
While prokaryotes and viruses have explicit naming conventions and the existence of approved names appearing on lists, for other microorganisms, multiple accepted names are commonplace. The International Code of Nomenclature for Algae, Fungi, and Plants (Shenzhen Code, 2018) is often used as a source for eukaryotic algae (http://www.iapt-taxon.org/nomen/main.php). Many collections use a preferred name for their deposits, but collections may differ on which of several accepted names is preferred. Occasionally collections may use an incorrect designation based on the depositor's evaluation without independent confirmation. When a recipient algal strain is obtained from a collection, it is best for submitters to determine all the synonyms under which a strain might be listed. If they suspect a collection may retain an incorrect species designation for a strain provided to the submitter, it would be useful to EPA if the submitter includes relevant information in their possession that may enable resolution of any naming conflicts. Online services such as Algaebase (www.algaebase.org) can provide assistance for the naming of existing algal species. Sources such as this can provide links to relevant literature and may be cited to confirm the taxonomic designation. For isolates obtained from the environment, it may be more challenging to determine if the strain matches an existing species or is a new species. Most often, for eukaryotic algae, there are morphological components used for taxonomic designations. However, modern systematics often rely on nucleic acid sequence analyses to supplement traditional phenotypic descriptors. This polyphasic approach is generally seen as preferred to reliance on just traditional phenotypic or genotypic approaches. Some strains may be difficult to identify and thus EPA recommends that the submitter contact the Agency for assistance if needed.

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. EPA recommends providing details from which the recipient microorganism was obtained, if applicable, and the following:

a. Gram reaction (applicable to cyanobacteria only)
b. Specific growth rate (under various temperatures and light conditions)

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

B. GE ALGA CHARACTERIZATION

This section is intended to describe information EPA finds useful in identifying the taxonomy of the subject microorganism[5] and the taxonomy of the donor organisms[6] (as required at C.F.R. § 725.12). As previously mentioned, the subject microorganism is the subject of the microbial submission. The donor organisms are those that contribute genetic material to the subject microorganism, or those that contribute genetic material to intermediate microorganisms[7] used to construct the subject microorganism.

Although EPA focuses its risk assessment on the intergeneric nucleic acids, knowledge of all introduced nucleic acid sequences (both intra- and intergeneric sequences) allows the Agency to better assess differences between the subject microorganism and the recipient. Other manipulations such as deletions, mutagenesis, directed evolution, etc. are also important in allowing a full comparison of the subject microorganism to the recipient microorganism such that EPA may assess any altered characteristics of the subject microorganism.

1. Taxonomy of the Subject Microorganism

The principles of taxonomic designation previously addressed for the recipient

[5] Subject microorganism is the subject of the microbial submission.
[6] Donor microorganisms are those which contribute DNA to the subject microorganism, or those which contribute DNA to intermediate microorganisms used to construct the final subject microorganism.
[7] Intermediate microorganisms do not contribute DNA, but may be used to temporarily contain a vector used to construct the subject microorganism, to aid in a triparental mating, etc.

microorganism apply to that for the subject microorganism as well (Sec. II.A.1). While highly unlikely, if genetic manipulations are so extensive that a subject microorganism might be more appropriately assigned to a different taxon than the recipient microorganism, it is recommended that the submitter provide data supporting the new taxonomic designation of the subject microorganism equivalent to that which was provided for the designation of the recipient microorganism (Section II.A.).

Likewise, if through synthetic biology the subject microorganism is entirely or largely composed of synthetic sequences, it may not sufficiently resemble any existing species such that a 'recipient microorganism' name can be given. In those cases, it is recommended that identification of all contributors to the genome of the subject microorganism be described to the extent possible, along with full descriptions of synthetic sequences that may be relevant to taxonomic assignment [as required at 40 C.F.R. § 725.155(d)].

2. Taxonomy of the Donor Organisms/Synthetic Sequences

Taxonomic characterization of donor microorganisms which contribute intergeneric nucleic acids to the subject microorganism or provide intrageneric nucleic acids that may affect the expression, stability, or transfer capabilities of the intergeneric sequences should be given [as provided at 40 C.F.R. § 725.155(d)(2)(iii)]. EPA recommends that characterization include identification of a genus, species, and strain designation for each donor organism.

C. GENETIC MODIFICATIONS

1. Construction of the Subject Microorganism

This section describes the methods and source organisms used to produce the subject microorganism [as required by 40 C.F.R. § 725.155(d)(2)]. A preferred approach consists of a flow diagram(s) with explanatory text. Diagrams and text describing the names, functions, and sources of 1) donor organisms, 2) recipient strain, and 3) vector nucleic acids which have been manipulated to produce the subject microorganism are recommended.

a. A brief summary of the construction strategy or genome editing techniques used is recommended. Information on why the genetic manipulations were done and their effect(s) on the subject microorganism relative to the recipient microorganism is useful to include in the summary.

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, including GenBank® accession numbers

4) Stability of gene integration
5) Restriction sites used
6) Location of endogenous gene(s) homologous to the introduced nucleic acid sequences that could promote mobility/transfer of the introduced genetic material
7) Characterization of the insertion site for the introduced genetic material
8) Use of antibiotic resistance marker genes
9) Characterization of gene silencing/RNAi technology employed and description of gene(s) that are downregulated
10) Stability of gene silencing
11) 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 modifications. For plasmids that have been illustrated in their entirety earlier, a diagram of a linear portion of the plasmid representing only the changes is recommended. EPA would find it helpful for these intermediate construct illustrations to be sufficiently detailed to trace and verify the origins of intergeneric genetic material shown in the final genetic construct[8] 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 recipient strains, plasmids, cosmids, etc.

g. If sequences are chemically synthesized, providing the GenBank® accession number for the sequence on which it is based is recommended. Stating whether or not the sequence was codon-optimized for the recipient microorganism is also recommended.

h. References for pertinent literature

2. Final Genetic Construct

EPA recommends providing an illustration of the final genetic construct which is in the subject microorganism. The final construct is the term that describes the introduced genetic material and includes such sequences as structural genes, vector DNA, and marker genes. It is recommended that the legend which accompanies the final genetic construct illustration focus on the intergeneric genetic material, and intrageneric nucleic acid sequences that could affect expression or genetic transfer of the intergeneric genetic material. EPA finds it useful for introduced intrageneric structural genes, promoters,

[8] Final construct is the term which describes the DNA sequences contributed by the donor microorganisms, and where applicable, the recipient's DNA sequences immediately flanking the inserted donor microorganisms' DNA. The final construct includes such sequences as structural genes (usually genes which encode an enzyme that is of commercial importance), vector DNA, and marker genes.

leaders, repressors, marker genes, transposons or transposon fragments, other gene fragments, and cloning sites (which affect the expression, stability, or mobility of the intergeneric genetic material) and restriction sites used to be identified.

D. POTENTIAL HUMAN HEALTH EFFECTS OF THE GE ALGA

Potential health effects of the GE algal strain on workers (conducting fermentations, harvesting, and applying or manipulating the microorganisms in the field), the general population, and consumers are assessed by EPA. This section identifies information which is used to assess the potential for the subject microorganism to cause pathogenicity, toxicity, or allergenicity to humans, including the effects to potentially exposed and susceptible subpopulations under the conditions of use.

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. Toxicity of triglycerides/lipids or bioproducts produced
e. Relevant route of exposure of toxins or secondary metabolites (e.g., dermal absorption, inhalation, ingestion)
f. Pathway(s) for toxin formation (e.g., polyketide synthetase, non-ribosomal peptide synthetase)
g. Genetic regulation of toxin production

3. Immunological Effects of the GE Alga or its Products

a. Allergy (e.g., 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. Volatile Compounds

a. Methane derivatives
b. Volatile fatty acids

5. **Contaminants in Ponds**
    a. Detection of pathogenic microorganisms
    b. Methods to prevent growth of pathogenic microbial contaminants

E. POTENTIAL ECOLOGICAL EFFECTS OF THE GE ALGA

Potential effects of the subject strain 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, are assessed by EPA. In order to examine these potential effects, it is helpful to provide a review of the ability of the 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 subject microorganism with other microorganisms, its role in biogeochemical cycles, and potential effects on aquatic food webs if the 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 II.D.2.)
    b. Toxicity of triglycerides/lipids or bioproducts produced
    c. Relevant route of exposure (e.g., ingestion, dermal absorption, inhalation)
    d. Host range (e.g., toxicity to aquatic species, terrestrial animals)
    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 and erosion of carapace of American horseshoe crab by green algae)

3. **Pathogenicity/Toxicity to Plants**
    a. e.g., *Cephaleuros* red rust is a genus of green algae pathogenic to red algae
    b. Other adverse effects on plants

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 (i.e., tendency to aggregate)

5. **Potential Effects on Primary Productivity**
    a. Changes in photosynthesis rate (e.g., carbon fixation/Calvin cycle and 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 of the alga to organisms that consume it
   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 from CO_2 Source (e.g., flue gas) and Water Sources**
    a. Metals in algal biomass
    b. Metals in gas, liquid, and solid waste streams
    c. Metals in algal bioproduct(s)

F. FATE OF THE GE ALGA

The transfer or transport mechanisms that may result in exposures of the subject microorganisms to other organisms in the environment is part of EPA's assessment. This section identifies information which is helpful in assessing the potential for the subject microorganism to survive in the environment which affects human and environmental exposures to the microorganism, and thus, the likelihood of potential effects occurring. This section also addresses the ability of an algal strain to out-compete indigenous microorganisms.

1. **Survival in Potential Aquatic and Terrestrial Receiving Environments**
   a. Survival relative to the 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 subject microorganism

c. Effects on microbial community structure

G. INFORMATION APPLICABLE TO SMALL-SCALE FIELD TESTS

This section provides information regarding small-scale field tests or small-scale environmental introductions that EPA recommends be described in a submission.

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 subject microorganism
6. Sampling procedures
7. Measurement of methodologies and verification of quality assurance/quality control
8. On-site containment practices
9. Termination and mitigation procedures
10. Recordkeeping and reporting test results

H. MANUFACTURING PROCESS DESCRIPTION AND PRODUCTION VOLUME

This section provides recommendations on submitting information on the use [as required at 40 C.F.R. § 725.155(g)], the production volume of microorganisms [as required at 40 C.F.R. § 725.155(f)], and the manufacturing process descriptions for various algal production platforms [as required at 40 C.F.R. § 725.155(h)(ii)]. Providing information on the design and materials used for photobioreactor systems and open ponds is also recommended. This section also discusses the recommendations on submitting information on by-products from microbial production [as required at 40 C.F.R. § 725.155(e)]. Also note that Appendix A describes current algae manufacturing processes and identifies potential points of releases into the environment from a variety of algae manufacturing platforms.

1. Heterotrophic Fermentation

a. Uses and annual production/processing volumes for each use for 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/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. Light source (e.g., natural, artificial, hybrid) and radiation measurements
    d. Size/volume/cell density - and whether batch or continuous culture
    e. Number of harvests per year and time between harvests (batches)
    f. Number of microorganisms harvested - production of alga and contaminants/pathogens
    g. Harvesting technologies
    h. PBR material (e.g., thickness, mil, tensile strength)
    i. Integrity/weatherability of materials used in PBRs
    j. Longevity/replacement time of PBRs
    k. Junctions of inlet and outlet tubing and potential for leaks
    l. Biofuel or bioproduct produced (may need Premanufacture Notice for the chemical)
    m. Amount and source of CO_2 and potential contaminants (e.g., metals in flue gases)
    n. Amount and sources of supplied nutrients
    o. Water source (e.g., freshwater, salt water, wastewater, recycled water)
    p. Water characteristics (e.g., N and P concentrations, salinity, temperature, presence of heavy metals, arsenic, other contaminants)
    q. Characteristics of algogenic organic material (AOM)
    r. Distance to surface and underground water sources
    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. Light source (e.g., natural, artificial, hybrid) and radiation measurements
    e. Number of harvests per year and time between harvests (batches)
    f. Number of microorganisms harvested – production of alga and contaminants/pathogens
    g. Harvesting technologies
    h. Pond construction materials
    i. Use of liners
    j. Use of berms
    k. Circulation system and rate and potential for bioaerosols
    l. Biofuel or bioproduct produced (may need Premanufacture Notice for the chemical)
    m. Amount and source of CO_2 and potential contaminants
    n. Amount and sources of supplied nutrients
    o. Water source (e.g., fresh water, salt water, wastewater, recycled water)
    p. Water characteristics (e.g., N and P concentrations, salinity, temperature, presence of heavy metals, arsenic, other contaminants)

q. Characteristics of algogenic organic material (AOM)
r. Distance to surface and underground water sources
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 Site 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. Sunlight - annual total and seasonal distribution
f. Frequency and severity of storms
g. Potential for catastrophic weather events (e.g., hurricanes, tornados, location in flood zone)

I. EXPOSURES TO THE GE ALGA

This section describes the information EPA typically uses for evaluating exposures to potentially exposed subpopulations (e.g., workers), the environment, and the general population (including susceptible subpopulations) [as required at 40 C.F.R. § 725.155(h)(iii)].

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 (hours per shift)
   6) Personal protective equipment (PPE) used

EPA recommends that companies fill out the following table which specifies the type and duration of exposure for their specific manufacturing platform.

Table 1. Occupational Exposure Assessment Information

Worker Activity	PPE/ Engineering Controls	Maximum # of Workers Exposed per Shift	Number of Shifts per Day	Number of Days per Year (days/yr.)	Maximum Activity Duration (hrs./shift)	Monitoring Data, if available (CFUs/day)

b. Inhalation exposure (CFUs/day)
c. Dermal exposure (CFUs/day)

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. In the absence of inactivation efficiency information or experimental data, only a 99% reduction in numbers of viable cells is assumed as a reasonable worst-case estimate.
   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

Consumer exposures including susceptible subpopulations 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 limit of detection in various environmental media
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 effects are observed
c. Handling of spills or leaks

III. REFERENCES

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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) photoautotrophic outdoor photobioreactors (PBRs), or (3) photoautotrophic outdoor open/raceway ponds. The following descriptions of current algae production platforms can serve as a guide for the type of information EPA recommends be included in a submission.

I. Closed-System Fermentors

While microalgae are typically grown photoautotrophically, 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)

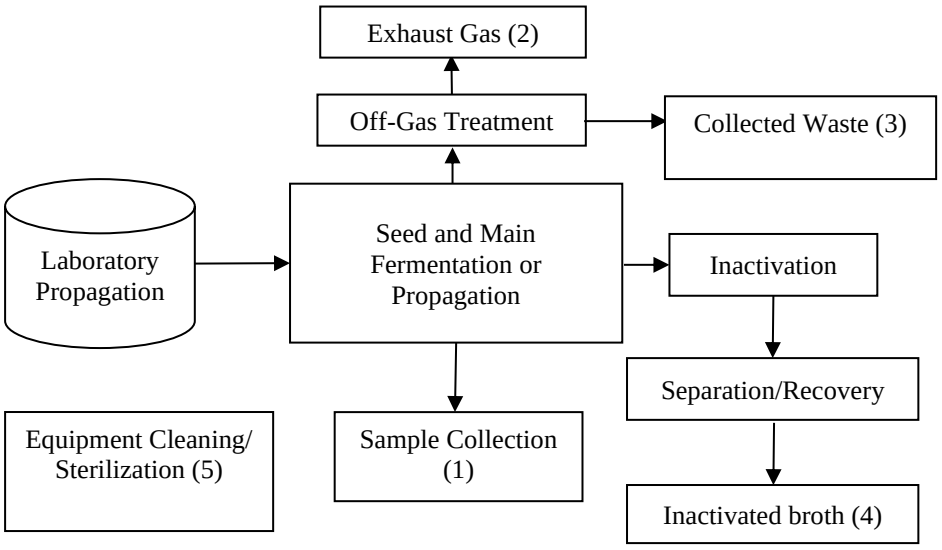
- heat treatment

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

The following diagram (Fig. 1) presents a general process flow diagram for industrial fermentation that includes potential release points from closed-system fermentors.

Figure 1.



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

The following photo (Fig.2) is an example of closed-system fermentors[9].

Figure 2.

II. Photobioreactors (PBRs)

Photobioreactors allow for photoautotrophic growth of algae outdoors where 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

PBR use generally consists of 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 having light and dark intervals to enhance the growth rate.

[9]OSHA Technical Manual, Section IV: Chapter 5, Ethanol Processing, Figure II.12. (https://www.osha.gov/dts/osta/otm/otm_iv/otm_iv_5.html#fig2-12).

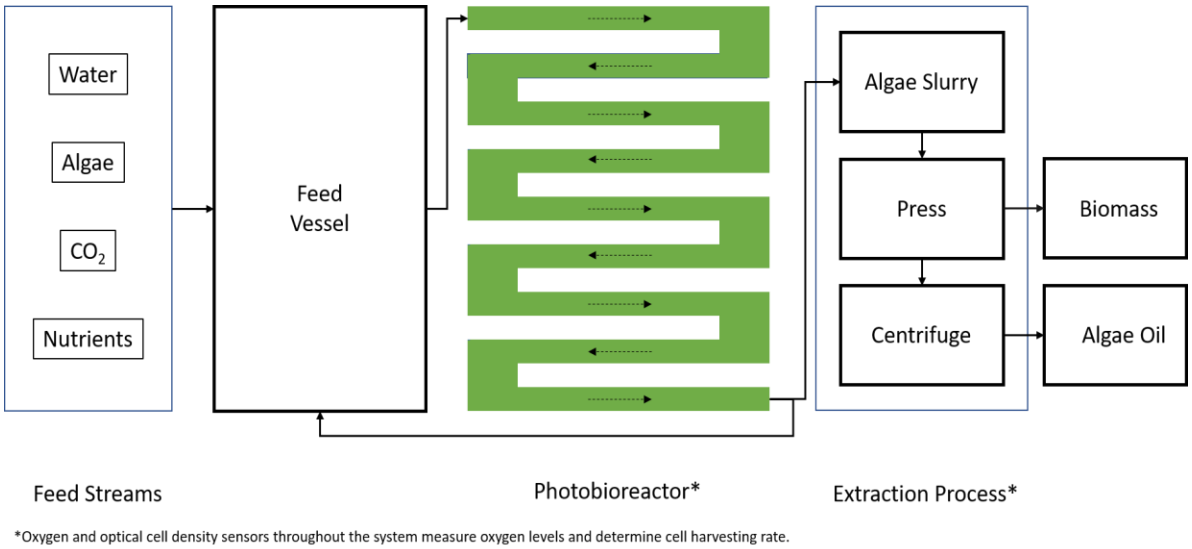
4. After the algae have completed the flow through the PBR, the cells are passed back to the feed vessel. As the cells progress through the hoses, oxygen sensors determine how much oxygen has built up and this oxygen is released in the feeding vessel itself. Optical cell density sensors also determine 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).

The following diagram (Fig. 3) is a schematic of the PBR manufacturing process.

Figure 3.



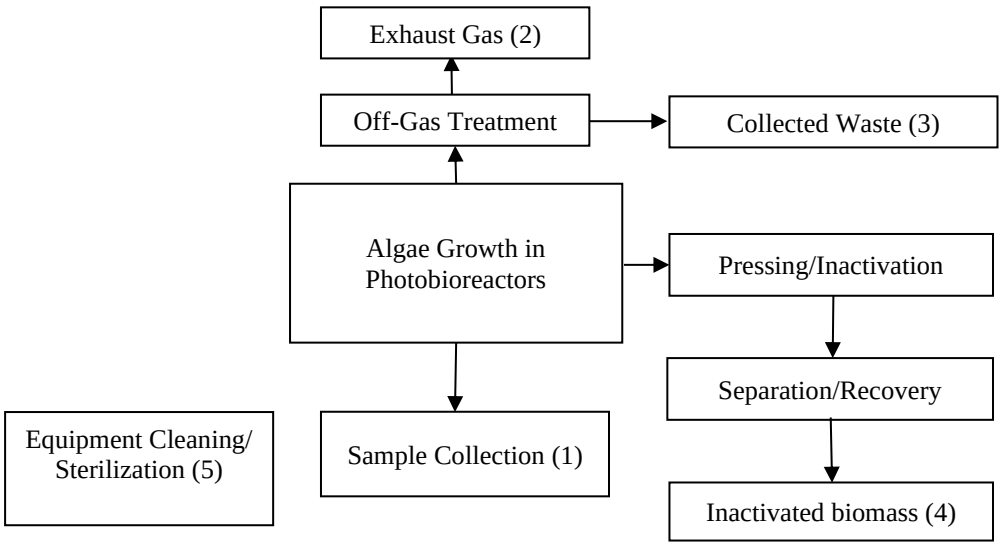
PBRs can come in many different setups, including, but not limited to (Oilgae, 2010):

- 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

The following figure (Fig. 4) presents a general process flow diagram for a PBR system that includes potential release points.

Figure 4.



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

The following photo (Figure 5) is an example of flat panel PBRs[10].

[10] Photo courtesy of the Arizona Center for Algae Technology and Innovation (AzCATI) at Arizona State University.

III. Open/Raceway Ponds

Open pond systems are the most common system of photoautotrophic cultivation of naturally-occurring algae in the United States used 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 algal oils. Raceway ponds resemble a racetrack and often use paddle wheels or other mechanical aeration devices to keep the algae circulating (ABO, 2011).

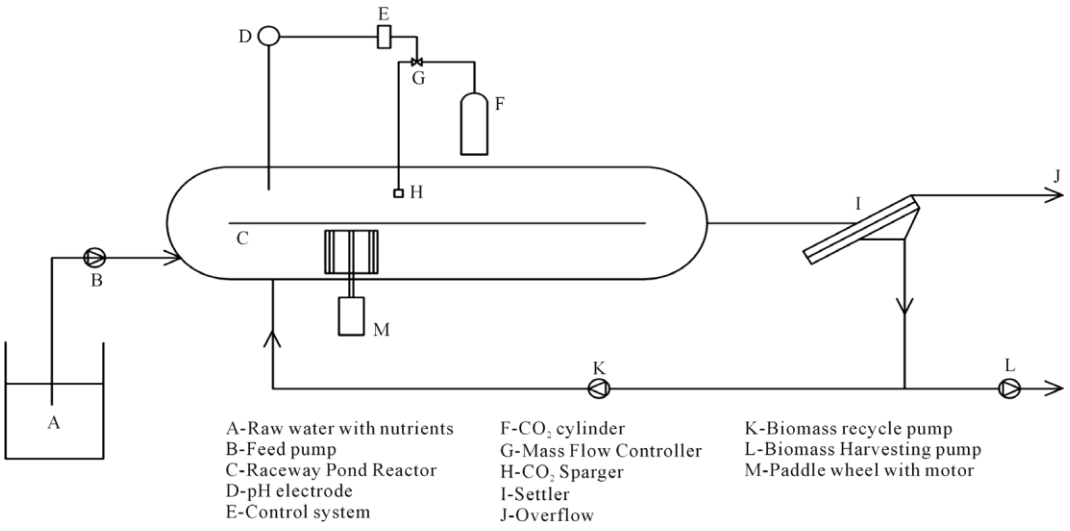
A. Process Description

In a typical open/raceway pond system, algae are added to the pond with constant mixing and circulation to maintain growth productivity. Mixing and circulation are provided via motorized paddle wheels. 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 (Li, 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/oils are converted into biodiesel, jet fuel, or other oil-based products. The residues can be used for other bioproducts (ABO, 2011; ABO, 2015).

The following diagram (Figure 6) is a schematic of a raceway pond[11].

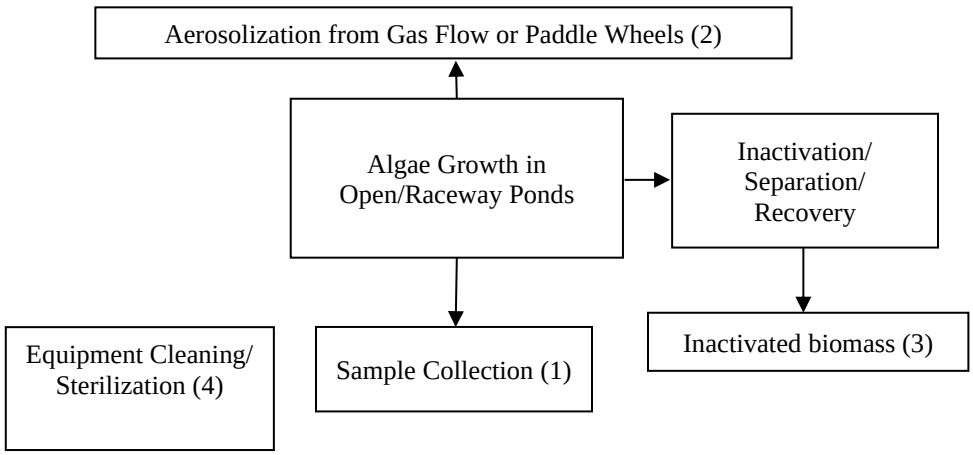


[11] http://file.scirp.org/pdf/AJPS_2015093014342112.pdf. Used with permission.

B. Flow Diagram and Potential Release Points

The following figure (Fig. 7) presents a general flow diagram and for open/raceway ponds that includes potential release points.

Figure 7.



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

The following photo (Fig. 8) is an example of a raceway pond[12].

Figure 8.

[12] Photo courtesy of the Arizona Center for Algae Technology and Innovation (AzCATI) at Arizona State University. Photo taken by Mark Segal, formerly at EPA.

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