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Research Needs for Novel Engineered Microbes & Biopesticides Intended for Open Release into the Environment

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Research Needs for Novel Engineered Microbes & Biopesticides Intended for Open Release into the Environment

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This document has been reviewed in accordance with U.S. Environmental Protection Agency policy and approved for publication.

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List of Acronyms

ARS	Agricultural Research Service
BPPD	Biopesticides and Pollution Prevention Division
BRAG	Biotechnology Risk Assessment Research Grants
CBI	Confidential Business Information
CCTE	Center for Computational Toxicology and Exposure
CEMM	Center for Environmental Measurement and Modeling
CESAR	Center for Environmental Solutions and Emergency Response
CPHEA	Center for Public Health and Environmental Assessment
CSS	Chemical Safety for Sustainability
DOE	Department of Energy
dsRNA	Double-Stranded Ribonucleic Acid
eDNA	Environmental Deoxyribonucleic Acid
EPA	U.S. Environmental Protection Agency
ERDC	U.S. Army Engineer Research and Development Center
FFDCA	Federal Food, Drug, and Cosmetic Act
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
GDP	Gross Domestic Product
GEM	Genetically Engineered Microorganism
HGT	Horizontal Gene Transfer
JGI	Joint Genome Institute
MPCA	Microbial Pest Control Agents
MIT	Massachusetts Institute of Technology
NASEM	National Academies of Sciences, Engineering, and Medicine
ncAAs	Noncanonical Amino Acids
nsAAs	Nonstandard Amino Acids
NCBI	National Center for Biotechnology Information
NCD	New Chemicals Division
NRP	National Research Program
NSF	National Science Foundation
OCSPP	Office of Chemical Safety and Pollution Prevention
OPPT	Office of Pollution Prevention and Toxics
OPP	Office of Pesticide Programs
ORD	Office of Research and Development
ORAU	Oak Ridge Associated Universities
ORISE	Oak Ridge Institute for Science & Education
OSAPE	Office of Science Advisor, Policy and Engagement
PESD	Pacific Ecological Systems Division
PIPs	Plant Incorporated Protectants
R10	U.S. Environmental Protection Agency, Region 10
RIDL	Release of Insects with Dominant Lethality
RNAi	Ribonucleic Acid Interference
STAR	Science to Achieve Results

StRAP	Strategic Research Action Plan
TSCA	Toxic Substances Control Act as amended by the Frank R. Lautenberg Chemical Safety for the 21st Century Act
USACE	U.S. Army Corps of Engineers
USDA	U.S. Department of Agriculture
XNAs	Xenonucleic Acids

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1 Introduction

1.1 Executive Summary

Recent advances in biotechnology and biomanufacturing are positively impacting many sectors of society and the bioeconomy. Updated scientific information and assessment tools are needed to ensure the safety and sustainability of novel products of biotechnology that have unique properties and uncertain risks.[1, 2] In July 2021, seventy-seven individuals (Appendix A) participated in a virtual workshop hosted by the EPA Office of Research and Development to discuss research needs for engineered microbes and biopesticides intended for open release into the environment. Government and external subject matter experts gathered to discuss the needs in terms of empirical experiments, monitoring, models, databases, research capacity, collaborations, and priorities. The workshop sessions (Appendix B) were initiated with plenary talks from experts including awardees from the 2020 EPA Science to Achieve Results (STAR) grants for *Assessment Tools for Biotechnology Products.*[3] Follow-on Breakout Group discussions were focused on four biotech product categories, which were of greatest interest to OCSPP and ORD:

- Engineered Microbes-1; widespread open uses, biofertilizers, ocean bioremediation, etc.
- Engineered Microbes-2; semi-contained uses, algae ponds, biomining, biosensors, wastewater treatment, localized bioremediation, microbial enhanced oil recovery, etc.
- Biopesticides-1; population control/elimination, *Wolbachia**, release of insects with dominant lethality (RIDL), gene drives, etc.
- Biopesticides-2; plant incorporated protectants (PIPs), microbial pest control agents (MPCA), double-stranded ribonucleic acid (dsRNA), biochemicals, etc.

This report on the workshop findings delineates research priorities that are relevant to OCSPP TSCA and FIFRA/FFDCA risk assessments as well as those made by other organizations on emerging products of biotechnology derived through synthetic biology, genome editing and/or metabolic engineering. Overall, the report identifies important biotech research questions facing the Agency and in turn, it can be used to develop a strategy to focus ORD's future research and collaborations in this area.

* *Wolbachia* are bacteria that have been used in biological pest control.

1.2 Background and Goals

Over the last decade, the US bioeconomy has rapidly increased and as of 2016 it was responsible for at least 5.1 percent of the U.S. GDP.[1] The global bioeconomy is projected to become worth $4 trillion to $30 trillion dollars in the future.[2] This explosive growth has been largely driven by advances in genome editing, synthetic biology, and metabolic engineering, which along with other transformational techniques are being used to generate novel products of biotechnology that do not occur in nature. Globally, the application of modern biotechnology is improving the efficiency and sustainability of many sectors of the broader economy including medicine, pharmaceuticals, cosmetics, clothing, food supply, agriculture, production of chemicals, fuels, pesticides, environmental remediation, and defense. There are various research efforts underway to engineer microbial, animal, plant, and synthetic/cell-free products. While some are being designed for use under containment, other products are planned for open release into the environment. When these products have unique properties and uncertain risks, they pose regulatory challenges. This is true for many novel biotechnology products such as engineered microbes and biopesticides. Safety questions remain regarding

potential impacts to human health and the environment from these products. To realize the potential benefits of novel biotechnology while avoiding unintended consequences, updated scientific information and assessment tools will be required. This agrees with recent recommendations for additional resources to support risk assessments and regulatory decision-making for emerging biotech products.[2, 4, 5]

The goals of the virtual workshop were to identify specific research needs related to the uncertain risks of biotech products to human health and the environment based on input from EPA Office of Pollution Prevention and Toxics (EPA/OPPT), Office of Pesticide Programs (EPA/OPP), Office of Research and Development, (EPA/ORD), and voluntary perspectives offered by Biotech STAR Grant awardees, and outside experts. This workshop also built on research areas and recommendations identified in recent reports [4-10] and the EPA STAR Grant RFA, EPA-G2020-STAR-C1: Assessment Tools for Biotechnology Products.[3]

1.3 Workshop Objectives

- Familiarize participants (Appendix A) with recently identified novel biotech risk research needs.
- Review current EPA biotech risk assessment resources; EPA/OPPT and EPA/OPP; including trends of product submissions (Appendix C).
- Gain perspectives on upcoming biotech risk research from recent STAR Grant awardees and outside experts (Appendix C).
- Conduct Breakout Group discussions based on biotech product intended uses. Each group considered research needs in at least the following contexts:
    - Empirical experiments and monitoring
    - Models and reference databases
    - Research capacity (staff and infrastructure), collaborations and priorities

1.4 Charge Questions

Each Breakout Group was asked to respond to the same following charge questions to guide the identification of research needs. The entire set of questions were considered in the context of the central novel biotech product category being discussed. Breakout Groups were given the latitude to focus on issues related to the questions that they saw as most pertinent for their biotech product category:

- What empirical experiments are needed to fill data gaps for this biotech product category? Describe potential experiments and sources of reference samples.
- How could results from the experimental work identified by the group transform future biotech risk assessments beyond what is possible today?
- What monitoring efforts are needed to fill data gaps for this biotech product category? Where applicable, describe potential monitoring sites and logistics/requirements anticipated to conduct the monitoring.
- How could results from the monitoring efforts identified by the group transform future biotech risk assessments beyond what is possible today?

- What models and reference databases are needed for improved risk assessments in this biotech product category? Describe potential upgrades to existing models and databases or those that could be created *de novo*.
- How could the models and databases identified by the group transform future biotech risk assessments beyond what is possible today?
- What are the anticipated staffing requirements for implementation of the research needs identified above?
- What is the ranked priority of research needs identified above?

2 Cross-Cutting Research Priorities

Section 2 describes the research priorities that were identified by participants, most of which were shared among the findings from the Breakout Groups. Several overlapping research needs arose from the consideration of the four biotech product categories. These priorities delineate areas of research and development that are essential to support risk assessments of engineered microbes and biopesticides by EPA, other Agencies/Departments, academic labs, and product developers. The research priorities are also areas in need of additional funding support.

2.1 General considerations for experimental testing and monitoring

Testing for the performance and safety of biotech products intended for release into the environment has often followed the same paradigm since the 1990s: lab microcosms → greenhouse/mesocosm → small trial field tests → large scale field test. Rigid adherence to these scaled-up steps is not realistic for all current engineered microbes and biopesticides. For example, testing highly mobile engineered invertebrates within small, confined spaces may interfere with their normal behavior and interactions. On the other hand, open field testing of engineered organisms that are not easily contained may inadvertently result in wide-spread releases into the environment. When scaled-up experimental steps are employed , there are fundamental elements that are necessary for this phased approach to provide useful information on applicable products.

- Mesocosms can be valuable tools for running controlled experiments under containment. Where mesocosm experiments are practical intermediate testing steps for biotech products, they should be designed with clearly defined metrics to address specific hypotheses regarding risks. More often, mesocosm experiments should involve communities of organisms that simulate many real-world interaction complexities. The need for clear metrics and specific hypotheses extends to all aspect of experimental testing and monitoring.

- No matter what the scale of the system, baseline data will be needed for communities and populations that are utilized in experimental testing. Likewise, baseline data on potentially exposed natural communities and populations should be collected before and after release/application of products for monitoring projects. To the extent possible, the baseline data should be derived from multiple timepoints and spatially distributed locations.[5, 10] Additional funding is needed to support projects that collect baseline data for ecological risk assessments.

- Updated tracking methods are needed for safety experiments with biotech products being tested under containment and for post-release monitoring of their persistence, performance, and efficacy. Tracking methods are likely to be specific to product types.[9]

- Early stakeholder/community engagement is needed and very important when choosing locations for biotechnology field testing, especially at large scale sites. Likewise, monitoring endpoints should include those driven by stakeholder/community concerns.[5, 11-13]

- When confined testing interferes with the normal behavioral or ecological interactions of engineered organisms (i.e., gene drive insects, rodents, etc.), then testing in isolated or remote locations such as islands is a preferable alternative option for large scale field testing prior to open release/application.

2.2 Toxicity/allergenicity of biomolecules from biotech products

A primary concern with released products of biotechnology is that their unique characteristics may result in generation of biomolecules with unintended toxicity/allergenicity to exposed humans and other organisms in the environment.

- Data are needed on the toxicity and/or allergenicity of biomolecules for both engineered microbes and biopesticides. In the case of engineered microbes, the biomolecules may be intentionally expressed or incidentally released as degradation products. For biopesticides, the compounds of concern also include those that are expressed or released, as well as formulated biomolecules intended for direct applications (i.e., exogenously applied dsRNA).
- For certain products, toxicity testing should include expanded routes of potential exposure to match product designs and intended uses. For example, routes could include ocular, respiratory and subcutaneous (injection by insect bite) exposures. Overall, toxicity test designs should reflect the anticipated route(s) of environmental exposure.
- Data are needed on impacts to target and non-target organisms. This applies to engineered microbes and biopesticides. Formulation of products may also need to be considered; for example, formulation can potentially alter toxicity of dsRNA if it impacts the uptake or stability of the biomolecule.[14-19]

2.3 Environmental persistence and spread

While some current biotech engineering efforts are intended to make products more durable (e.g., nucleic acid modifications and formulations), others are intended to limit persistence and spread (e.g., biocontainment strategies). Previous examples of transgene flow from engineered crops to sexually compatible species and establishment of transgenic plants in the wild highlight the need for assessing and managing the risk of unintended persistence and dispersal of released biotech products.[20-24]

- Additional research is needed on how novel biotech products persist and are prone to spread in the environment after they are released.[5, 13] This research will be closely tied with the development of updated tracking methods and monitoring projects. Exposures and hazards from unintended persistence and spread will also need to be assessed.

2.4 Stability and efficacy of biocontainment strategies

The emergence of biotechnology products designed to carry out specific processes in the environment has also given rise to creatively engineered biocontainment strategies to ensure that the products only function in the space and/or time intended.

- Empirical data are needed on the stability and efficacy of biocontainment strategies in genetically engineered microorganisms (GEMs), such as engineered auxotrophy for xenonucleic acids (XNAs) or noncanonical amino acids (ncAAs), genome recoding and kill switches.[25-28] XNAs are artificial nucleic acid analogs that may have modified backbones, sugars, or nucleobases. ncAAs (also known as nonstandard amino acids) are amino acids that are not usually incorporated into proteins during translation. While ncAAs may occur naturally in certain organisms, they may also be synthetically produced. Kill switches are genetic circuits that are engineered into organisms so that when there is an exit/escape from a permissive environment, the lethal components of the circuit are expressed.

- Data are also needed on the stability and efficacy of modified synthetic gene-drive configurations for population suppression of pests, such as insects that are disease vectors.[29-33]

2.5 Horizontal gene transfer

Another concern with released products of biotechnology is the potential for horizontal gene transfer (HGT), which is movement of genetic information between genomes that may include transfer between different species. Some biocontainment strategies such as engineered auxotrophies in GEMs are designed to reduce the likelihood of HGT.

- Data from experiments and monitoring are needed on HGT rates and effects.[5, 9] This effort will likely benefit from increased use of metagenomic sequencing of environmental deoxyribonucleic acid (eDNA). In this context, metagenomics is the analysis of genetic material extracted from the community of organisms present in environmental samples by sequencing.

2.6 Advanced models and databases

Updated mathematical models and databases that are validated will continue to be increasingly important tools for timely risk assessments of novel biotech products.[5, 9] Models are needed for simulations and predictions that range in scale from population to molecular interactions. Model recommendations include:

- Models for microbial community perturbations
- Models for microbial source tracking
- Models for assessing risks of products released in the environment
    - Bayesian probabilistic networks
    - Spatially explicit simulations
- Models for predicting consequences of pest population elimination
- Models for predicting targets and effects of biomolecules (e.g., dsRNAs and proteins) beyond homology across entire sequence.
- Models for off-target molecular effects, such as unintended RNAi caused by sequence matches in non-target organisms or partial sequence matches with alternate transcripts in target organisms.
- Models for predicting toxicity/allergenicity of synthetic biomolecules
- Development of a risk assessment dashboard of modeling tools

Well-maintained, curated, and validated databases supported by long-term funding are needed also to efficiently access the rapidly expanding volumes of relevant information on products and organisms. Database needs include:

- Databases for biocontainment strategies and organisms
- Databases for basic biology and life history parameters of commonly engineered macro-organisms (e.g., *Aedes aegypti*), which are potentially generalizable to risk assessments across products
- Databases for tiered-testing protocols and results

- Development of biotech product ontologies for communication clarity between databases
- Creation of databases that can be widely utilized by risk assessors and developers with protections against disclosure of CBI

2.7 Capacity and collaborations

There was agreement that an influx of new talent with relevant knowledge, skills and abilities will be beneficial for Agencies/Departments conducting research to support risk assessments on novel biotech products. The recommendations for increased capacity include:

- Hire a mix of new technical support staff, postdocs, and Principal Investigators.
- Encourage retention of talented fixed-term staff through more opportunities to become permanent employees.
- Streamline the federal on-boarding process for ORISE/ORAU students or use different routes such as institutional training grants.
- Increase funding opportunities and hires for underrepresented groups.
- Give students opportunities to do rotations in various parts of Agencies and Departments.
- Upskill locals/citizen scientists where field testing would occur, rather than just bringing in grad students, postdocs, and experts.
- Increase federal funds for biotech risk research within Agencies and Departments.

There were also points of consensus regarding collaborations:

- Increase collaborations between ecological scientists and model/database developers.
- There is a need for more collaborations across agencies to leverage resources.
- Government collaborations with external labs will be important for timely R&D success.
- Additional funding should be made available for competitive grants like the STAR program.
- More flexibility is needed to allow EPA biotech researchers to pursue funding from external sources like the USDA Biotechnology Risk Assessment Research Grants (BRAG) program.

3 Breakout Session Summaries

Section 3 presents consolidated points made and questions raised within the discussion sessions for each Breakout Group. The groups consisted of EPA regulators, EPA researchers, and non-EPA researchers. The items listed in sub-sections below do not necessarily represent a consensus opinion, but rather capture individual inputs, and reflect the major concepts that were brought up in the discussions.

3.1 Engineered Microbes-1; widespread open uses, biofertilizers, ocean bioremediation, etc.

3.1.1 Empirical experiments and monitoring

- The Engineered Microbes-1 group first identified some potential product application types including
    - Environmental surveillance of plant pathogens
    - Biofertilizers for soil enhancement
    - Biosensors for munitions detection
    - Climate change applications like increased carbon capture
    - Ice nucleation in clouds
- The group discussed the need for experiments that involve scaling-up from small to relatively large that simulate open release scenarios. There was recognition that there are considerable difficulties in predicting what's going to happen in the environment from wide-spread release of engineered microbes. For example, various interactions might happen resulting in unexpected effects.
- There could be various molecules produced by the GEMs that have effects on other prokaryotic or eukaryotic non-target organisms. There is a need for experiments that determine what happens to the engineered microbes when they're degraded in the environment. These include potential effects of nucleic acids and other biomolecules that are released from the GEMs into the environment after kill switches are activated.
- There is a need to know more about the stability of engineered biocontainment strategies. When such GEMs are released into the environment, how is the biocontainment going to change the effectiveness of the organisms at performing the primary functions that they were designed to carry out?
- There is the need for ecological models and baseline community/population data before introducing the organisms into the environment. The *in silico* work could be used to simulate large-scale systems before moving out of the lab to open release.
- Basic ecological information about the interactions between GEMs and other organisms would be needed to parameterize the models.
- Metagenomic data from experiments could be used more often to inform hazard identification. How do other organisms interact with particular types of genetically engineered organisms?

- It will be challenging to design specific experiments that will inform risk assessments on GEMs intended for large-scale release into heterogenous environments. It largely depends on what type of GEM you might be working with.
- Identifying potential hazards of GEMs may be more important than the exposures.
- The old way of doing things back in the 90s from the lab to the greenhouse, microcosms to the mesocosm may not be relevant for some newer products anymore.

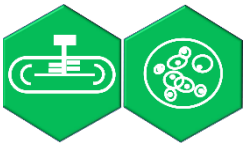
3.1.2 Models and reference databases

- Where possible, bioinformatics could be used as a first cut to determine what lab experiments should be done.
- There are many types of bioinformatic databases currently available:
    - Sequence databases
    - Databases for similarity searches and more sophisticated analyses
    - Whole genome assemblies with annotations
    - Molecular folding and 3D structure predictions
- Those databases include:
    - UniProt – Europe: gene annotation information
    - Swiss proteins – human reviewed
    - Algorithm that adds annotations eventually moved into the reviewed section – part of Swiss proteins
    - KEGG (Kyoto Encyclopedia of Genes and Genomes): modules – metabolic or genetic modules; intermediate metabolites
    - NCBI SRA (National Center for Biotechnology Information, Sequence Read Archives) and GenBank
    - Patric (Pathosystems Resource Integration Center)
    - VFDB (Virulence Factors Database)
    - Allerbase
    - CARD (Comprehensive Antimicrobial Resistance Database)
    - Natural Product Database (https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp)
    - Allergen on-line
    - AllerCatPro
- Models are needed for:
    - Determining what is a risk to the community – baseline and time series
    - Dose/response
    - Evaluation of potentially toxic, allergenic, or reactive metabolites and products of GEMs
    - Perturbations of soil microbial community composition – this is under development but is not widely available
    - Models for probability of widespread escape
        - Will depend on if it's a small-scale use of the organism or a widespread dispersal – crop dispersal or other
    - Microbial source tracking models

3.1.3 Research capacity, collaborations, and priorities

- There is a need for an infusion of PIs, postdocs, and technical support for implementation of the research needs.
    - Masters/PhD level
    - Additional new positions could be created through the *Pathways* or other programs
    - Hiring of more permanent EPA staff is advised and would reduce turnover of staff.
- The knowledge, skills, and abilities for new staff must match the research needs.
    - Specific knowledge will be needed for certain sub-fields.
    - There should be increased coordinated planning with the EPA program offices.
    - It will be important to form collaborations with researchers from internal and external organizations.
- Some independent efforts by Agencies/Departments are needed, however, there should also be expanded work in collaboration with external organizations.
    - There is a need for better communication of what is under the biotech purview of EPA and other agencies.
    - Increase the dialog with external partners and researchers on what information and tools are needed on the regulatory side.
    - There is a need for clearer coordination of research between ORD, programs, and outside institutions.
    - Examples of potential USDA biotech work include:
        - Biofertilizers
        - Genetically engineered microbes
- There should be increased activity of inter-Agency working groups.
- Agencies/Departments should expand funding of research by external organizations through contracts, Cooperative Research and Development Agreements, Materials Licensing Agreements, and extramural grants.

3.2 Engineered Microbes-2; semi-contained uses, algae ponds, biomining, biosensors, wastewater treatment, localized bioremediation, microbial enhanced oil recovery, etc.



3.2.1 Empirical experiments and monitoring

- Participants acknowledge there are many research needs for semi-contained GEMs that overlap with those for large-scale release.
- For semi-contained systems, there is potential for unintentional release of GEMs into the environment, so updated methods for detection and tracking are required.
    - The main example that was used for the semi-contained case was algal growth in open ponds with potential for algal escape/dispersal into the environment.
    - Most semi-contained uses are assessed on a case-by-case basis and take into account location where testing will occur (e.g., the arid heat of the Arizona desert or in Florida during hurricane season). However, it is difficult to find the baseline data to make assessments in such different environments.
- The safety metrics for contained experiments need to be clearly defined to address specific hypotheses.
    - Assessments for the safety level of genes of interest can be based partially on available literature and some exposure models, but there's not very much defined for hazard metrics.
    - Having a standard set of practices used to monitor GEMs in the environment would be helpful, and this would allow other groups in the future to build off of successes instead of starting from scratch on each new monitoring effort.
- There is a need to better understand toxicity and other risks by the synthetic biology technologies being presented by new products.
    - More experiments that capture the effects on whole communities in that toxicity assessment would be helpful. The potential synergistic effects between organisms, and the potential toxicity when a synthetic genetic construct is transferred to another microorganism within that community (Horizontal Gene Transfer, HGT) may be studied in microcosm or mesocosm experiments.
- Many new technologies use noncanonical amino acids (ncAAs), such as applications for biocontainment. The efficacy and stability of these systems needs to be further investigated.
    - Biocontainment strategies can be designed to add an extra comfort level to reduce concerns about horizontal gene transfer. If those strategies are effective, even if there is HGT to a natural population, those organisms should not gain novel activity.
    - Microorganisms with kill switches should really be assessed for their long-term community effects, for example in the bioremediation realm.
    - Even if you are going to introduce a GEM with a kill switch for a short-term remediation effort, we really need to assess the starting microbial community and then measure how the GEM affects the community while active. But also, there is the need to continue tracking the GEM

throughout its lifecycle to see how the microbial community has changed after the GEM is deactivated by the kill switch.
    - If the GEM is going to be used to remove a pollutant, that by itself may cause changes to the microbial community, so we need to know whether that change is all beneficial and watch for unanticipated adverse effects such as the production of toxic metabolites.
    - At what level do we know that the genome recoding becomes incompatible for HGT and at what point does the recoding make GEMs resistant to infection by viruses?
    - How many mutations would it take for the biocontainment to fail? Future experiments are needed to be directed at this question.

3.2.2 Models and reference databases

Models for predicting consequences of population elimination

- Lab domestication of strains should be considered – most lab strains are adapted for lab growth in ideal conditions, and when thrown back into the wild, they underperformed due to decreased fitness.

- If metabolic community models are to be based on genome sequencing, metagenomic sequencing from eDNA may be required first in order to parameterize the models with broad representation of the diversity of microbial genes present within a community.

- There are challenges in creating community metabolic models because we don't *a priori* know the functions of all genes. Even after identifying the genes and their potential functions, consortium modelling (simulating the activities of diverse microorganisms that can act together as a community) is very challenging without expression data.

GEM Databases

- Databases for interactions among microbes in an environment (e.g., soil or water bacteria) can be useful to understand the potential for HGT in different settings. For example, it would be useful to document which taxa utilize naturally occurring control elements and how they function in native microbial communities. This could potentially involve machine learning for functions of mobile elements.

- A big challenge may be keeping databases updated with no guarantee of continued funding. Basic maintenance can cost hundreds of thousands of dollars a year. This cost can be more substantial than previously discussed but could be feasible with multi-agency collaboration, along with synbio organizations/industry.

- Who is the audience for GEM databases? Who benefits?

    - Databases can benefit both Agencies/Departments and industry – they may save time and resources for industry to not follow a path that may be less likely to be approved due to known risk concerns.

- Creation of centralized databases on biotech product submissions must be careful to prevent disclosure of CBI.

Implementing databases of GEMs – what would that take?

- Ideally a collaborative effort by agencies dealing with GEMs in order to cover a range of categories

- Some in the scientific communities are likely unaware of EPA interests in synthetic biology research and developing guidance.
    - The synthetic biology community can greatly contribute toward EPA goals.
    - Collaboration and outside input are necessary given the need for funding:
        - Projects may find support in places like DOE, JGI, MIT Lincoln Lab, NSF, etc.
- Maintaining a database of GEMs may not require great expense if it is done using infrastructure that is already in place, e.g., NCBI.
- What is the feasibility of open source for GEM databases?
    - If they are Python based, there is better memory management with large databases.
    - GenBank is used regularly, and there is some degree of trust, accuracy needs to be considered.
    - Wiki pages have been utilized for similar projects:
        - Pros: spreads out ownership on everyone
        - Cons: quality of data will be an obstacle to be considered
    - Curation can be challenging, but it depends on what level of detail from the database will be utilized.
- If employing multiple preexisting databases, there will potentially be issues when databases are interacting with one another. Is it common to have translation problems on how information is shared? There is a need for developing ontologies to use similar characteristics/terms.

Data from toxicology experiments at community level for databases and models

- Data collected from small scale experiments could be shared in databases or used to parameterize cross-lab/Agency models to expand upon those types of experiments.
- Complications arise with complexity of chemical mixtures, with many variables needing to be assessed
    - Superfund sites for example, are often mixtures of contamination.
    - Maybe the focus should be on the 'worse offenders' contaminants, most likely to affect microbial communities.
    - If complex models are to be built, high-throughput platforms will be crucial.
    - Integration of existing resources is key

Model of 'simplified' microbial communities

- From an experimental point of view, the limitation is how much can we actually screen. In terms of resource development, again high throughput data collection and analyses will be critical for identifying gene transfer events and other interactions.
- Consider when it would be temporally appropriate to screen for the risk. It may not be useful to simply screen for risk at one time point. On the contrary, this must be revisited at various stages of the microbial community succession. Are there community shifts correlated to exposures that occur later that can change the risk of the GEM?
- In terms of horizontal gene transfer, there is much data on which genes/loci are prone to these transfer events, but frequency data is less precise. GEM functions often depend on exact modifications. There is a need for funding to assess how frequency of HGT may affect risk.

3.2.3 Research capacity, collaborations, and priorities

- Collaboration between academia, government agencies, and institutions will be important to develop tests for products in mesocosms or in field studies. It would be great to have industry input, but proprietary issues may hinder that**.**

3.3 Biopesticides-1; population control/elimination, *Wolbachia*, release of insects with dominant lethality (RIDL), gene drives, etc.

3.3.1 Empirical experiments and monitoring

Laboratory, mesocosm and/or field

- There are challenges to building gene drives that would be feasible for field testing (e.g., those that are containable, those with developed remediation methods).
- There are synthetic gene drives in the lab, but there are uncertainties about how to take the next steps and go to field releases.
- Semi-caged trials may not accurately capture behavioral aspects of the modified species for gene drives.
- One pathway for gene drives would be to first conduct experiments in the lab or with models, then move to the field. The focus could be on testing split drives or other similar configurations to make the gene drive more self-limiting. This would allow for testing of individual drive components and the opportunity to gather data on specific risk hypotheses prior to potentially releasing a gene drive.
- In terms of field trials themselves, there has been considerable discussion on various types of containments: population structure, genetic (e.g., split drive), geographic (e.g., islands). Community engagement can also play a role in the test location chosen.
- Testing the penetrance of the genetic construct in different genetic backgrounds may be important.
- Protein toxicity/allergenicity are largely evaluated from an oral exposure scenario, so different tools may be needed to evaluate exposure via subcutaneous injection instead, which is likely more relevant for mosquitoes, particularly if female mosquitoes are released.

Monitoring

- There was discussion of what exactly needs to be monitored and proxies for monitoring (e.g., temperature may impact performance of *Wolbachia*).
- Monitoring endpoints could include measuring the penetrance of the product vs. population levels.
- The types of things that you may want to monitor or test for can also be driven by community concerns.

3.3.2 Models and reference databases

Need basic life history information

- Modelers and experimenters/field biologists need increased crosstalk.
- There was not much discussion on the need for better modeling techniques/capabilities in academia, but more on the need for better data to use as input parameters for the models.

- Specifically, data are needed on life history and species interactions. Basic biological parameters related to developmental time, use of metabarcoding techniques to evaluate blood meals/predator gut contents to determine species interactions, etc.
- This type of data is critical as it will not only inform risk assessments for specific products but will also give important information as to which model parameters are generalizable/can be pulled from the literature and which need to be related to a certain location.
- Often in a risk assessment, the data is generated by the developer as it is product specific, but for these technologies many of the risk assessment questions/analyses will be generalizable across products (e.g., role of *Aedes aegypti* in the food web, interactions between *Ae. aegypti* and *albopictus*, population genetic structure of *Ae. aegypti*)
- Because we know this is a need and we have a good idea of the types of species we will most likely be evaluating in the near future, this seems like a good opportunity for EPA to play a role either in generating the data, compiling/reviewing the existing data, or providing funding for others to gather the data.

Need a place to store/compile/curate data

- The data just mentioned also needs to be stored somewhere that makes it more accessible/useful.
- There are current databases available that may potentially serve as resources, but curation/management is lacking.
- Funding or maintenance of a relevant database was also identified as a current research gap.

Need for collaboration and info sharing on modeling techniques

- Finally, although there wasn't much discussion about the need for better modeling techniques/capabilities in academia, it was noted that there is a desire to see up-to-date modeling used in EPA risk assessments.
- This was identified as a potential collaborative opportunity to bridge modeling capabilities in academia with those used in EPA.
- It was also noted that EPA will need training in these complex models in order to be able to properly evaluate and analyze them.

3.3.3 Research capacity, collaborations, and priorities

Regulatory resources/info for developers

- For newer developers, it would be useful to have a clearer picture of what the regulatory process involves and what researchers need to provide.
- EPA encourages pre-submission meetings and visiting the EPA website for guidance.
- Researchers would find it useful for EPA to develop improved guidance and find ways to disseminate it. It was felt that the EPA website was not the easiest to navigate.

Infrastructure requirements

- Use academic settings to scale up technology to demonstrate efficacy where possible. There is a lack of infrastructure for such purposes, so some technologies have done so via industry and private investments.
- This may be especially applicable for biopesticide products that are not meant for general consumers but are more likely to be sold to countries or regions or mosquito control districts.
- Another option would be for EPA to run a facility where researchers could send their products to be tested in scaled up experiments.

Collaborations

- Interdisciplinary collaborations are needed and there should be more calls for such proposals.
- Collaborations between modelers and ecologists/field biologists are needed.
- Social community engagement will be essential in order to elucidate important risk considerations from community members.

Funding for training

- Is there an EPA role or option for increasing funding opportunities/training programs for underrepresented groups?
    - It would be nice if this were also broader than typical grad students/post docs or bringing in local experts. This could involve upskilling the people that are in the locations where the field testing may take place as a form of research capacity building (molecular techniques, analysis techniques). Products and/or practices associated with novel technologies that are developed locally by local communities have reduced chances to be rejected due to mere unfamiliarity or due to perceived lack of transparency.
- There is always room for external collaboration, but the Agency also needs to bring in new blood/talents not currently represented. Efforts towards recruiting talent with specific knowledge of the types of techniques would be useful.
    - Developing a workforce pipeline would also help.
    - Create transitional funding for those interested in academic research who want to go more applied, and then list these funding/opportunities. Finding this information currently can be difficult for some PhD advisors.
    - Also, create opportunities to work with EPA regional offices/ORD labs to give people those transitional/applied experiences.
    - The onboarding process for ORISE/ORAU students is slow and can delay students' ability to complete projects. Find ways to reduce turnaround time for training.
    - Opportunities might be generated with institutional grants or multi-campus training grants.
    - One could bring in a student and take them to different parts of the Agency (rotational development program) to give someone the perspective/depth.
    - Expand the federal postdoc program with mind to diversity. There is a need for a more diverse workforce.
    - There needs to be a track for rotational students/postdocs to stay on with Agency.

- It would be useful to introduce relevant topics in university courses/graduate programs to recruit people to the pipelines. FDA has the 'feed your mind' initiative. Is there something like this EPA can do?

3.4 Biopesticides-2; plant incorporated protectants (PIPs), microbial pest control agents (MPCA), dsRNA, biochemicals, etc.

3.4.1 Empirical experiments and monitoring

- The session focused on microbes, exogenously applied double-stranded RNA, and plant-incorporated protectants.
- Empirical data collection and monitoring were viewed as two sides of the same coin.
- Before field studies can begin, decisions have to be made regarding the monitoring requirements, which will inform the experimental design.
- There is need for mesocosm experiments that better reflect the real-world environment as opposed to something that's more artificial, e.g., experiments using a very limited number of species.
- With microbes that might persist even though they're engineered to not survive long with a kill switch or synthetic auxotrophy, the questions are:
    - Is persistence something that we're concerned about?
    - What are the risk implications of that persistence?
- Just because a microbe is short-lived and might not persist in the environment for a long time, it may have a lasting impact depending on what it's exposed to. Meaning that even with kill switches, the use of orthogonal pathways is not necessarily obstacle free.
- From a risk assessment standpoint, there is a need to know how to short circuit the worst-case scenario (i.e., horizontal gene transfer and persistence).
- Even if an organism is designed to preclude HGT, what information can be provided to support the risk assessment that the risk of HGT is fairly low under field trials?
- In the future, there will probably be many new strains of engineered prokaryotic and eukaryotic microbes that would not be easily contained in field testing scenarios. This will make monitoring increasingly important, where there are concerns.
- Another consideration with certain regulatory actions is the defined timeline for a decision. It might be 90 days to approve a field trial with an engineered microbe. So, if a mesocosm study is added as a requirement prior to getting into the field, that will significantly change the considerations on timing for the developer. Note that 40 CFR part 172 essentially dictates what EPA's authorities are and what the Agency is intended to ask for in terms of information prior to authorizing a small-scale field release.
- More data are needed on various modifications of double-stranded RNAs that are used for stabilization and that may alter persistence, target specificity, uptake, and efficacy of the dsRNA, for example:
    - Fluorinated RNAs that are significantly more stable.
    - Additions of surfactants in the end-use formulation
    - Incorporation of RNAs within liposomes or micelles

- Specifically, more work could be done to study how the formulations change the persistence, target specificity, uptake, and efficacy of exogenously applied dsRNA products.
- Regarding GEMs, more information is needed about their persistence in the environment.
- Even though some of these are designed to preclude or drastically reduce already low probability events like horizontal gene transfer, there is a need for more data about the other microbes in the soil where the field tests might be conducted and the possibility for HGT.
- How do we detect engineered microbes months after release to see if in fact they are functioning? This will require development of new tracking methods, such as sensitive PCR methods and sequencing metagenomics to look for evidence of this.

3.4.2 Models and reference databases

- It would be useful to have databases for toxicity, considering if the homology falls under guidelines that would trigger certain concerns. Some databases we have aren't easily searchable. The absence of centralized databases can be frustrating.
- Reference databases used to support the risk assessment process for proteinaceous PIPs and proteins produced in microbes: EPA has two allergenicity databases we rely on. There are numerous databases on the toxicity side, but not one singular. What else could we use if we had it?
- A new classification of pesticidal proteins that is based on not just homology but also structural similarities has recently been published.[34] Knowing that a certain protein looks like other things is helpful. A database published recently reflects updated nomenclature for pesticidal proteins and provides similarity based on structure. More and more of the PIPs EPA is going to be seeing are no longer just 3 domains.
- Finding genes in the RNAi pathway with homologous sequences, can be a first step in risk assessments.
- Given the amount of databases that have toxicity information within them, would those people be willing to share their information if ORD was to create a centralized database? Is that doable given the number of databases out there? What type of resources would ORD need to pull that off?
- There is a potential challenge of assessing off-target effects for dsRNA products. Tools like DS Finder may be helpful, although there are limitations based on genome quality.
- It may be useful to have models or tools that are able to predict the potential off-target effects of dsRNA.
- There's a lot of small RNAs that affect gene expression and also have post-transcriptional effects. But there's not much useful in terms of tools for RNAi predictions for risk assessors. If there is homology within some non-target organism, maybe it comes down to annotating more genomes.
- For non-target organisms in bioassays, you may have to extend the assay based on the life stages to see the effects of a toxicant.

3.4.3 Research capacity, collaborations, and priorities

- ORD's role in doing research involves addressing needs of other parts of EPA (i.e., OPPT, OPP) and external partners.
- ORD has been encouraged to do collaborative work that helps the Program Offices.
- There is a need for more capability to assess stability of products and how that changes their potential hazards and exposures.
- Collaboration with other Agencies is encouraged lately. Everybody's funding is limited. If we can emphasize interest from other partners when pitching proposals for projects/funding, that is very helpful. The more collaborative a proposal, the more compelling.
- It would be helpful if ORD could pursue additional funds from biotech grant programs like those at USDA (i.e., BRAG). Persistence of dsRNA relative to nontarget effects could make a good project.
- USDA is open to collaborations with other Agencies, although access for ORD researchers to BRAG funds has been constrained.

4 Conclusion

As development of emerging biotechnology products is enhanced by molecular techniques including genome editing, use of synthetic elements, and metabolic engineering, risk assessors will be challenged in assessing both human health and environmental impacts. In many instances, risk assessments are based in part on existing information from available literature including the use of established databases to query for similarities to known comparators. The expansion of novel product types and intended uses will require greater empirical data generation by researchers and product developers plus the use of mathematical models to aid in risk assessments.

Workshop participants sought to identify the most pertinent research priorities regarding engineered microbes and biopesticides to be released into the environment. The workshop focused on areas needing attention for risk assessors evaluating products under TSCA and FIFRA/FFDCA oversight, which will help to guide further research in EPA/ORD and collaborating labs toward addressing some of the knowledge gaps. The key research needs identified were:

- Refined use of laboratory and mesocosm experiments
- Collection and utilization of baseline data on communities
- Improved tracking methods for engineered organisms and synthetic constructs
- Increased stakeholder engagement for field testing
- Alternative options for stages of field testing
- Toxicity and/or allergenicity of biomolecules from biotech products
- Environmental persistence and spread of products
- Stability and efficacy of biocontainment strategies
- Detection and impacts of horizontal gene transfer
- Development and use of advanced models and databases
- Recruitment and retention of new researchers
- Establishment of collaborations between Agencies, Departments, and external partners

The overlap among the challenging priorities above and those previously identified in reports on biotechnology products [4, 5, 9, 10] reinforces the point that substantial research efforts and investment are needed to support timely and accurate regulatory decision making by the Agency and other organizations. An iterative process is anticipated wherein research projects funded through the EPA STAR granting program conducted primarily in academic institutions plus those performed in ORD and other collaborating laboratories lead to gains in our understanding that are reflected in future guidelines and approaches to risk assessments and risk management decisions.

Appendix A: Workshop Organizers, Presenters, and Attendees

Name	Affiliation	Designation
Omar Akbari	University of California, San Diego	STAR Grantee/Presenter
Souhail Al-Abed	EPA/ORD/CESER	
Frank Antwi	EPA/OCSPP/OPP	
Natalya Baranova	EPA/OCSPP/OPP	
Heidi Bethel	EPA/ORD/CSS	Breakout Group Notetaker
Lauren Boldrick	EPA/R10	
Michael Bollman	EPA/ORD/CPHEA	
Robert Burgess	EPA/ORD/CEMM	
Andrew Byro	EPA/OCSPP/OPP	Organizer
Giles Chickering	EPA/ORD/CESER	
Man-Yeon Choi	USDA/ARS	
Catherine Chuirazzi	EPA/ORD/CPHEA	Breakout Group Notetaker
Mary Beth Claude	EPA/OCSPP/OPP	
Steven Eikenbary	Integral	
Natalie Farny	Worcester Polytechnic Institute	STAR Grantee/Presenter
Courtney Gardner	Washington State University	
Mohamed Ghorab	EPA/OCSPP/OPP	
Marissa Giroux	EPA/ORD/CEMM	
Michael Gordon	Oregon State University	
Nicholas Guido	MIT Lincoln Labs	
Alfred Handler	USDA/ARS	
Paul Harten	EPA/ORD/CCTE	
Kay Ho	EPA/ORD/CEMM	
Scott Jenkins	EPA/ORD/CPHEA	
Susan Jennings	EPA/OCSPP/OPP	
Carlton Jones	EPA/ORD/CCTE	
Juan Jurat-Fuentes	University of Tennessee, Knoxville	
Scott Keely	EPA/ORD/CEMM	Organizer
Cassandra Kirk	EPA/OCSPP/OPP	
Barbara Klieforth	EPA/ORD/OSAPE	Organizer/STAR Grant EPA-G2020-STAR-C1 Contact
Tom Knudsen	EPA/ORD/CCTE	Organizer
Konstantinos Konstantinidi	Georgia Tech	STAR Grantee/Presenter
Todd Kuiken	Congressional Research Service	Presenter
Aditya Kunjapur	University of Delaware	Presenter
Victoria Kurker	EPA/OCSPP/OPP	
Richard Lance	USACE/ERDC	
Wayne Landis	Western Washington University	Presenter
Jared LeBoldus	Oregon State University	
Yoosook Lee	University of Florida	STAR Co-PI/Presenter
Blake Linder	Georgia Tech	STAR Co-PI/Presenter
Todd Luxton	EPA/ORD/CESER	
Cresten Mansfeldt	University of Colorado, Boulder	STAR Grantee/Presenter
John Marshall	University of California, Berkeley	STAR Co-PI/Presenter

Gwen McClung	EPA/OCSPP/OPPT	Organizer/Presenter/Breakout Group Lead
Mike Mendelsohn	EPA/OCSPP/OPP	
Tae Seok Moon	Washington University, St. Louis	STAR Grantee/Presenter
Ashley Nelsen	EPA/OCSPP/OPS	
Megan Nelson	EPA/OCSPP/OPPT	
Khoa Nguyen	EPA/OCSPP/OPPT	Organizer/Breakout Group Lead
Tonya Nichols	EPA/ORD/CESER	Organizer
Nina Ortiz	EPA/OCSPP/OPP	
Kimberly Parker	Washington University, St. Louis	STAR Co-PI
Leslie Paul	EPA/OCSPP/OPP	
Amanda Pierce	EPA/OCSPP/OPP	Organizer/Breakout Group Lead
Robyn Raban	University of California, San Diego	STAR Co-PI
Mizanur Rahman	EPA/OCSPP/OPPT	
Coral Reed	EPA/ORD/CPHEA	Breakout Group Notetaker
Jay Reichman	EPA/ORD/CPHEA	Organizer/Workshop Lead
Caroline Ridley	EPA/ORD/CPHEA	Organizer/Presenter
Sarah Samples	EPA/R9	
Greg Sayles	EPA/ORD/CESER	
Nathan Schumaker	EPA/ORD/CPHEA	
Max Scott	North Carolina State University	
Sanjiv Shah	EPA/ORD/CESER	
Geoffrey Sinclair	EPA/OCSPP/OPP	
Kimberly Slentz-Kesler	EPA/ORD/CCTE	
James Smith	EPA/ORD/CSS	Breakout Group Notetaker
Bonnie Smith	EPA/ORD/CPHEA	
Raymond St. Leger	University of Maryland	Presenter
Megan Stallard	EPA/OCSPP/OPP	
Darius Stanton	EPA/OCSPP/OPP	
Wiebke Striegel	EPA/OCSPP/OPP	Organizer/Presenter
Scarlett VanDyke	EPA/ORD/CSS	Breakout Group Notetaker
Katrina Varner	EPA/ORD/CEMM	
Michael Wade	Indiana University	
Chris Wozniak	EPA/OCSPP/OPP	Organizer/Presenter/Breakout Group Lead
Doug Young	EPA/ORD/CCTE	Organizer

Appendix B: Workshop Agenda

Wednesday July 28, 2021

Pacific Time	Eastern Time	
6:00 AM	9:00 AM	Main Room Opens
6:30 AM	9:30 AM	Opening remarks & participant introductions: Jay Reichman, EPA/ORD
7:00 AM	10:00 AM	Presentations: Caroline Ridley, EPA/ORD; Todd Kuiken, Congressional Research Service
7:50 AM	10:50 AM	Break
8:00 AM	11:00 AM	Presentations: Gwen McClung, EPA/OPPT; Chris Wozniak, Wiebke Striegel & Amanda Pierce, EPA/OPP; Raymond St. Leger, University of Maryland
9:00 AM	12:00 PM	Lunch
10:00 AM	1:00 PM	Presentations: Aditya Kunjapur, University of Delaware; Konstantinos Konstantinidi, Georgia Tech; Tae Seok Moon, Washington University in St. Louis
11:00 AM	2:00 PM	Break
11:10 AM	2:10 PM	Session 1 - Empirical Data & Monitoring: All Breakout Groups
12:30 PM	3:30 PM	Summary of Session 1 Findings: All Breakout Groups
1:10 PM	4:10 PM	Day 1 Debrief: Jay Reichman, EPA/ORD

Thursday July 29, 2021

Pacific Time	Eastern Time	
6:00 AM	9:00 AM	Main Room Opens
6:30 AM	9:30 AM	Introduction of Day 2 activities: Jay Reichman, EPA/ORD
6:40 AM	9:40 AM	Presentations: Wayne Landis, Western Washington University; Omar Akbari, University of California, San Diego; Natalie Farny, Worcester Polytechnic Institute
7:40 AM	10:40 AM	Break
7:50 AM	10:50 AM	Presentation: Cresten Mansfeldt, University of Colorado, Boulder
8:10 AM	11:10 AM	Break
8:20 AM	11:20 AM	Session 2 - Models & References Databases: All Breakout Groups
9:40 AM	12:40 PM	Lunch
10:40 AM	1:40 PM	Summary of Session 2 Findings: All Breakout Groups
11:20 AM	2:20 PM	Break
11:30 AM	2:30 PM	Session 3 - Research Capacity, Collaborations & Priorities: All Breakout Groups
12:50 PM	3:50 PM	Summary of Session 3 Findings: All Breakout Groups
1:30 PM	4:30 PM	Day 2 Debrief: Jay Reichman, EPA/ORD

Appendix C: Presentation Abstracts

- Caroline E. Ridley, US Environmental Protection Agency, Office of Research and Development

    - Novel Biotechnology Research Needs: A Review of Recent Reports

    There have been multiple, recent efforts to identify research needs around assessment of biotechnology and synbio. In this presentation, the most relevant given their scope will be reviewed:1) National Academies of Sciences, Engineering, and Medicine. 2017. Preparing for Future Products of Biotechnology. Washington, DC: The National Academies Press. 2) Warner et al. 2019. Synthetic biology: research needs for assessing environmental impacts. Environmental Laboratory, Engineer Research and Development Center, U.S. Army Corps of Engineers. ERDC/EL TR-19-10. 3) U.S. EPA. 2020. Assessment tools for biotechnology products. Request for Applications EPA-G2020-STAR-C1, Science to Achieve Results Program. U.S. EPA, Washington, DC 3-5.

- Todd Kuiken, Congressional Research Service

    - Beyond Containment: Novel Biotechnology Applications Near and Far

    Utilized by an expanding group of actors, emerging biotechnologies are complex and increasingly applications are being developed for intentional release into the environment. With the advent of synthetic biology, CRISPR, gene drives and other gene editing technologies; understanding the scientific, technical, ethical, and societal implications around biotechnologies is more important than ever. The recent report from the International Union for Conservation of Nature stated that; "these new and rapidly evolving technologies create exciting opportunities in many fields, including new kinds of conservation, but they also raise serious questions and complex challenges". Understanding these complexities requires a convergence of disciplinary study and thinking. Integrating knowledge from a variety of fields, cultures, and ethics. This talk will examine these complexities and how scientists, technology developers, funders, businesses, regulators, and society at large should contemplate their development and use.

- Gwendolyn McClung, US Environmental Protection Agency, Office of Pollution Prevention and Toxics

    - Research Needs of the Biotechnology Program in the Office of Pollution Prevention and Toxics

    The Toxic Substances Control Act (TSCA), which was amended by the Frank R. Lautenberg Chemical Safety for the 21st Century Act in 2016, gives EPA authority to regulate the manufacture, import, processing, distribution in commerce, use, and disposal of new chemicals, including "new microorganisms". Under TSCA, "new microorganisms" are defined as those that are "intergeneric", i.e., microorganisms formed by the deliberate combination of genetic material originally isolated from organisms of different taxonomic genera. Microorganisms constructed with synthetic sequences not identical to that which would be derived from the recipient genus are also considered to be intergeneric.

    EPA assesses the risks of intergeneric microorganisms intended for a variety of commercial uses such as closed system fermentation for production of enzymes, specialty chemicals, commodity chemicals, and textiles. TSCA applications also include fuel production, biomass conversion, waste treatment, biofertilizers, biosensors, bioremediation, biomining, and others. There are generally two types of submissions for genetically engineered (GE) microorganisms, a Microbial Commercial

Activity Notice (MCAN) for microorganisms intended for commercial use, and a TSCA Experimental Release Application (TERA) for those microorganisms intended for introduction into the environment for commercial R&D. Information to be provided in the submissions includes descriptions of the recipient and donor organisms, details of the genetic modifications, potential human health and environmental effects, conditions of use, production volume, worker exposures, environmental releases, environmental and general population exposures, and the expected survival of the microorganism in the environment. Currently, EPA typically determines the hazard of a microorganism by obtaining information on the recipient microorganism from the scientific literature and then evaluating how the genetic modifications applied to the recipient affects its characteristics and behavior.

More novel biotechnology products such synthetic biology microorganisms pose increased challenges for risk assessment. Little is known on the stability of various biocontainment strategies such as the recoding of microorganisms, use of noncanonical amino acids, or use of xenonucleic acids over time. Of particular interest to the Agency's risk assessment process is the stability of these biocontainment strategies and their potential interactions of synbio microorganisms once in the environment. The de novo synthesis of entire genomes also results in the loss of comparators since there is no "recipient" microorganism to base a scientific literature search on. Much research is needed on various synbio microorganisms and their biocontainment strategies, as the scientific community is rapidly developing these microorganisms.

The views expressed in this abstract are those of the authors and do not necessarily represent the views or the policies of the U.S. Environmental Protection Agency.

- Chris Wozniak, Wiebke Striegel and Amanda Pierce, US Environmental Protection Agency, Office of Pesticides Programs

    - Risk Assessment for Biotech Pesticides and Future Considerations

    Pesticides created using biotechnology are regulated using EPA's existing statutory authorities for pesticides. For the past twenty-five years, most biotechnology pesticide products have been classified as plant-incorporated protectants (PIPs) and are used in crop plants that have been genetically modified to protect themselves from disease or insect pests. EPA's risk assessment framework for PIPs has been applied to other biotechnology pesticides, like genetically modified mosquitoes, and can be applied to additional emerging technologies in the future. Future considerations for biotechnology pesticides include gene drive applications and the increased use of modeling, assessing the use of alternative nucleotides in synthetic biology, and containment strategies for genetically modified microbes.

- Raymond J. St. Leger, University of Maryland

    - Stages in Development of a Biocontrol Product Focusing on Risk, Monitoring, Stakeholder Engagement and Regulation

    We have applied genomics and DNA technology to insect pathogenic fungi in order to study infectious processes in general, and to provide new possibilities for solving insect pest problems. We have engineered narrow host range fungi that incorporate genes for scorpion and/or spider toxins, thereby greatly increasing their ability to kill some of the worst agricultural and disease

vector pests. We have also used genes from other fungi as well as extremophile bacteria in order to greatly increase fungal survivability in host environments. In field trials with varied transgenic fungi, we have showed that their application to corn seeds boosts yield by >35%. We are currently investigating long-term issues such as the evolvability of transgenic microbes, and the needs, feasibilities, and realities of monitoring, detecting and culling engineered microbes that escape or are deliberately released into the environment. We are also working, particularly in Burkina Faso, with developing integrated control strategies based on community engagement, in order to translate laboratory discoveries to the field.

- Aditya Kunjapur, University of Delaware

    - Risk Assessment for a Microbial Biocontainment Technology with Unprecedented Effectiveness: Synthetic Auxotrophy

    The incorporation of non-standard amino acids (nsAAs) within protein sequences offers the benefits of an expanded repertoire of chemical functionality in proteins, a means to control protein expression at the translational level, and a means to achieve biological containment of an organism by synthetic auxotrophy. Synthetic auxotrophy is when an organism is engineered to depend on a synthetic nutrient for its growth. It has been engineered in the model bacterium *Escherichia coli*, which does not exhibit observable escape when a particular combination of three auxotrophic markers is chosen. However, the effectiveness of synthetic auxotrophy outside of batch mono-culture contexts is unknown, as is the ease of transferring nsAA incorporation technology to other industrially relevant bacteria. This talk highlighted our latest characterization of *Escherichia coli* synthetic auxotrophs, which includes continuous evolution to evaluate the long-term stability of synthetic auxotrophy and direct addition to mammalian cell cultures to evaluate the robustness of synthetic auxotrophy in new contexts. Finally, this talk also described our successful efforts to engineer nsAA incorporation in the gram-positive microbe *Bacillus subtilis*. *B. subtilis* is a model system for both the study of bacterial cell biology and for industrial uses as a probiotic, rhizobacterium, efficient protein secretor, and spore-former. To enable greater understanding and control of proteins in *B. subtilis*, we achieved incorporation of many distinct non-standard amino acids within proteins. We used these systems to achieve click-labelling, photo-crosslinking, and translational titration. Synthetic auxotrophy in *E. coli* and eventually in *B. subtilis* could serve as an enabling safeguard for biochemical production in microbes applied in new contexts.

- Konstantinos T. Konstantinidi, Georgia Tech, EPA STAR Grant PI

    - Metagenome-Based Comparisons of Decay Rates and Host-Specificity of Fecal Microbial Communities for Source Trackings

    Nucleic acid based approaches for identifying and tracking microbes through the environment, including those that are products of synthetic biology (synbio), promise high levels of sensitivity and throughput. Further, these approaches are well suited to assess the ecological effects of synbio organisms on the natural microbial communities, including whether such organisms are capable of passing their genes to indigenous populations. However, nucleic acid based approaches are not without challenges. For instance, while several bioinformatics methods have recently been proposed to determine in situ abundance of a target organism or gene and determine its presence

or absence (i.e., limit of detection), these methods have not yet been standardized, and it is not clear how the data from different approaches can be compared. Further, detection of a target organism in a background of a complex microbial community such as those that are typically found in environmental samples remains challenging because the frequency of false-positives remains essentially unknown. False-positives may result from inadequate identification of regions of the genome that are either too highly conserved to be diagnostic (e.g., rRNA genes) or prone to frequent horizontal genetic exchange (e.g., mobile elements). To overcome the false-positive signal, a large library of sequence data representing different pollution inputs (e.g., sewage vs. animal feces vs. synbio organisms) need to be built and curated. Such a library will also help to advance the highly-related field of Microbial Source Tracking (or MST) (i.e., identification of the sources of pollution), which is central to the mission of environmental compliance agencies. In this talk, I will summarize our efforts to develop such a bioinformatics pipeline and its associated library of genome sequences and discuss the validation of the pipeline based on laboratory freshwater mesocosms perturbed with material from specific (known) pollution sources such as animal feces and sewage, to simulate contamination events. Our approach allowed source partitioning, or the ability to estimate to what extent a source(s) is contributing to the contamination present. These capabilities represent a novel problem-solving framework that may be especially useful to site-specific environmental monitoring of not only fecal pollution but also monitoring of the release of synbio organisms into the environment.

- Tae Seok Moon, Washington University in St. Louis, EPA STAR Grant PI

    - Multi-Input CRISPR-Based Kill-Switches for Engineered Microbes

    Probiotic microbes have become an effective framework for diagnostic and therapeutic technologies.[35, 36] However, there are safety concerns associated with using genetically engineered organisms for medical applications. Probiotic microbes have the potential to evolve growth advantages over natural microbes and characteristics that are harmful to the host or to the outside environment. To mitigate these concerns, we engineered the probiotic *Escherichia coli* Nissle 1917 to survive only when and where it is needed using CRISPR-based kill-switches (CRISPRks).
    We first designed a CRISPRks that induces cell death by expressing Cas9 and genome-targeting guide RNAs in response to the chemical inducer anhydrotetracycline. This design allows cell killing to occur while the microbe is in the gut in response to oral administration of the chemical. We optimized the efficiency and stability of the CRISPRks by combining four genomic Cas9 expression cassettes with three plasmid-based guide RNA expression cassettes, removing the antibiotic dependence for maintenance of the guide RNA plasmid, and knocking out genes involved in DNA recombination and mutagenesis. Using this optimized circuit in vitro, we achieved more than a 9-log reduction in cell number and demonstrated genetic stability for up to 28 days of continuous growth. This high killing efficiency was maintained in vivo, where we achieved complete elimination of the probiotic 24 hours after oral administration of the inducer. This is the first time on-demand elimination of an engineered microbe has been demonstrated in vivo. We next modified our chemically inducible-CRISPRks to also induce cell death in response to ambient temperatures below 33'C. This two-input design induces cell killing either in response to oral administration of the chemical or when the microbe is excreted from the body in response to the reduced environmental

temperature. This two-input circuit achieved more than a 9-log and 7-log reduction in cell number in vitro after exposure to the chemical inducer and temperature downshift, respectively.[37] Future directions will include incorporating the CRISPRks in microbes engineered to diagnose and treat diverse medical conditions.[35, 36] Our CRISPRks strategy provides a template for future microbial biocontainment circuits. The sensor and killing mechanism employed in the kill-switch are well characterized and functional in many microbes, allowing the CRISPRks design to be broadly utilized. In addition, the temperature-sensing module can be easily replaced with sensors that recognize alternative signals, allowing comparable kill-switches to be created for applications beyond medicine.

- Wayne G. Landis, Ethan A. Brown, Steven Eikenbary. Institute of Environmental Toxicology and Chemistry. Huxley College of the Environment, Western Washington University.

    - Scenario Specific Ecological Risk Assessment of Synbio Using the Bayesian Networks and Spatially Specific Ecological Modeling

    The approach of this presentation was to report scenario specific case studies for the risk assessment framework as outlined in NASEM (2017) [6] Chapter 6. In this chapter the Source-Stressor-habitat (location)-Effect-Impact model was applied to the risk assessment for organisms with gene drives in order to estimate both the efficacy of their use and risks to non-target endpoints.[38] Two different scenarios were examined: (1) the use of a gene-drive mouse to control invasive rodents on South Farallon Island, and (2) the use of a gene-drive mosquito to reduce the incidence of human disease in Ponce, Puerto Rico. Both case studies are hypothetical in nature-but both used site-specific information as to habitat, distribution of the organisms in the study sites, and the use of pesticides in the region. The MGDriveE model, developed by Sánchez et al.[39] and modified for the specific species was used to estimate the population dynamics of the gene drive and the host population. The Bayesian network relative risk model (BN-RRM) [40] was the conceptual structure used to estimate risk.

    In the case of the mouse-Farallon island scenario it was assumed that a CRISPR-Cas9 gene drive would be used to transmit the Sox9 gene to females causing sterility. The goal was to eradicate the mouse population to a level as to not provide food for the burrowing owls. The burrowing owls are attracted to the island because of the high mouse population, but they consequently feed on the Ashy Storm Petrel, a threatened species. The BN-RRM model assumed 95 percent homing for the gene drive and that no rodenticide was applied to the island. After 10 years it was found that the mouse population did reach the goal of having a 95 percent probability of reaching a zero state.

    In the case of the mosquito-disease-Ponce scenario the goal was to reduce the incidence of Dengue and Zika in the region. Both diseases are carried by Aedes aegypti. The study area has extensive information on land use, habitat, disease incidence, human habitation and other factors used to build the Bayesian network-based model. Information was also available on pesticide use. Different homing rates for the gene drive introduced to the mosquitos to create sterility and the frequency of release. Resistance to the drive was also incorporated into the model. There were 36 different combinations of input variables, and each scenario was run 50 times using MGDriveE parameterized with appropriate error terms. A probability distribution of each outcome was derived. The most important factor in the risk analysis was found to be the accuracy of the homing drive, since inserts not in the same reading frame cause resistance to the drive.

The drive did cause a decrease in incidence of Dengue to below the threshold 80 percent of the time. Pesticide use slightly improved this probability to 82 percent. However, resistance to the drive remains an issue. A primary uncertainty in this analysis is the lack of information on the ecological effects of the reduction in mosquito populations has to other wildlife or to agriculture in the region.

These studies do demonstrate the feasibility of estimating the probabilities of outcomes when a gene drive is inserted into a wild population. In calculating the impacts on wildlife, the issues seem to be (1) an understanding of the performance of gene drives in the wild and (2) a lack of basic ecological information on how the alterations in the population dynamics of the target affects the non-target species.

- Omar S. Akbari, University of California, San Diego, EPA STAR Grant PI

    - Development of a Data-Driven Model for Assessing Benefits and Risks of the pgSIT Approach for Aedes aegypti Eradication in Hawaii

    In this presentation our team describes the six main objectives of our EPA funded project. These consist of, Objective 1: Develop a transgene detection technology to aid in monitoring performance and risk assessments of synthetic technologies in the field; Objective 2: Collect mosquito life history parameters for Hawaii strain; Objective 3: Collect ecological data in a candidate field site; Objective 4: Collect biotic and abiotic environmental data (meteorological + landscape) and assess the relationship of these variables to the relative density of *A. aegypti*; Objective 5 Modeling release scenarios to build confined small-scale field release scenarios; Objective 6: Risk assessment to address gaps in knowledge for further study.

- Natalie G. Farny, Worcester Polytechnic Institute, EPA STAR Grant PI

    - Microbial Community Models for Measuring Survival and Persistence of SynBio Microbes in Soil

    Many synbio organisms have been developed in recent years for soil-related applications including biosensing, bioremediation, and pathogen control. However, it is unclear how to deploy these synbio solutions safely, as it is difficult to predict survival and persistence of a genetically engineered microbe (GEM) in the soil environment. The overarching goal of the work presented here is to understand, predict, and control the relationships between natural soil microbial communities (SMCs) and GEMs. We hypothesize that changes in dominant SMC species affect the survival and persistence of GEMs, and that understanding these relationships will permit us to manipulate SMCs to control GEMs. Here we describe the problem of GEM survival and persistence in a native soil environment, and demonstrate the rapid decline of engineered organisms in soil. Further, we have shown the poor performance of genetic circuit function in soil and soil-simulated conditions. Our future experiments will aim to: 1) to apply our current understanding of the composition of natural SMCs to create laboratory model SMCs to measure survival and persistence of GEMs; and 2) Assess the capacity of dominant SMC phylotypes to control the survival and persistence of GEMs. We will further discuss plans investigate the possibility of applying native soil microbes as a biocontainment strategy for GEMs. Our work aims to achieve safer and better-

informed deployment of GEMs for contaminant detection and bioremediation of contaminated soils, resulting in improved human and environmental health.

- Cresten Mansfeldt, University of Colorado, Boulder, EPA STAR Grant PI
    - Structuring EcoGenoRisk : Bioinformatic Mining of Expanding Genomic Databases to Predict Ecological Hazards and Risks

    Synthetic biological (synbio) products pose a unique challenge for effective assessment and mitigation of risks. This project focuses on developing an ecological risk assessment for the release of a full synthetic microbial cell (a subset of all synbio organisms) into an environmental matrix by developing EcoGenoRisk, a Python-based bioinformatic tool. This software package will satisfy three primary aims: (1) identify those genomes within public databases that negatively respond to the product, display the most similarity genetically to the synbio microorganism, and/or harbor similar or competing pathways, in combination, defining the susceptible populations; (2) identify environmental habitats that are likely to harbor the susceptible populations; and (3) quantify both the likelihood of the synbio microorganism to arrive in the susceptible habitat and the extent of structural or functional disruption experienced by the resident microbial community. Therefore, the development of EcoGenoRisk focuses on three subfunctions: HazID, EnvCen, and RiskQ. HazID identifies the susceptible community members within a population. EnvCen then quantifies the population of different environmental matrices that are likely susceptible. Finally, RiskQ then quantifies the overall ecological risk of a synbio microorganism released into various environmental matrices. Each of these functions requires database development and curation, employing the resources of public bioinformatic and chemoinformatic archives. Combined, EcoGenoRisk will provide the user with an ability to query genetic databases for predicted risks. The open-source development of EcoGenoRisk will ensure that users may incorporate the software into other bioinformatic pipelines and link with existing EPA ecological risk assessment tools.

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