8-1-2022

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Regulatory Toxicology and Pharmacology 133 (2022) 105195

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

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https://doi.org/10.1016/j.yrtph.2022.105195
Received 12 November 2021; Received in revised form 19 May 2022; Accepted 25 May 2022
Available online 2 June 2022

ARTICLE INFO

Handling Editor: Dr. Lesa Aylward

Keywords:
Animal testing
Aquatic toxicology
Ecotoxicology
Ecotoxicity testing
New approach methodologies
Non-animal methods
Regulatory requirements
U.S. federal agencies

ABSTRACT

U.S. regulatory and research agencies use ecotoxicity test data to assess the hazards associated with substances that may be released into the environment, including but not limited to industrial chemicals, pharmaceuticals, pesticides, food additives, and color additives. These data are used to conduct hazard assessments and evaluate potential risks to aquatic life (*e.g.*, invertebrates, fish), birds, wildlife species, or the environment. To identify opportunities for regulatory uses of non-animal replacements for ecotoxicity tests, the needs and uses for data from tests utilizing animals must first be clarified. Accordingly, the objective of this review was to identify the ecotoxicity test data relied upon by U.S. federal agencies. The standards, test guidelines, guidance documents, and/or endpoints that are used to address each of the agencies' regulatory and research needs regarding ecotoxicity testing are described in the context of their application to decision-making. Testing and information use, needs, and/or requirements relevant to the regulatory or programmatic mandates of the agencies taking part in the Interagency Coordinating Committee on the Validation of Alternative Methods Ecotoxicology Workgroup are captured. This information will be useful for coordinating efforts to develop and implement alternative test methods to reduce, refine, or replace animal use in chemical safety evaluations.

Abbreviations

ADC	Animal Damage Control Act
APHIS	USDA Animal and Plant Health Inspection Service
ARS	USDA Agricultural Research Service
AWIC	ARS Animal Welfare Information Center
BGEPA	Bald and Golden Eagle Protection Act
CERCLA	Comprehensive Environmental Response, Compensation, and Liability Act
CFR	U.S. Code of Federal Regulations
CWA	Clean Water Act
DOD	U.S. Department of Defense
DOI	U.S. Department of the Interior
EcoWG	ICCVAM Ecotoxicology Workgroup
EDSP	Endocrine Disruptor Screening Program
EPA	U.S. Environmental Protection Agency
EPA-HQ	EPA Headquarters
FDA	U.S. Food and Drug Administration
ESA	Endangered Species Act
FFDCA	Federal Food, Drug, and Cosmetic Act
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FLPMA	Federal Land Policy and Management Act of 1976
FQPA	Food Quality Protection Act
FS	USDA Forest Service
GMA	General Mining Act of 1872
ICCVAM	The Interagency Coordinating Committee on the Validation of Alternative Methods
ISO	International Organization for Standardization
MPRSA	Marine Protection, Research, and Sanctuaries Act
MBTA	Migratory Bird Treaty Act
NAM	New Approach Methodologies
NCR	National Research Council
NEPA	National Environmental Policy Act
NPDES	National Pollutant Discharge Elimination System
NIEHS	National Institute of Environmental Health Sciences
NIST	National Institute of Standards and Technology
NICEATM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
NRI	natural resource injury
NTP	National Toxicology Program
NWRSAA	National Wildlife Refuge Systems Administration Act
NWRC	APHIS National Wildlife Research Center
OCLSA	Outer Continental Shelf Lands Act
OCSPP	EPA Office of Chemical Safety and Pollution Prevention
OECD	Organisation for Economic Co-operation and Development
OPA	Oil Pollution Act of 1990
OPP	EPA Office of Pesticide Programs
OPPT	EPA Office of Pollution Prevention and Toxics
ORD	EPA Office of Research and Development
OST	EPA Office of Science and Technology
OW	EPA Office of Water
OWM	EPA Office of Wastewater Management
QPL	Qualified Products List
SMRCA	Surface Mining Control and Reclamation Act
SDWA	Safe Drinking Water Act
TMDLs	Total Maximum Daily Loads
Tox21	Toxicology in the 21st Century
TSCA	Toxic Substances Control Act
USDA	U.S. Department of Agriculture
USGS	U.S. Geological Survey
WET	whole effluent toxicity
WQS	water quality standards

1. Introduction

Multiple agencies of the United States (U.S.) federal government are charged with protecting human and animal health, natural resources, and/or the environment (16 U.S.C. § 661-667e, 1934; Fairbrother, 2009) and/or assessing the impact of human activity on the environment (42 U.S.C. § 4321 et seq., 1969). These agencies include, but are not limited to, the U.S. Department of Defense (DOD), U.S. Department of the Interior (DOI), U.S. Environmental Protection Agency (EPA), U.S. Food and Drug Administration (FDA), National Institute of Environmental Health Sciences (NIEHS), and the U.S. Department of Agriculture (USDA). Other agencies, like the National Institute of Standards and Technology (NIST), develop and use reference materials and standards related to measurements of environmental quality.

To carry out these activities, the federal agencies determine the hazards and risks presented by substances that may enter the environment, including but not limited to industrial chemicals, pharmaceuticals, pesticides, food additives, and color additives. Where critical data are absent, agencies use standardized ecotoxicity tests to assess hazard, risk, and environmental impacts. These tests are currently performed on live organisms using U.S. standardized and internationally harmonized test methods. Such testing has been the backbone of chemical safety assessments for decades and has served the purpose of gaining an understanding of chemical toxicities to inform regulatory decision-making.

Ecotoxicity tests include a broad spectrum of procedures, with differing species, exposure media, and effects measurements. In this

context, the standard ecological toxicity tests can be used to demonstrate whether contaminants are bioavailable, assess toxic effects of individual chemicals and the aggregate toxic effects of all contaminants in a medium (*e.g.*, discharged effluent from a facility into a receiving water), and can characterize the nature of a toxic effect on the organism *(e.g.*, survival, reduced growth, impaired reproduction, and behavioral changes).

For the most part, the vast array of aquatic toxicity tests is highly standardized, straightforward to conduct, and have been widely used since the 1970s, while standardized sediment toxicity test procedures must also consider bioavailability in different sediment types. Soil testing with invertebrates and standard soils is well established, but higher order terrestrial organism tests with plants and vertebrates are more difficult and more expensive to conduct. Results generated from these standardized tests are used for many regulatory practices such as evaluating new chemical registration, evaluating potential toxicity of existing chemicals in commerce, developing remedial goals, application in developing water quality criteria, and monitoring in the environment. Although these tests have proven to be useful for informing U.S. regulatory decision-making, tests in vertebrates and invertebrates used for evaluating chemical product registrations are resource intensive and raise ethical concerns associated with using animals for this purpose. Given the large number of chemicals produced each year, it is difficult to keep pace with chemical safety evaluations using these traditional test methods which have long been recognized as a limitation for risk assessments due to advances in the development and rapid production of new chemistries.

Furthermore, test methods are developed and standardized with specific organisms that have been selected to serve as model organisms, typically chosen for their availability, adaptability to laboratory testing, potential to be tested at different life stages, low-cost of maintenance, historical data, their potential to serve as representatives of broader populations and life cycles. The choice of model species should consider their "domain of applicability" and conservation or the sharing of toxicity-relevant biological traits between model species and ecological target species" (Segner and Baumann, 2016). This remains a challenge in the use of model organisms in toxicity testing where it is traditionally assumed that the test organism is representative of other species based on a qualitative understanding of species relatedness.

The focus of this paper is to identify U.S. federal agency applications, the need for, and/or requirements for ecotoxicity testing methods. The identification of the routinely used methods in ecotoxicology is an important step toward identifying and prioritizing potential tests or toxicities that may be targeted for developing alternative methods. This review was prepared by the Ecotoxicology Workgroup (EcoWG) under the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) (National Toxicology Program, 2021a). Among ICCVAM's member agencies are those that have statutory mandates to protect the environment and biota such as plants, invertebrates, fish, and wildlife, including threatened and endangered species, as well as agencies interested in assessing the effects of chemicals on diverse species as part of broader research or operational goals.

Many of the tests used to assess hazard and risk are currently performed on live organisms and may cause pain or distress. Federal facilities conducting ecotoxicity testing are required to comply with the regulatory requirements and guidelines for humane animal care depending on species and funding. These include but are not limited to: the Animal Welfare Act (AWA (7 U.S.C. § 2131 et seq., 2012),); U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training (NIH OLAW, 2018); the Public Health Service Policy on Humane Care and Use of Laboratory Animals (Office of Laboratory Animal Welfare, 2015); the "Guide for the Care and Use of Laboratory Animals" (National Research Council, 2011); and the "Guide for the Care and Use of Agricultural Animals in Research and Teaching" (Federation of Animal Science Societies, 2010).

An Institutional Animal Care and Use Committee (IACUC) has oversight of the live animal work under the AWA, PHS policy, and other applicable regulations and guidelines. The IACUC reviews and approves work conducted with live animals. As part of the process, the IACUC has imperatives to ensure pain and distress are minimized as much as scientifically justifiable while accomplishing ecotoxicity testing needs (Carbone, 2011, 2019). The IACUC is also required to ensure alternatives to procedures that cause greater than momentary or slight pain/-distress are considered by the Principal Investigator (9 CFR § 2.31 (d)(1)(ii), 2004).

Ecotoxicology work with wildlife species may also require compliance with the other Federal regulations such as the Endangered Species Act; and compliance with State regulations to obtain permits.

An approach to toxicity testing envisioned to be more efficient, predictive, and economical than animal use was proposed over a decade ago by the National Research Council (National Research Council, 2007, and has gained international support (Andersen and Krewski, 2009; Krewski et al., 2014). This approach, which uses *in chemico*, *in vitro,* and *in silico* new approach technologies/methodologies (NAMs) that can inform hazard and risk assessments, has been adopted by the U.S. Interagency Toxicology in the 21st Century (Tox21) Consortium (Tox 21 Consortium, 2020). Use of NAMs is gaining acceptance for some regulatory testing applications including endocrine activity (U.S. EPA, 2015a) and skin sensitization (U.S. EPA, 2018).

U.S. government activities to support the development of NAMs and increase confidence in their use for a broad range of U.S. regulatory needs are being guided by the 2018 "Strategic Roadmap for Establishing New Approaches to Evaluate the Safety of Chemicals and Medical Products in the United States". Development of the Strategic Roadmap was coordinated by ICCVAM. ICCVAM facilitates the development, validation, and regulatory acceptance of NAMs and other approaches that replace, reduce, or refine (Russell and Burch, 1992; Tannenbaum and Bennett, 2015). ICCVAM membership is comprised of 17 U.S. federal agencies that use, generate, or disseminate toxicological and safety testing information(National Toxicology Program, 2021b). ICCVAM's EcoWG is actively pursuing the application of NAMs to ecotoxicity testing scenarios.

The Strategic Roadmap (ICCVAM, 2018a) describes three goals to be addressed in development and implementation of NAMs:

- Connecting end-users with the developers of NAMs,
- Fostering the use of efficient, flexible, and robust practices to establish confidence in new approach methods, and
- Encouraging the adoption and use of NAMs and other approaches by federal agencies and regulated industries.

To address the goals of the Strategic Roadmap, implementation plans for toxicity areas are developed (ICCVAM, 2018b). While such summaries of testing and information needs have been developed for nanomaterials (Petersen et al., 2021), human acute systemic toxicity (Strickland et al., 2018), skin and eye irritation (Choksi et al., 2019), and skin sensitization (Strickland et al., 2019), no such summary exists for ecotoxicity.

In this paper, the EcoWG (which is sponsored by DOD, DOI, and EPA, and includes representatives from these agencies as well as from FDA, NIEHS, NIST, and USDA) defines specific ecotoxicity testing and information gathering approaches relevant to the regulatory or programmatic mandates of the agencies participating in the EcoWG. This summary is not intended to be a compendium of all statutes which require testing, or all test methods used to evaluate toxicity to ecosystems, nor is it intended to be a complete survey of all U.S. agencies, offices, or divisions that require or utilize ecotoxicity testing. However, by collating this information, we believe that the U.S. and international efforts to develop and implement alternative methods for ecotoxicity testing will be enhanced, along with harmonization of ecotoxicity testing and regulatory requirements.

Table 1
U.S. statutes and regulations which consider ecotoxicology test data and applicable agencies.

U.S. statute/regulation[a]	Abbreviation	Applicable Agency
Animal Damage Control Act[b]	ADC	DOI, USDA
Bald and Golden Eagle Protection Act	BGEPA	DOI, USDA
Comprehensive Environmental Response, Compensation and Liability Act	CERCLA	DOD, DOI, EPA
Clean Water Act	CWA	DOD, DOI, EPA
Endangered Species Act	ESA	DOI, EPA, USDA
Federal Insecticide, Fungicide, and Rodenticide Act	FIFRA	DOI, EPA, USDA
Federal Land Policy and Management Act of 1976	FLPMA	DOI
Food Quality Protection Act	FQPA	EPA
General Mining Act of 1872	GMA	DOI
Marine Protection, Research and Sanctuaries Act	MPRSA	DOD
Migratory Bird Treaty Act	MBTA	DOI
National Environmental Policy Act	NEPA	DOI, FDA, USDA
National Wildlife Refuge System Administration Act	NWRSAA	DOI
Oil Pollution Act of 1990	OPA	DOD, DOI, EPA
Outer Continental Shelf Lands Act	OCLSA	DOI
The Organic Act Establishing the U.S. Geological Survey as a Research Entity	None	DOI
Toxic Substances Control Act	TSCA	EPA, USDA

[a] Copies of the laws cited in this table can be obtained from web locations available in Table S1.
[b] On August 1, 1997, the Animal Damage Control program was officially renamed to Wildlife Services (Hawthorne, 2004).

2. Use of ecotoxicity data by select U.S. federal agencies

EcoWG members were surveyed to determine which statutes, guidelines, and methods were relevant to their agencies. Responses included tests conducted in single celled organisms such as algae and cyanobacteria as well as plants. Since one of the objectives of this document is to identify opportunities for regulatory uses of non-animal replacements for ecotoxicity tests, single celled organismal and plant tests were not included in the detailed results of that survey which are provided in the supplemental materials (Tables S1 and S2) and are summarized in Table 1 and Table 2. Eighteen different U.S statutes were identified that either require or make use of ecotoxicity data (Table 1). While several of these statutes govern the activities of a single agency, others are more broadly applicable to the activities of multiple federal agencies. To address these statutory requirements, an even greater number of U.S. and international ecotoxicity test guidelines and guidance documents have been developed. These tests include invertebrates and vertebrates (Table 2), and the majority are used to identify risks to aquatic, avian, or terrestrial organisms. Further details of the statutes and regulations under which these tests are carried out, along with the scope and endpoints measured by each of the tests are included in Table S1.

The test guidelines in Table S2 are broadly divided into toxicity endpoint groups (*e.g.*, acute toxicity, bioaccumulation, etc.), and taxa (*e.g.,* amphibian, avian, fish, etc.). Table 2 represents the number of tests per endpoint that use representatives of a given taxa. Chronic/Growth/Reproduction and Acute toxicity tests in invertebrates, fish, pollinator, and avian species are most commonly requested across the U.S. federal agencies, followed by bioaccumulation tests which use organisms in diverse taxa. EPA requests the majority of ecotoxicity test data conducted using the guidelines listed in Table S2.

There are a variety of testing and information requirements based on diverse scenarios addressing different agency needs. These differing needs are discussed in the subsequent sections.

2.1. U.S. Department of Defense

2.1.1. Department of the Air Force

The Air Force performs natural and cultural resource management and evaluates environmental stressors under authority of the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA (42 U.S.C. § 9601 et seq., 1980);); Clean Water Act (CWA (33 U.S.C. § 1251–1387, 1972);); the National Environmental Policy Act (NEPA; (42 U.S.C. § 4321 et seq., 1969); the Oil Pollution Act (OPA; (33 U.S.C. § 2701 et seq, 1990)); and the Resource Conservation and Recovery Act (42 U.S.C. § 6901 et seq., 1976) (Table 1).

The Air Force evaluates potential threats and impacts to human health and the environment for emerging contaminants through the Environmental Impact Analysis Process (32 CFR § 989, 1999). The challenge with the Air Force's ecotoxicity needs is that they are specific to individual bases, sites, and scenarios.

2.1.2. Department of the army

The Army has many needs for understanding the toxicity of environmental stressors on ecological species (CERCLA, OPA, NEPA, and CWA Table 1).

The Army uses ecotoxicity testing to understand the potential hazards of new materials, including energetic compounds, compounds used in signaling and obscurants, and nanomaterials. The specific compounds and tests to be performed are determined on a case-by-case basis depending on the needs and the potential uses. Ecotoxicity testing can also be required for site assessment, which is also determined on a case-by-case basis. An example of Army ecotoxicity activities would be the evaluation of dredged material proposed for disposal under the Marine Protection, Research and Sanctuaries Act to prevent toxicity and bioaccumulation that could affect human health and the environment (33 U.S.C. §1401 et seq., 1988). There are other uses for ecotoxicity data within the military mission; however, these are very specific to those programs and those needs may change on an ongoing basis.

The Army performs natural resource damage assessments (U.S. Army, 2020) to identify natural resource injuries (NRI) that are regulated under CERCLA, OPA, and CWA. A NRI is defined as any adverse and measurable change to a natural resource, where the term natural resource is defined to include land, fish, wildlife, biota, water, air, groundwater, drinking water supplies, and other similar resources. This definition creates a nexus with the Endangered Species Act (ESA; (U.S. EPA, 2014a)) when the NRI may involve or affect threatened and

Table 2
Number of test guidelines by type/taxa.

Test Type[a]	Amphibians	Aves	Bees	Fish	Invertebrates	Mammals	Cross-taxa	Number of Test Guidelines by Test Type
Acute		2	4	7	12	2		27
Bioaccumulation				2	2		1	5
Chronic/Growth/Reproduction	4	3	2	14	25	6		54
Field testing			2				2	4
Microcosm							3	3
Number of Test Guidelines by Taxa	4	5	8	23	39	8	6	

[a] The numbers presented for each taxa represent the number of test guidelines per test type that use members of the specified taxa, e.g., there are two acute toxicity test guidelines that use mammals.

endangered species directly or indirectly.

As noted for the Air Force, the Army's ecotoxicity assessment needs tend to be scenario specific. For instance, the Army may want to develop a training area for long-range precision fires (*e.g.*, artillery). To do that, the Army will study the natural resources in the area and determine whether these new activities will result in a nuisance or hazard to any existing threatened and endangered species. The Army seeks to limit its liability under CERCLA, which establishes responsibility for remediation of releases of chemicals that may affect public health or the environment. As a result, the Army will need to perform some ecotoxicity testing to ascertain the level of injury that may result to species of concern. With threatened and endangered species, this becomes even more challenging, as there may not be enough animals to use for testing without causing a significant impact to the existing population. However, in rare instances testing may be undertaken with appropriate permissions. Thus, any alternatives to the use of animals will help fill knowledge gaps that may not be filled through species extrapolation from currently available test methods.

2.2. U.S. Department of the interior

The mission of the DOI is broad and includes the generation of scientific information to assist in the conservation and management of the nation's natural resources. DOI acts as the steward of roughly 20% of the Nation's lands through management of national parks, wildlife refuges, and other land management units. Of its nine technical bureaus, at least two conduct ecotoxicity tests (U.S. Geological Survey, U.S. Fish and Wildlife Service), and others (*e.g.*, Bureau of Land Management, Bureau of Reclamation, National Park Service) either conduct ecotoxicity tests directly or indirectly use such data in natural resource management decisions.

Part of its stewardship responsibility requires DOI to play a major role in the management of fish, wildlife, and threatened and endangered species. DOI conducts a wide array of ecotoxicological research, damage assessment, restoration, and registration studies under no fewer than 20 statutes and regulations. The primary drivers of these activities are CERCLA, migratory bird hunting regulations (16 U.S.C. § 703, 1918; 50 CFR § 20.134, 1996), the Bald and Golden Eagle Protection Act (16 U.S.C. § 688-688d, 2018), NEPA, OPA, CWA, ESA (Table 1), and the Surface Mining Control and Reclamation Act (30 U.S.C. § 1201-1328, 1977; DOI, 2018).

Under CERCLA, CWA, and the OPA, the DOI Natural Resource Damage Assessment and Restoration Program (DOI, 2015) identifies injury to resources. Activities include (but are not limited to) biomonitoring for contaminant exposure and potential adverse effects in field settings and detailed toxicological characterizations of environmental contaminants and polluted matrices (*e.g.*, water, sediment, effluent, soil) in controlled exposure studies with invertebrates, fish, and wildlife. The data generated are used by the Department of Justice in establishing claims upon a responsible party and determining the nature of restoration, rehabilitation, replacement, or acquisition of the equivalent, of the natural resources.

DOI has regulatory authority for registration of alternative shot and shot-coatings that replace the highly toxic lead historically used in the hunting of waterfowl and coots (DOI, 2013; Perry et al., 1997). The tiered-testing protocol conducted by registrants generates data that are submitted for review to the U.S. Fish and Wildlife Service at various stages of the approval process. The protocol incorporates concepts of reduced animal use in the testing, review, and registration process, as existing data can be used to approve the candidate shot or shot-coating for use.

In view of the uncertainty in extrapolating potential adverse effects of contaminants among taxa (see Section 3.1), DOI and in particular the U.S. Geological Survey, undertakes exposure and effects studies with model invertebrate, fish, and wildlife species to determine actual versus perceived hazard of a range of environmental contaminants (*e.g.*, pesticides, industrial compounds, pharmaceuticals, metals). Such studies use statistical methods and designs to minimize the number of animals, frequently incorporating sublethal and minimally invasive endpoints to obtain comprehensive toxicity and mechanistic data. Some endpoints require whole animal tests (*e.g.*, avian eggshell thinning, flight energetics). However, several ongoing activities use cell-based or early life-stage systems for which test species are not classified as animals by current statutes. In addition, DOI undertakes field biomonitoring efforts with invertebrates, fish, and wildlife to obtain exposure and effects data on natural resources in various settings where the potential for pollution is substantial. Such field biomonitoring studies often utilize sublethal, minimally-invasive and even non-invasive sampling from biota to assess exposure and adverse effects.

DOI also has responsibility for restoring and preserving fish and freshwater mussel populations in the United States and has a nationwide system of hatcheries to carry out this responsibility. Research and product approval activities on therapeutic agents for use in aquaculture have been undertaken in the past. The array of laboratory studies and clinical field trials required for product approval by FDA can be costly to undertake and market demand is limited. There is, however, an ongoing effort for development of pest and invasive fish control agents that seeks to replace traditional toxicity tests using whole fish assays with a high-throughput alternative to screen compounds. This effort entails an initial *in silico* step of pre-screening a chemical databank to select molecules possessing characteristics identified as predictive criteria for potential toxicity to various species of fish, followed by cytotoxicity screening in fish cell lines. This two-stage procedure is being used to identify species-specific candidates for detailed animal or acceptable alternative methods testing.

2.3. U.S. Environmental Protection Agency

EPA administers several environmental statutes to ensure protection of human health and the environment and is responsible for maintaining and enforcing national standards under applicable environmental laws and working with states and tribes who enforce state and tribal laws (U.S. EPA, 2013a). The two primary offices within EPA that implement environmental statutes for which toxicity data generated on ecological species are considered and, in some cases, required to meet regulatory requirements are the Office of Chemical Safety and Pollution Prevention (OCSPP) and the Office of Water (OW). Together, these offices work to protect the environment from potential risks from pesticides, toxic chemicals, and other compounds. The laws that are important drivers of ecological effects testing include: the Federal Fungicide, Insecticide, and Rodenticide Act (7 U.S.C. § 136 et seq, 1996)(FIFRA), the Toxic Substances Control Act (TSCA (15 U.S.C. § 2601 et seq., 2016);), and the Clean Water Act (33 U.S.C. § 1251 101 (a) et seq, 1972; 33 U.S.C. § 1251 102 et seq, 1972). In addition, the 1996 Food Quality Protection Act (FQPA (7 U.S.C. § 136, 1996);), amendments to the Federal Food, Drug, and Cosmetic Act (FFDCA (21 U.S.C. §301 et seq, 2002);), and the Safe Drinking Water Act (SDWA (42 U.S.C. § 201, 1996; 42 U.S.C. § 300f, 1974);) mandate that EPA screen chemicals for endocrine activity, which includes, at some screening tiers, whole animal ecotoxicity tests. To conduct this screening, EPA established the Endocrine Disruptor Screening Program (EDSP).

EPA program-specific ecotoxicity testing needs are described in more detail below. EPA's Office of Research and Development (ORD) developed the Agency's test procedures detailed in Title 40 of the Code of Federal Regulations (CFR) Part 136.3 (40 CFR § 136.3, 2002) and in final published guidance toxicity test methods. In some cases, these were reviewed by EPA Headquarters' (EPA-HQ) Office of Water and EPA Regional offices. These methods are used by the Office of Water,

including for regulation. While EPA ORD is not responsible for administering any environmental laws, it does use ecotoxicity data and may conduct *in vivo* testing for a variety of programs as well as for the development of NAMs as animal alternatives and validation of those methods.

2.3.1. Office of chemical safety and Pollution Prevention

OCSPP implements FIFRA and TSCA, as well as sections of FFDCA, via its program offices and uses ecological effects data in its regulatory decision-making. OCSPP program offices with ecotoxicity testing needs include the Office of Pesticide Programs (OPP) and the Office of Pollution Prevention and Toxics (OPPT).

2.3.1.1. Office of Pesticide Programs. OPP uses toxicity data in its ecological risk assessments to inform pesticide registration decisions and determinations of the effects of regulatory decisions on nontarget organisms including federally listed threatened and endangered species (U.S. Fish and Wildlife Service, 2020) under the ESA. OPP's procedures for conducting pesticide risk assessments are described in the "Overview of the Ecological Risk Assessment Process" (U.S. EPA, 2014a) and are typically based on the most sensitive species tested for each taxon. OPP grants a registration to allow a pesticide's distribution, sale, and use only after the application for registration meets the scientific and regulatory requirements. These data requirements apply to any person, entity, or any company that registers pesticides under FIFRA or seeks a tolerance or tolerance exemption under FFDCA.

In evaluating a pesticide registration application, OPP assesses a wide variety of potential environmental effects associated with use of the product. Registrants must generate scientific data necessary to define properties (*e.g.*, product chemistry, performance, toxicology, ecological effects, human exposure, spray drift, environmental fate) and potential adverse effects to a variety of taxonomic groups of organisms (Table 2). The data allow OPP to evaluate whether a pesticide could have adverse effects on nontarget organisms and federally listed threatened or endangered species, including terrestrial and aquatic vertebrates and invertebrates from exposure as a result of registered uses of a pesticide.

FIFRA provides EPA with considerable authority to establish or modify data needs and timing for individual pesticide registration actions to achieve statutory and program objectives. Data requirements for pesticide registration actions are codified in 40 CFR Part 158 (2012), informally referred to as "Part 158". These regulations provide OPP with substantial discretion to make registration decisions based on what OPP determines to be the most relevant and important data for each regulatory action.

The studies required under FIFRA Part 158 provide the scientific basis for effects characterization to evaluate the potential risks associated with specific pesticide uses. There are additional data "requirements" relevant to mandatory screening of pesticides for the potential for endocrine disruption under FFDCA 408(p)(3) unless a pesticide is exempted under FFDCA 408(p)(4). Table 2 represents the general breadth of requirements commonly encountered for registration decisions. There is considerable flexibility available to OPP in implementing Part 158; for example, additional data can be required (Section 158.75), alternative approaches can be accepted, and studies can be waived (Section 158.45). OPP's goal is to acquire adequate information to reliably support pesticide registration decisions that are protective of human health and the environment. This goal also includes avoiding the generation and evaluation of data that do not materially influence the scientific certainty of a regulatory decision and ensuring that high-quality science is used to support regulatory decisions while avoiding unnecessary use of time and resources, data generation costs, and animal testing. To address these goals OPP staff have been provided with "Guiding Principles for Data Requirements" to focus on the information most relevant to the assessment (U.S. EPA, 2013a,b).

OPP is also responsible for developing, maintaining, and evolving the EDSP with the goal to screen chemicals for potential endocrine bioactivity and interactions with hormone systems in humans and other nontarget vertebrate organisms. EPA utilizes a two-tiered screening approach. The Tier 1 battery of assays consists of five *in vitro* and six *in vivo* assays, four conducted in a model rat species, one conducted in a model fish species, and one conducted in a model amphibian species (U.S. EPA, 2008). Tier 2 consists of three non-mammalian test guidelines, which complete the 890 test guideline series, and also utilizes two existing mammalian test guidelines (U.S. EPA, 2015b).

There remain five *in vivo* Tier 1 assays without proposed NAMs (Table S2), which assess male rat reproductive toxicity (U.S. EPA, 2009a), female and male rat sexual maturation (U.S. EPA, 2009b; 2009c), fish reproduction (U.S. EPA, 2009d), and amphibian development (U.S. EPA, 2009e). Also included in Table S2 are all the Tier 2 tests, which include three non-mammalian 890 test guidelines (890.2100 Avian Two-Generation Toxicity Test in the Japanese Quail (U.S. EPA, 2015d), 890.2200 Medaka Extended One Generation Reproduction Test (U.S. EPA, 2015h), and 890.2300 Larval Amphibian Growth and Development Assay (U.S. EPA, 2015c)) and both mammalian test guideline options (EPA 870.3800 Reproduction and Fertility (U.S. EPA, 1998), and OECD 443 Extended One-Generation Reproductive Toxicity Study (OECD, 2018)). EPA may also accept other scientifically relevant information in lieu of 890 test guidelines to inform Tier 2 testing needs (U.S. EPA, 2009f).

EPA remains committed to and focused on the goals of the EDSP21 Work Plan to develop, validate, and adopt NAMs to screen chemicals for endocrine bioactivity faster and better, with lower cost and the use of fewer animals, while remaining protective of human and wildlife health (U.S. EPA, 2011).

2.3.1.2. Office of Pollution Prevention and Toxics. OPPT has authority under TSCA to regulate the manufacture (including import), processing, distribution in commerce, use, and disposal of chemical substances within the United States. OPPT uses data submitted under TSCA to carry out mandates including risk assessments and risk management activities. In characterizing the hazard of a new or existing chemical substance under specific conditions of use, OPPT considers effects on both human health and the environment. Special considerations are made for chemical substances or mixtures predicted to be persistent in the environment, bioaccumulative, and toxic (PBT). These substances present unique concerns to ecological species because they can remain in the environment for long periods of time and can accumulate in organisms. Certain substances regulated by other U.S. agencies or EPA offices under the authority of separate federal statutes are excluded from TSCA risk management, including, among others, color additives, drugs, food, and pesticides.

The 2016 Frank R. Lautenberg Chemical Safety for the 21st Century Act amended TSCA (15 U.S.C. § 2601 et seq., 2016) and expanded EPA's authority relating to chemical substances. The Lautenberg Act amendments require the Agency to:

- Make risk determinations and address identified unreasonable risks as required for new chemical substances before market entry;
- Prioritize and categorize existing chemical substances as low- or high-priority substances for risk evaluation;
- Perform risk evaluations on high-priority substances to determine if they pose an unreasonable risk of injury to health or the environment;
- Take regulatory action to address the identified unreasonable risks.

TSCA does not require a base set of human health or environmental effects data to be submitted with a new chemical substance submission or prior to evaluating risks of existing chemicals. However, TSCA does give EPA the authority under Section 4 (U.S. EPA, 2014b) to require

testing or information development, as necessary, for prioritization of chemicals or to assess risk for new and existing chemicals. Additionally, under Section 5(e) (U.S. EPA, 2015e), EPA can require testing of a new chemical substance prior to its commercialization. The types of ecotoxicity data considered most relevant for TSCA risk evaluations include aquatic toxicity data across several trophic levels (e.g., fish, invertebrates, and aquatic plants) and terrestrial toxicity data for at least two trophic levels (e.g., soil invertebrates and mammalian or avian species).

An additional legislative mandate added by the 2016 amendments to TSCA (TSCA Section 4(h)15 U.S.C. § 2603(h), 2016) requires EPA to consider non-traditional data and to promote the development and implementation of alternative test methods. Specifically, the amendments call for the reduction and replacement, to the extent practical and scientifically justified, of vertebrate animal use in toxicity testing. EPA is required to consider existing information before requesting tests using vertebrate animals (Section 4(h)(1)). While the amended TSCA does not identify the specific hazards for which animal alternatives should be considered, Section 4(h)(2)(A) directs EPA to consider NAMs before EPA requests or requires toxicity testing: 1) computational toxicology and bioinformatics, 2) high-throughput screening methods, 3) testing of categories of chemical substances, 4) tiered testing methods, 5) *in vitro (i.e.*, cell-based*)* studies, 6) systems biology, 7) new or revised methods identified by validation bodies such as ICCVAM or the Organisation for Economic Co-operation and Development (OECD), and 8) industry consortia that develop similar information or approaches. Consideration of NAMs in ecological hazard evaluations is ongoing for both new and existing chemical substances.

2.3.2. Office of Water

In the United States, the CWA has been the cornerstone of surface water quality protection, and OW has three program offices that implement the CWA. The Office of Science and Technology (OST), the Office of Wastewater Management (OWM), and the Office of Wetlands, Oceans and Watersheds (OWOW) that work with states, tribes, and other stakeholders to help ensure our nation's waters can be used for fishing, swimming, and drinking water and can support healthy and sustainable biological communities. OST does not require ecotoxicity testing but does manage several programs that utilize ecotoxicity data (*i.e.*, water quality criteria (U.S. EPA, 2015f, 2013c) and water quality standards (WQS) (U.S. EPA, 2014c)). OWOW manages the CWA program under which states develop Total Maximum Daily Loads (TMDLs) for waterbodies and submit them to EPA for review and approval or disapproval. TMDLs may use water quality criteria in the development of these TMDLs to ultimately achieve the water quality standards. OWOW also manages the listing program under CWA Section 303(d), under which states assemble and evaluate water quality-related data and information to determine whether water bodies are impaired and require a TMDL under applicable WQS. OWOW collaborates with states and tribes on water quality monitoring, supporting state and tribal monitoring and assessment programs under CWA Sections 106(e) and 305(b) to report on the extent of waters that support the CWA goal that water quality provides for healthy biological communities and recreational activities. OWOW programs do not require use of toxicity testing, but the results are incorporated into state assessment decisions when available. OWM oversees a range of programs promoting effective and responsible water use, treatment, disposal, and management, including the National Pollutant Discharge Elimination System (NPDES; (U.S. EPA, 2015g, 2014d)) regulatory and permitting program which requires whole effluent toxicity (WET) testing as part of its monitoring of permitted effluent discharges for determining the reasonable potential for excursions of state or tribal water quality standards (40 CFR Part 122.44(d)(1), 2003) and NPDES WET permit limit compliance monitoring (40 CFR Part 122.41(j), 2003).

Under CWA Section 304(a), EPA develops and publishes criteria for surface waters to protect various designated uses, including those associated with aquatic life. From time to time, these criteria, which are not regulatory, are revised based on the latest scientific knowledge. States and authorized tribes may adopt EPA CWA Section 304(a) criteria into their WQS or may adopt their own criteria that differ from EPA's recommendations using scientifically defensible methods, subject to EPA's approval. States implement EPA-approved criteria as part of their regulatory WQSs, and exposure is considered by states in permits and listing decisions.

OW/OST uses available, reliable aquatic toxicity data, including data found in publicly-available literature and data generated through the activities of other EPA offices, to develop ambient water quality criteria (U.S. EPA, 2015f) for aquatic life. These criteria are developed following procedures in the "Guidelines for Deriving Numerical National Water Quality Criteria for Protection of Aquatic Organisms" (Stephen et al., 1985; U.S. EPA, 2015f, 2015i). Acute aquatic life criteria are based on data for at least eight families of aquatic organisms, three vertebrates and five invertebrates. Chronic test requirements are of similar scope with different durations/species. Acute and chronic life data are used by OW to generate a sensitivity distribution of genus average data to estimate criteria that are statistically protective of approximately 95% of aquatic genera. OST has initiated work to examine the use of NAMS to address gaps in available data for aquatic life criteria development, which would reduce the need for animal tests for this purpose.

CWA Section 301 made it unlawful to discharge any pollutant from a point source into navigable waters unless authorized under a NPDES permit as provided in CWA Section 402. As required under CWA Section 301(b)(1)(C), NPDES permits must include water quality-based effluent limitations to implement any applicable state and tribal WQS. To protect water quality, EPA recommends using "whole effluent toxicity" (WET) tests in NPDES permits together with requirements based on chemical-specific water quality criteria (U.S. EPA, 2015f) to ensure that the state or tribal criteria in the WQS for aquatic life protection are met. Under the NPDES program WET testing is used to assess whether there are toxic impacts to aquatic life at a level that would result in an excursion of state or tribal WQS. As described in 67 FR 69951 (U.S. EPA, 2002), for potentially regulated entities, EPA and authorized states, territories, and tribes, issue permits that comply with the technology-based and water quality-based requirements of the CWA. If EPA has "approved'' (*i.e.*, promulgated through rulemaking) standardized and promulgated test procedures in 40 CFR Part 136 for a given pollutant, the NPDES permitting authority must specify one of the approved testing procedures or must use an EPA-approved alternate test procedure as directed by the permitting authority (40 CFR Part 122.21 (j)(5), (viii)) for monitoring pollutant discharges as required under a NPDES permit. Aquatic toxicity test methods designed specifically for measuring WET (U.S. EPA, 1994a; 1994b, 1993, 1995), the 821 methods cited herein, are codified in 40 CFR Part 136 (40 CFR 136, 2016; U.S. EPA, 2002) and employ a suite of standardized freshwater, marine, and estuarine plants, invertebrates, and vertebrates to estimate acute and short-term chronic toxicity of effluents and receiving waters (methods specified in (40 CFR § 136.3(a) - Table IA, 2002; 40 CFR § 136.3(a) - Tables II, 2002)).

2.3.3. Directive to reduce animal testing

EPA released its "New Approach Methods Work Plan: Reducing Use of Animals in Chemical Testing" in June 2020 (U.S. EPA, 2020a), with an updated version released in December of 2021 (U.S. EPA, 2021). In this work plan, the Agency described the objectives:

- Evaluate regulatory flexibility for accommodating the use of NAMs;
- Develop baselines and metrics for assessing progress;
- Establish scientific confidence in NAMs and demonstrate application to regulatory decisions;
- Develop NAMs that fill critical information gaps; and

- Engage and communicate with stakeholders to incorporate their knowledge and address concerns as EPA moves away from mammalian testing.

2.4. U.S. Food and Drug Administration

FDA is responsible for protecting the public health by ensuring the safety, effectiveness, and security of human and veterinary drugs, biological products, and medical devices, and by regulating the safety of our nation's food supply, color additives, and products that emit radiation. FDA is also responsible for regulating the manufacturing, marketing, and distribution of tobacco products, and for reducing tobacco use by minors. FDA-regulated products account for about 20 cents of every dollar spent by U.S. consumers (FDA, 2019).

As part of its responsibilities, FDA considers the potential environmental effects of agency actions, following policies and procedures set forth in NEPA and as codified in 21 C.F.R. § 25 (1997). NEPA and its implementing regulations in 40 CFR Parts 1500-1508 (2011) provide tools such as the environmental assessment (EA), the environmental impact statement (EIS), and categorical exclusions (for which neither an EA nor EIS are required) to evaluate the potential for environmental impacts. However, NEPA does not strictly specify the methods or approaches used to evaluate potential impacts with these tools. This allows flexibility for each agency to determine testing requirements based on the needs of their proposed action(s).

Under NEPA, FDA typically assesses or prepares prospective EAs and claims of categorical exclusion prior to approval or market authorization of regulated products (*e.g.*, drugs, biologics, food additives, tobacco products, medical devices). The overall goal of these assessments is to determine whether an agency action (*e.g.*, approval and subsequent marketing of a regulated product) will have a significant impact on the environment, in which case an EIS is prepared. FDA has published Guidance for Industry documents (FDA, 2006a; 2006b, 2001, 1998) that contain recommendations on how to prepare an EA, including data requirements and tiered approaches for ecotoxicity testing. These documents recommend the use of validated test methods and guidelines, many of which are published by the Test Guidelines Programme of the OECD (Table S2). FDA also accepts the use of other validated test guidelines, such as those published by EPA. However, FDA guidance documents are not binding on FDA or the industry, and FDA often considers alternative approaches on a case-by-case basis. Findings obtained through use of alternative methods, including NAMs (*e.g.*, *in chemico*, *in silico,* or *in vitro* assays), need to be correlated to an apical or population-level endpoint (*e.g.*, mortality, growth, or reproduction) for the data to be used in regulatory risk assessments. FDA also encourages the use of published literature, when available, in place of original laboratory studies. Generally, multiple independent literature studies with adequate methods, analyses, and consistent findings should be provided to replace a validated and well-controlled laboratory study conducted under Good Laboratory Practices (40 CFR § 160, 2002).

Some alternative methods are currently being used in limited cases. For instance, the FDA Center for Drug Evaluation and Research may consider alternative methods on a case-by-case basis to screen human drugs for possible endocrine-active signals in the environment (FDA, 2016).

2.5. National Institute of standards and technology

NIST is regularly involved in the development of reference materials, test methods, and documentary standards that support other agencies with fulfilling ecotoxicity testing needs. For example, NIST has produced a broad range of standard reference materials for samples from various environmental media (*e.g.*, sewage sludge, soil, sediment, water (NIST, 2010)), and has quantified the concentration of various organic and inorganic pollutants in these matrices. These materials can be used as part of a quality control system for laboratories that are testing the concentration of different chemicals in environmental samples, for verifying the performance of an extraction method during ecotoxicity testing, or for developing NAMs. In addition, NIST has performed method development and interlaboratory testing to evaluate methods to quantify contaminants in different matrices. (Reiner et al., 2011; Schantz et al., 2015; Wise et al., 1988).

In recent years, a concerted effort has focused on developing reference materials and standards related to the potential environmental and human health risks of nanomaterials. NIST was tasked with developing these materials and standards as part of the National Nanotechnology Initiative in 2012 (National Nanotechnology Initiative, 2011). Research in this area has led to the production of a wide range of reference materials (*e.g.*, gold nanoparticles, silver nanoparticles, single-wall carbon nanotubes, titanium dioxide nanoparticles, and silicon nanoparticles (NIST, 2010)). In addition, methods have been developed for quantifying different carbon and inorganic (*e.g.*, gold) nanomaterials in matrices such as soil and water (Bustos et al., 2015; Deng et al., 2017; El Hadri et al., 2018), and evaluating the release of nanomaterials from consumer products (Jacobs et al., 2016; Nguyen et al., 2017). The robustness of ecotoxicology methods (*e.g.*, an ISO *C. elegans* assay (Hanna et al., 2018, 2016), OECD test guidelines (Petersen et al., 2015), and bioaccumulation tests (Bjorkland et al., 2017)) have been rigorously evaluated with recommended protocols and control tests provided to identify and minimize potential artifacts. Lastly, a cell viability assay has been developed and evaluated using an interlaboratory comparison (Elliott et al., 2017; Rösslein et al., 2015), and is now available as a standard issued by the International Organization for Standardization (19007:2018(en) Nanotechnologies — *In vitro* MTS assay for measuring the cytotoxic effect of nanoparticles, (ISO, 2018)).

2.6. U.S. Department of Agriculture

Like other federal agencies, USDA must comply with all relevant environmental statutes related to actions they may fund, authorize, or regulate. USDA uses ecotoxicology data primarily to meet its regulatory requirements under these environmental statutes (Table 1). Compliance under NEPA includes the preparation of EAs, EISs, and categorical exclusions. The evaluation of USDA actions under these three processes can include the use of ecological toxicity data to determine the extent of potential effects to fish and wildlife from a proposed action by a USDA agency. In addition, the ESA requires the use of ecotoxicology data to determine if an action proposed by USDA could impact a listed species. The data used in these types of analyses include measuring effects in nontarget fish and wildlife from chemical and non-chemical stressors. These data may originate as part of a regulatory requirement or are obtained from publicly available peer-reviewed journals and other published documents.

The USDA Animal and Plant Health Inspection Service (APHIS) is a registrant for several types of compounds for control of pest species (*e.g.*, avicides, rodenticides) that require the development of ecotoxicology data for registration under FIFRA. Data submissions that characterize the effects of a pesticide to nontarget fish and wildlife are required as part of the FIFRA evaluation process under EPA OPP when registering a pesticide.

The USDA Forest Service National Technology and Development Program evaluates and qualifies wildland fire suppressants and retardants. Once a fire chemical meets all Forest Service (FS) requirements, it is added to a Qualified Products List (QPL) and becomes available for use by federal wildland firefighting agencies. The evaluation requires the development of mammalian and aquatic toxicity data for use in risk assessments and environmental consultation tied to the required Environmental Impact Statement on aerially-applied fire retardant. These studies are conducted by other third-party laboratories.

Both APHIS and FS prepare human health and ecological risk assessments for proposed pesticide use for many of its programs. Court decisions in the 1980's required the FS to perform risk assessments

particular to USDA's proposed uses, beyond those conducted by EPA for pesticide registrations. These risk assessments use available ecological toxicity testing data available through the pesticide registration process or available in the peer-reviewed literature and other publicly available documents, to make estimates about risk to terrestrial and aquatic nontarget fish and wildlife for pesticides and other chemicals that may be used in specific agency activities.

USDA may also use and develop ecotoxicology-related data as part of its research activities. One example is the Agricultural Research Service (ARS). The ARS is divided into four program areas that address 1) Nutrition, Food Safety and Quality, 2) Animal Production and Protection, 3) Crop Production and Protection, and 4) Natural Resources and Sustainable Agricultural Systems. Within each program, ARS supports research efforts to evaluate a wide variety of topics related to agriculture. As an example, in studies to determine impacts and identify solutions concerning bee exposure to multiple agrochemicals, ARS uses the expertise of environmental chemists along with data from EPA to develop and validate models of pesticide movement from nest-building materials into the nectar and pollen stores used to feed larvae.

The ARS also uses ecological-related effects data to characterize how various land and agricultural management practices can impact the environment, including fish and wildlife.

The USDA actively promotes and supports the use of alternatives to live animal use. For example, the APHIS National Wildlife Research Center (NWRC) uses alternate *in vitro* techniques to replace animal testing such as assessing metabolic pathways using liver microsomes. The NWRC also uses data generated from proteomic, metabolomic, and genetic databases, and computer modelling to meet research needs as alternatives to animal testing.

The Animal Welfare Information Center (AWIC), which is located within ARS' National Agricultural Library, was designated in 1985 under the AWA to serve as a resource to assist investigators in finding alternatives to animal testing. AWIC provides training in conducting literature searches for alternatives, specifically searches for *in silico*, *in chemico,* or *in vitro* techniques using a plethora of databases (USDA, 2021a). AWIC's services are provided at no cost and are available to all members of the research community. In addition, the AWIC website provides a list of peer-reviewed publications on alternatives to animal testing and other guidance designed to reduce, replace, and refine ecotoxicity testing using animals (USDA, 2021b).

3. Discussion

The preceding sections provide a synopsis of regulatory and non-regulatory testing needs of ICCVAM agencies for ecotoxicology testing data, which are still largely fulfilled by data from animal testing. Thus, replacing animal testing for ecotoxicology endpoints remains a long-term goal. While agencies work towards the long-term goal of replacement, opportunities exist to improve the utility of currently obtained data and reduce animal use by improving understanding of toxicity mechanisms and implementing testing waivers.

3.1. Challenges with cross-taxa and interspecies extrapolation

Consideration of the need and in some cases regulatory requirements for tests in Table 2, and the associated taxa employed in these specific test guidelines, suggest that there is a relatively narrow selection of surrogate test species being used to represent a large assemblage of species organized in relatively broad taxonomic groups. For example, data from the medaka one-generation test is extrapolated out to hundreds of other ray-finned fish species, and reproduction toxicity data for two species of precocial birds is used to make hazard inferences for all the precocial and altricial birds in North America. There are a number of technical, legal, historical, logistical, and financial reasons why only a few species of a given taxa are ever tested (Lillicrap et al., 2016). A challenge for the use of such data across a given taxonomic group is the expected difference in the relative sensitivity to the toxicant among the untested species. Under current data sets dependent upon *in vivo* testing, accounting for these interspecies differences is accomplished by several approaches:

- Reliance on the most sensitive species tested;
- Application of generic interspecies adjustment factors to available data sets derived from only a few species to approximate some level of protection based on a fixed position on the distribution of possible outcomes; or
- Application of chemical-specific species sensitivity distributions derived from larger multiple-species testing data sets.

Limiting assumptions for these approaches have been outlined by Forbes and Forbes (1993) and Forbes and Calow (2002), and include:

- The distribution of species sensitivities in natural ecosystems closely approximates the postulated theoretical distribution;
- The sensitivity of species used in laboratory tests provides a measure of the variability and range of the sensitivity distribution of species in natural communities;
- By protecting species composition, community function is also protected, and
- Interactions among species in communities/ecosystems can be ignored.

However, it should be noted that extrapolating data generated from laboratory animals to a broader suite of organisms in the environment has known uncertainties, including:

- Laboratory animal species may not exhibit the full suite of toxic effects of interest for the target species. For example, the protocols of routine studies with precocial bird species (*e.g.*, Ecological Effects Test Guidelines OCSPP 850.2300 (U.S. EPA, 2012)) do not evaluate potential effects of behavioral endpoints such as nest building, complex courtship behavior, egg incubation, and care of hatchlings. It is possible such studies are incomplete predictors of reproduction hazard for wild birds especially altricial species where many of the behaviors are critical to offspring production (Ar and Yom-Tov, 1978);
- Laboratory animals may not have the same sensitivity to toxicants as the species of interest (Brown et al., 2009);
- Laboratory animals may not occupy the same taxa or ecological niche, or may not have the same life cycles as the species of interest (Brown et al., 2014);
- Model organisms may be chosen for conveniences such as ease and low-cost of maintenance, rapid development, and high fecundity, rather than for appropriateness of the surrogate species (*i.e.*, functional homologies or toxicity relevant traits with the species of concern (Segner- and Baumann, 2016));
- Single-species laboratory tests using model organisms with limited genetic variability (Brown et al., 2009) do not reflect the genetic heterogeneity of wild populations;
- Tests on laboratory animals may not be able to accurately predict ecosystem responses (Cairns, 1988).

Advances in bioinformatics and the development of the concept of adverse outcome pathways (Ankley et al., 2010; Ellison et al., 2016; Jeong and Choi, 2017; Vinken et al., 2017) could be applied to strengthen the inferences made in ecotoxicity extrapolations by developing lines of evidence such as:

- An understanding of the genetic and biochemical evidence applicable to the uptake, distribution, and metabolic activation/inactivation of a given toxicant across the genetic variation among species within given taxa;

- Mechanism-of-action data relative to specific genetic inductions or receptor affinities and the resultant pathway to adverse outcomes; and
- The conservation of adverse outcome pathways across taxa, which can be explored using approaches in bioinformatics like EPA Sequence Alignment to Predict Across Species Susceptibility tool (LaLone et al., 2016).

Further, *in silico* methods to predict toxic effects will provide additional valuable information with respect to these lines of evidence important to cross-taxa extrapolation (Eng et al., 2017; Fuchsman et al., 2017; U.S. EPA, 2016a).

3.2. Waiving the need for certain ecotoxicity test data

Although few *in vitro, in silico,* or *in chemico* NAMs exist that have been assessed or routinely used to fully replace the use of animals for ecotoxicity testing, there are circumstances where the need for *in vivo* data for certain ecotoxicity tests can be waived, resulting in the reduction of animal use. Some circumstances where waivers may be used are described below.

Registrants of chemicals can request a waiver of data requirements or can bridge information from one data set to another. Waiver submissions must specify the data requirement for which a waiver is being sought and must include the supporting rationale why the requirement should be waived. Waiver requests can include suggestions for alternate means of obtaining the data. These actions create the opportunity to reduce animal use (U.S. EPA, 2020b) and avoid generating data that are not needed or are available through other means while still ensuring that regulatory decisions are suitably informed. For example, a retrospective analysis of avian acute-oral and sub-acute dietary test data for pesticide registration by EPA demonstrated that risk quotients used in decision-making were almost exclusively derived (>99%) from acute oral test (Hilton et al., 2019). Based on this analysis, EPA released guidance in April 2020 (U.S. EPA, 2020c) stating that the sub-acute avian dietary test requirement can be waived when deemed to provide little additional scientific information for environmental or public health. Likewise, fish bioconcentration test guidelines historically required that bioconcentration factors (BCFs) be determined at two exposure concentrations. Analysis of 236 fish BCF studies revealed that estimates did not differ significantly when more than one test concentration was used (Burden et al., 2014), and thus if the BCF value is less than or equal to 667, adequate BCF data may be obtained using one test concentration, as described in "Fish Bioconcentration Data Requirement: Guidance for Selection of Number of Treatment Concentrations." (U.S. EPA, 2020d), a supplement to EPA 850.1730 (U.S. EPA, 2016b).

In some instances, federal agencies may waive the need for ecotoxicity tests when existing data for risk assessment and regulatory decisions are adequate (*e.g.*, DOI, approval of candidate shot and shot-coatings used in hunting of waterfowl [Sec. 2.2]; FDA approval of human and veterinary drugs [Sec. 2.4]). Use of these waivers decreases animal usage by reducing the number of required tests.

4. Conclusions

The breadth of data needed to support U.S. Federal ecological risk-based decisions varies with each program. The ICCVAM EcoWG has identified key points to consider that are intended to aid U.S. federal agencies, academia, the regulated community, and other national, state, and local stakeholders in developing assays to refine or reduce the use of animals in ecological testing. The participating agencies have highly divergent needs ranging from chemical testing regimes, to water quality assessments for statutory regulatory requirements, to more specific scenario-based understandings to evaluate the potential impact of an agency action on the environment, or evaluate, reduce, or control natural resource damage. These divergent needs make it challenging to develop metrics for assessing progress in NAM development and use. In order to facilitate this process, in early 2020, ICCVAM established a Metrics Workgroup to identify ways to help the committee and its member agencies better monitor their progress across the range of their efforts to reduce animal use and report members' progress to the public. A report is available[6] that describes the recommendations of the Metrics Workgroup and provides resources that can be used to follow federal agency progress.

The diversity of agency testing needs coupled with the biological complexity of vertebrates makes it unlikely that a single animal test can be replaced with a single alternative test. Each federal agency must evaluate replacement methods in the context of program needs to determine the extent to which each method provides information equivalent to the whole animal test targeted for replacement. For example, validating approaches that extrapolate *in vitro* or *in silico* results to population-level effects may prove to be a challenge in some cases. Similarly, new animal assays, such as the medaka extended one generation reproduction test (OECD, 2015; U.S. EPA, 2015h), represent a challenge to NAMs development in that they introduce new ecological effects endpoints for consideration in regulatory decision-making. Continued development of animal assays may add to the list of animal methods that are potential candidates for replacement and may expand the complexity of analyses needed to support the move to use *in silico* and *in vitro* assays. However, the examples described herein demonstrate that agencies are motivated to identify opportunities to implement alternatives to animal testing in appropriate contexts. The EcoWG and participating agencies will monitor advances already realized in the context of human health protection (ICCVAM, 2020) to determine their applicability to ecotoxicity testing. Future EcoWG activities include preparation of a review of available and applicable NAMs for acute fish toxicity testing to justify its immediate prioritization as a target for replacement.

Distribution statement

Approved for public release; distribution unlimited.

Funding

This project was funded in part with federal funds from the NIEHS, National Institutes of Health under Contract No. HHSN273201500010C to ILS in support of the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods.

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This article has been peer reviewed and approved for publication consistent with USGS Fundamental Science Practices (https://pubs.usgs.

[6] https://ntp.niehs.nih.gov/go/903258.

gov/circ/1367/). ILS staff provide technical support for the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods, but do not represent NIEHS, the National Toxicology Program, or the official positions of any federal agency. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. government.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank DOI Solicitor Lindsey Spinelli for guidance on DOI statutes and regulations listed in Table S1, Jayne-Anne Bond for providing information on Air Force testing needs, and Amy Blankinship, Debra Denton, William Eckel, Douglas Ethan Harwood, Jo Ellen Hinck, Jean Holmes, Tamara Johnson, Michael Lowit, Moira A. McKernan, Laura Phillips, Katrina White, and Austin Wray for commenting on the earlier draft of this manuscript.

In addition, we thank the USDA Animal Welfare Information Center for creating a web page containing information about ecotoxicity testing (USDA, accessed 2020b) in response to this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yrtph.2022.105195.

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