
UNITED STATES
ICCVAM
Advancing Alternatives
to Animal Testing

Alternate models for acute fish toxicity testing: a survey

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Agency for Toxic Substances and Disease Registry • Consumer Product Safety Commission • Department of Agriculture • Department of Defense
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Summary

- Aquatic toxicity testing assesses the adverse effects of chemicals and other environmental stressors on aquatic organisms.
- It involves different media or environments (i.e., marine, freshwater), and a large number of species.
- Common tests include acute and chronic exposures, often standardized by agencies and international bodies such as the US EPA or OECD.
- There is interest and social pressure worldwide to develop alternative methods.
- Here, we summarize the use of the Acute Fish Toxicity Test, which is often requested as part of the registration of new substances and has lethality as an endpoint.
- We then explore the potential of alternate models that could provide hazard information and potentially replace that test.

Acute Fish Toxicity Testing

Fish Acute Toxicity Test

INTRODUCTION

1. OECD Guidelines for Testing of Chemicals are periodically reviewed to incorporate scientific progress, changing regulatory needs, and animal welfare considerations. The revision of this Guideline (originally adopted in 1981, updated in 1984, 1992), reflects also updates on a series of recommendations from the OECD Fish Toxicity Testing Framework 2011 (OECD, 2012), and includes:

- Alternative methods: in the interest of animal welfare and efficient use of resources, it is important to avoid/reduce the use of animals whenever possible and appropriate. Therefore, before carrying out a fish acute toxicity test according to this guideline, it should be considered whether reliable information on fish acute toxicity could be derived with alternative methods in a weight-of-evidence approach, such as the use of QSAR, read-across, fish embryos (OECD 2013), fish cell lines and others. Alternatively, the use of the threshold approach (OECD, 2010) or the limit test as described in § 30 of this guideline may be sufficient. Where testing on fish is required (i.e., alternative methods currently may not be sufficient for all jurisdictions and testing needs. Therefore; make sure the tests fulfil the regulatory requirements), alternative methods such as those listed above can be considered for range finding
- A specification that testing the minimum concentration causing 100% and the maximum concentration causing 0% mortality are not mandatory requirements (e.g. no need to test additional concentrations just to demonstrate 0 and/or 100% mortality).
- guidance on the circumstances under which a water control is required when solvent is used (OECD, 2018).
- the introduction of estuarine and marine fish species in the recommended species list.
- the enhanced recording of visible abnormalities (also referred to as sublethal clinical signs) that fish may display during the exposure in order to improve our

PRINCIPLE OF THE TEST

3. The fish are exposed to the test chemical for a period of 96 hours, under either static, semi-static or flow-through conditions. Mortalities and visible abnormalities related to appearance and behaviour are recorded. Where possible, the concentrations to kill 50% of the fish (LC_{50}) are determined.

ability to predict chemical toxicity and minimise suffering of animals in the future analogously to those described in Guidance Document No. 19 on the recognition, assessment, and use of clinical signs as humane endpoints for experimental animals used in safety evaluation for mammalian studies (OECD, 2000).

2. Definitions used in this Test Guideline are given in Annex 1.

ANNEX 2

TABLE 1: RECOMMENDED FISH SPECIES, TOTAL LENGTHS AND TEST CONDITIONS

Species[6]	Temperature[7] (°C)	Salinity[8] (‰)	pH	Hardness (mg/L $CaCO_3$)	Photoperiod (hours light)	Recommended length range[9] (cm)
Danio rerio *Zebrafish*	21-25	<0.2	6.0-8.5	40- 250, preferably <180	12-16	1-2
Pimephales promelas Fathead minnow	21-25	<0.2	6.0-8.5	40-250, preferably <180	12-16	1-3
Cyprinus carpio Carp	20-24	<0.2	6.0-8.5	40-250, preferably <180	12-16	2-4
Oryzias latipes Japanese Medaka	23-27	<0.2	6.0-8.5	40-250, preferably <180	12-16	1-2
Poecilia reticulata Guppy	21-25	<0.2	6.0-8.5	40-250, preferably <180	12-16	1-2
Lepomis macrochirus Bluegill	21-25	<0.2.	6.0-8.5	40-250, preferably <180	12-16	1-3

[6] If other species are used, the rationale for the selection of the species must be reported together with any adaptations to the test guideline's recommendations. It is suggested that the species is selected on the basis of their ready availability, ease of maintenance, and historical use in safety testing.
[7] Where culture temperature differs from the recommended range, the acclimatization period should be used to acclimatize the fish to the desired test temperature.
[8] For any given test this shall be performed to ± 2‰, e.g. 17±2 =15-19‰, 31±2 =29-33‰.
[9] Test fish must be juveniles when used in this test (before reaching sexual maturity). If fish of sizes other than those recommended are used, this should be reported together with developmental stage (juvenile, sub-adult, adult stage) and the rationale.

Oncorhynchus mykiss Rainbow trout	10-14[10]	<0.2	6.0-8.5	40-250, preferably <180	12-16	3-6
Gasterosteus aculeatus Three-spined stickleback	13-19	0-35	6.0-8.5	40-7500	12-16	1-2
Cyprinodon variegatus Sheepshead minnow	23-27	15-35	6.0-8.5	3000-7500	12-16	1-2
Dicentrarchus labrax European sea bass	18-22	15-35	6.0-8.5	3000-7500	12-16	4-8
Pagrus major Red sea bream	18-22	30-35	6.0-8.5	5000-7500	12-16	2-4

TABLE 1: Clinical signs observed in fish, compiled from publications (CCAC, 20015; Rufli, 2012; Drummond et al, 1986 and Midtlyng et al, 2011) and TG203 score sheets provided by individual laboratories. Non-shaded rows are the major categories of visible abnormality for which recording has been mandatory in TG203 since 1992. Shaded rows are optional explanatory sub-categories.

Clinical sign	Definition	Synonyms
LOSS OF EQUILIBRIUM (sub-categories below)		
Abnormal horizontal orientation	Loss of balance displaying as abnormal horizontal orientation/posture in water column	Keeling, lost righting reflex
Abnormal vertical orientation	Head-up or head-down posture	
Loss of buoyancy control	Floating at surface or sinking to the bottom	
ABNORMAL SWIMMING BEHAVIOUR (sub-categories below)		
Hypoactivity	Decrease in spontaneous activity	Torpid, apathy, lethargy, weak, immobility, inactivity, ceased swimming, quiescent
Hyperactivity	Increase in spontaneous activity	Erratic swimming, skittering
Corkscrew swimming	Rotation around long axis; erratic movements, often in bursts	Rolling, spiralling, spiral swimming, tumbling, circling movements
Convulsions	Abnormal involuntary and uncontrolled contraction of muscles	Seizures, twitching, muscle spasms, shaking, shuddering, vibration
Tetany	Rigid body musculature (intermittent or permanent)	Paralysis
Irritated skin behaviours		Flashing, scraping, rubbing
Abnormal surface distribution/behaviour	Abnormal depth selection, close to water/air interface	Jumping, surfacing; on/at/near/just below surface/top
Abnormal bottom distribution/behaviour	Abnormal depth selection, close to base of tank	Diving, sounding; Lying on/ orientation to / collecting at / near / just above bottom
Over-reactive to stimulus	Flight (startle) or avoidance response to: visual (hand passing over top of tank, light beam), tactile (touch) or vibration (tank rapped lightly) stimulus	Hyperexcitability; hyperactivity after stimulus/threat
Under-reactive to stimulus		Not responsive to external stimulation; inactivity after stimulus/ threat
Loss of schooling / shoaling behaviour	Individual fish show loss of aggregating and social interactions	Isolation, social isolation
Dense schooling / shoaling behaviour	Increase in clumped association of fish	Crowding
ABNORMAL VENTILATORY (RESPIRATORY) FUNCTION (sub-categories below)		
Hyperventilation	Increased frequency of opercular ventilatory movements, with possible open mouth and extended operculae	Rapid/strong respiratory rate/ function. Heavy gill movements, strong ventilation, strongly extended gills, abnormal opercular activity, operculae spread apart, mouth open
Hypoventilation	Decreased frequency of (and possibly shallow) opercular ventilatory movements	Reduced/laboured/weak/slow respiration/respiratory action/ventilation
Irregular ventilation	Irregular opercular ventilatory movements	Sporadic / spasmodic respiration / gill movement
Coughing	Fast reflex expansion of mouth and operculae not at water surface - assumed to clear ventilatory channels	Gasping, abnormal opercular activity, yawn
Gulping	Mouth (and opercular) movements at water surface, resulting in intake of water and air	Piping
Head shaking	Rapid lateral head movements	
ABNORMAL SKIN PIGMENTATION (sub-categories below)		
Darkened		Changed / increased / dark(ened) colour / pigmentation / melanistic markings
Lightened		Pallor, pale/changed/weak pigmentation
Mottled		Discoloured patches
OTHER VISIBLE (APPEARANCE & BEHAVIOUR) ABNORMALITIES (sub-categories below)		
Exophthalmia	Swelling within orbital socket(s) resulting in bulging of one or both eyes	Exophthalmos, exophthalmus, popeye, protruding eyeball
Oedema	Abdominal swelling due to accumulation of fluid. May cause protruding scales and/or fissure in abdominal wall	Distended/swollen/bloated abdomen/gut area; dropsy
Haemorrhage	Petechias (pinhead sized spots) and/or haematoma (area of blood) due to intradermal or sub-mucus bleeding	
Mucus secretion	Excess mucus production	Mucus build-up (pay close attention to eyes); increased secretion (mucus on skin or in water); mucus loss
Faecal (anal) casts	String of faeces hanging from anus or on tank floor	
Aggression and/or cannibalism		Aggression, direct attack, domination of choice tank locations, pick at or eat bodies of dead fish

Some examples of fish acute toxicity data requirements by sector and global region

Industry sector	Region	Example legislation	Acute in vivo test required for active substances (yes/no)	Species required/recommended, and product/formulation testing requirements
Biocides	EU	EU Biocidal Products Regulation (Regulation EU 528/2012)	Yes	• One freshwater (+marine species, if relevant) • Testing of products and ingredients may be required in some circumstances depending on the use pattern, relative sensitivity of other taxa compared to fish, and if the risk cannot be predicted/resolved based on the ingredients. • Exemptions can apply to active substance data, if 1) valid chronic (long-term) fish toxicity data are available or 2) in some rare cases, if negligible exposure is expected (attributable to the use pattern or properties of the active substance)

Burden et al, ET&C 2020

Some examples of fish acute toxicity data requirements by sector and global region

Industry sector	Region	Example legislation	Acute in vivo test required for active substances (yes/no)	Species required/recommended, and product/formulation testing requirements
Biocides	North America		Yes	• Cold freshwater, warm freshwater, marine; requirements can potentially be reduced dependent on use or expected exposure • Testing of ingredients
	Asia Pacific		Yes	• Cold freshwater; requirements can potentially be reduced dependent on use or expected exposure or country • Testing of products and ingredients
	Notes	For some product categories the data are already available from plant protection product registrations (e.g., certain fungicides and insecticides).		

Burden et al, ET&C 2020

Some examples of fish acute toxicity data requirements by sector and global region

Industry sector	Region	Example legislation	Acute in vivo test required for active substances (yes/no)	Species required/recommended, and product/formulation testing requirements
Human pharmaceuticals	EU	EU Human Pharmaceuticals (Regulation EC 726/2004)	No; considered not relevant because of long-term, low-level exposure	n/a
	North America	US Food and Drug Administration Center for Drug Evaluation and Research	Yes; action limit at expected environmental concentration >100 ng/L (if not an endocrine-disrupting compound), then a tiered approach if *Daphnia* or algae risk quotient <1000	Not specified

Burden et al, ET&C 2020

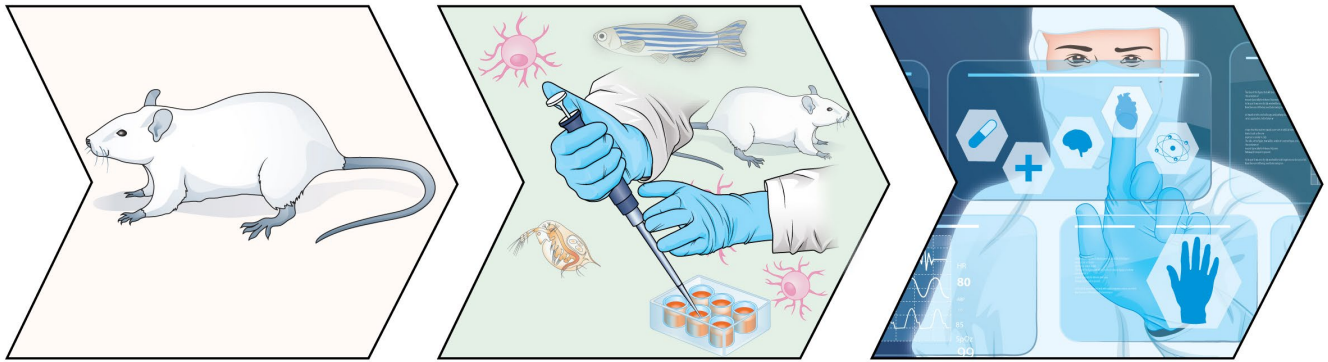
Some examples of fish acute toxicity data requirements by sector and global region

Industry sector	Region	Example legislation	Acute in vivo test required for active substances (yes/no)	Species required/recommended, and product/formulation testing requirements
Cosmetics	EU		No, although information on fish may be required on ingredients covered under REACH (>10 tonnes/yr); see Notes	
	Rest of world		Country-specific	Dependent on country; requirement in China for ingredients imported >1 tonne/yr to test with a native species
	Notes	Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products sets out a testing ban—prohibition on testing finished cosmetic products and cosmetic ingredients on animals and a marketing ban, prohibition on marketing finished cosmetic products and ingredients in the European Union which were tested on animals.		

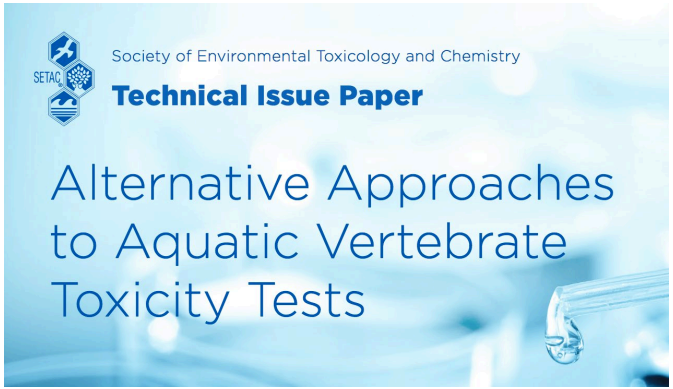
Burden et al, ET&C 2020

Alternative Approaches: the 3R-Principle

- **R**EDUCE the number of animals used in testing
- **R**EFINE any procedures to minimize pain, suffering, and distress
- **R**EPLACE the use of animals whenever possible



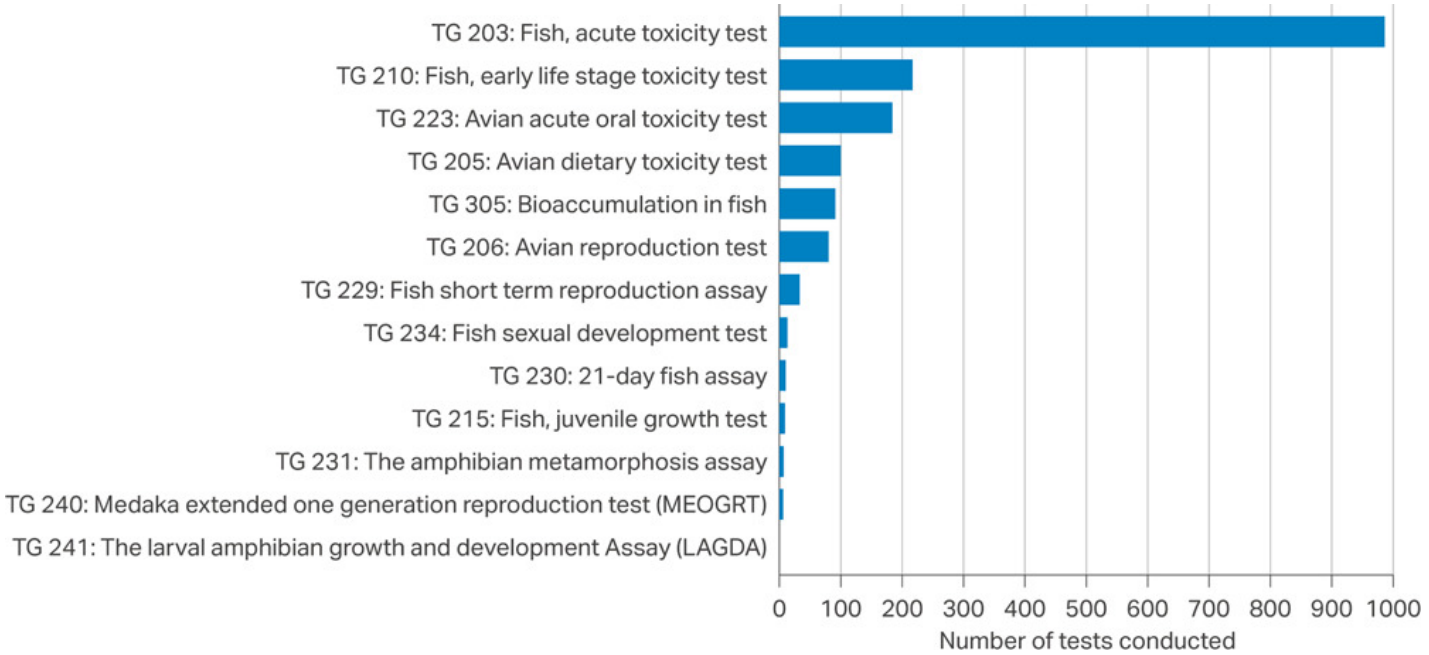
Moving towards the 6R-principle



- **R**eproducible and Reliable
- **R**elevant
- **R**egulatory accepted

Belanger et al, SETAC TIP

OECD test guideline vertebrate ecotoxicology studies conducted across 15 contract research organizations from 2014 through 2017



Burden et al, ET&C 2020

Considerations for in vitro methods

- Chemical space coverage
- Performance metrics:
  - Assay controls
  - Factors affecting assay results
- Proprietary information

Chemical space coverage

- The chemical space for fish acute toxicity testing is large (number of chemicals) and broad (variety of chemical properties), reflecting the wide range of regulatory (*e.g.*, pesticides, pharmaceuticals, and industrial chemicals) and associated research needs for toxicity data on compounds that may reach aquatic environments.
- NAM coverage will be limited by factors including but not limited to physiochemical properties and mode of action (MOA) of the chemical of interest and those used to develop an approach.
- For regulatory or research acceptance, NAMs approaches must establish the range of their “fit for purpose” to be applied in lieu of or as a supplement to whole-fish acute toxicity studies.

Performance metrics

- There are numerous factors to consider when evaluating the quality of results from a NAM for estimating the acute toxicity of a chemical to fish using *in vitro* laboratory methods
- It is critical to test in-process control measurements (e.g., positive chemical control, dilution water control [or negative control without test chemicals]), solvent control, and, in the case of *in vitro*-NAMs, a no-cells control with only the assay reagents) to measure key sources of variability each time the assay is performed to ensure consistent performance

Performance metrics:
Key control measurements in acute fish toxicity tests and NAMs

Guideline name	Control	Variation permitted	Citation
RTgill-W1	Difference in cytotoxicity between solvent and negative controls	≤10%	(ISO, 2019)
Freshwater Alga and Cyanobacteria, Growth Inhibition Test	Average specific growth rate in replicate control cultures	≤ 7%	(OECD, 2011a)
Algal Toxicity Test	Average specific growth rate in replicate control cultures	< 15	(U.S. EPA, 2012a)
Daphnia Acute Immobilisation Test	Immobilisation in dilution water and solvent controls	≤10%	(OECD, 2004a)
Aquatic Invertebrate Acute Toxicity Test, Freshwater *Daphnids*	Immobilisation in dilution water and solvent controls	≤10%	(U.S. EPA, 2016a)
Fish Acute Toxicity Test	Mortality of dilution water and solvent controls	≤10%	(OECD, 2019a)
Freshwater and Saltwater Fish Acute Toxicity Test	Mortality of dilution water and solvent controls	≤10%	(U.S. EPA, 2016a)
Fish Embryo Acute Toxicity (FET) Test	Mortality of dilution water and solvent controls	≤10%	(OECD, 2013a)
Fish Embryo Acute Toxicity (FET) Test	Positive control mortality	>30%	(OECD, 2013a)
Fish Early Life Stage (FELS) Toxicity Test	Hatching success of control groups	>66-80%[a]	(U.S. EPA, 2016b)
Fish Early Life Stage (FELS) Toxicity Test	Post-hatch success of control groups	>60-80%[a]	(U.S. EPA, 2016b)

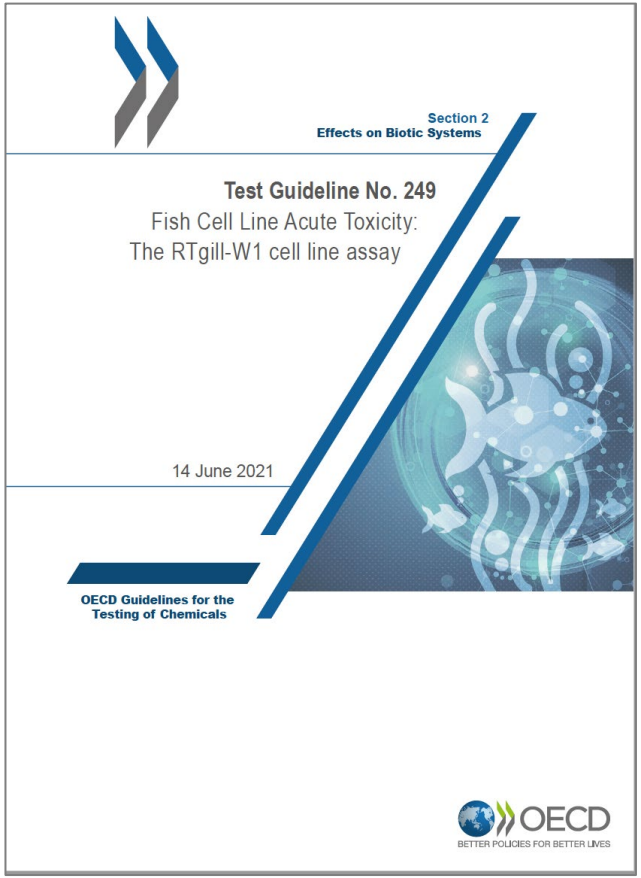
Proprietary information

- A critical issue when trying to validate and accept *in vitro* and *in silico* methods for potential replacement of fish acute toxicity testing is the presence of proprietary information.
- As many commercially available assays were originally intended for pharmaceutical candidate screening, most are at least partially proprietary.
- This might interfere with standard validation approaches, and the development of specific testing guidelines.

Good news

OECD recently released a formal guidance document for the use of a 24-well plate formatted RTGill viability assay as part of:

- Predictor of acute fish toxicity
- Range-finding and pre-screening before conducting the acute fish toxicity test or other fish-based testing
- Part of a WoE for hazard assessment.



Potential strategies and suggestions

- To expedite the development and use of NAMs, ICCVAM established a generalized framework for regulators and stakeholders that is used to enable development and establish confidence in the use of NAMs through coordinated efforts that address three strategic goals:
  - Connect end users with the developers of NAMs
  - Foster the use of efficient, flexible, and robust practices to establish confidence in new methods
  - Encourage the adoption and use of new methods and approaches by U.S. federal agencies and regulated industries
- However, each federal Agency and program must evaluate NAM approaches in the context of its own regulatory needs to determine if it is fit for purpose and whether adequate environmental protection can be maintained using the new tools within their specific framework.

Acknowledgements

ICCVAM EcoWG

EcoWG Members: Kristina M. Adams, Amy Bergdale, Raanan Bloom, Jennifer Brennan, Hoshing Chang, Carol Clarke, Debra Denton, William (Bill) Eckel (Co-chair), Karen Eisenreich, Michael Elias, Kellie Fay, Tara Flint, Natalia Garcia-Reyero (Co-chair), Kurt A. Gust, Douglas (Ethan) Harwood, Paula F.P. Henry, Katherine Horak, Wesley Hunter, Patrice Klein, Nicole Kleinstreuer, Kara Koehrn, Carlie Lalone, James Laurenson, Jessica K. Leet, Michael Lowit, Scott Lynn, Edward Perkins, Elijah Petersen, Laura Phillips, Barnett A. Rattner, Emily N. Reinke, Jim Warren, Marc Williams, Sarah Winfield

ILS Support Staff: Patricia Ceger; David Allen; Jonathan Hamm

Former EcoWG Members: Elyssa Arnold, Donna Judkins (Ret), Teresa Norberg-King (Ret), Edward Odenkirchen (Ret)