



Designation: ~~D6081~~—~~20~~ **D6081** – **22**

Standard Practice for Aquatic Toxicity Testing of Lubricants: Sample Preparation and Results Interpretation¹

This standard is issued under the fixed designation D6081; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ϵ) indicates an editorial change since the last revision or reapproval.

1. Scope*

1.1 This practice covers procedures to be used in the preparation of lubricants or their components for toxicity testing in aquatic systems and in the interpretation of the results of such tests.

1.2 This practice is suitable for use on fully-formulated lubricants or their components that are not completely soluble at the intended test treat rates. It is also suitable for use with additives, if the additive is tested after being blended into a carrier fluid at the approximate concentration as in the intended fully formulated lubricant. The carrier fluid shall meet the above solubility criterion, be known to be minimally toxic in the toxicity test in which the material will be tested, and be known to have a chemical composition similar to the rest of the intended fully formulated lubricant.

1.3 Samples prepared in accordance with this practice may be used in acute or chronic aquatic toxicity tests conducted in fresh water or salt water with fish, large invertebrates, or algae. This practice does not address preparation of samples for plant toxicity testing other than algae.

1.4 Standard acute and chronic aquatic toxicity procedures are more appropriate for lubricants with compositions that are completely soluble at the intended test treat rates (**1, 2, 3, 4, 5**).²

1.5 This practice is intended for use with lubricants or lubricant components of any volatility.

1.6 This practice does not address any questions regarding the effects of any lubricant or lubricant component on human health.

1.7 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.8 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.9 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

¹ This practice is under the jurisdiction of ASTM Committee **D02** on Petroleum Products, Liquid Fuels, and Lubricants and is the direct responsibility of Subcommittee **D02.12** on Environmental Standards for Lubricants.

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² The boldface numbers in parentheses refer to the list of references at the end of this standard.

***A Summary of Changes section appears at the end of this standard**

2. Referenced Documents

2.1 ASTM Standards:³

[D1129 Terminology Relating to Water](#)

[D1193 Specification for Reagent Water](#)

[D4175 Terminology Relating to Petroleum Products, Liquid Fuels, and Lubricants](#)

[E729 Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians](#)

[E943 Terminology Relating to Biological Effects and Environmental Fate](#)

3. Terminology

3.1 For definitions of terms used in this practice, see Terminologies [D1129](#), [D4175](#), and [E943](#).

3.2 Definitions:

3.2.1 *acute toxicity test, n*—a comparative toxicity test in which a representative subpopulation of organisms is exposed to different treat rates of a test material and is observed for a short period, usually not constituting a substantial portion of their life span.

3.2.2 *chronic toxicity test, n*—a comparative toxicity test in which a representative subpopulation of organisms is exposed to different treat rates of a test material and is observed for a period of time that constitutes a major portion of their life span.

3.2.3 *ecotoxicity, n*—propensity of a test material to produce adverse behavioral, biochemical, or physiological effects in non-human organisms or populations.

3.2.4 *effect load XX (ELXX), n*—a statistically or graphically estimated loading rate of test material that is expected to cause one or more specified effects in XX % of a representative subpopulation of organisms under specified conditions.

3.2.4.1 Discussion—

This terminology should be used instead of the standard ECXX when the test material is not completely soluble at the test treat rates.

3.2.5 *inhibition load XX (ILXX), n*—a statistically or graphically estimated loading rate of test material that is expected to cause a XX % inhibition of a biological process (such as growth or reproduction) that has an analog as opposed to a digital measure.

3.2.5.1 Discussion—

An example of a digital measure would be alive/dead. This terminology (ILXX) should be used instead of the standard ICXX when the test material is not completely soluble at the test treat rates.

3.2.6 *lethal load XX (LLXX), n*—a statistically or graphically estimated loading rate of test material that is expected to be lethal to XX % of a representative subpopulation of organisms under specified conditions.

3.2.6.1 Discussion—

This terminology should be used instead of the standard LCXX when the material is not completely soluble at the test treat rates.

3.2.7 *loading rate, n*—the ratio of test material to aqueous medium used in the preparation of ~~WAF, WSF~~, a water accommodated fraction (WAF), water soluble fraction (WSF), or mechanical dispersion and in the interpretation of the results of a toxicity study with a poorly water-soluble lubricant or lubricant component.

3.2.8 *mechanical dispersion, n*—a low energy aqueous medium produced by continuous stirring of the test solution and containing both dissolved and undissolved components of the test material.

3.2.9 *toxicity, n*—the propensity of a test material to produce adverse behavioral, biochemical, or physiological effects in a living organism.

3.2.10 *water accommodated fraction (WAF), n*—the predominantly aqueous portion of a mixture of water and a material poorly soluble in water, which separates in a specified period of time after the mixture has undergone a specified degree of mixing and which includes water, dissolved components, and dispersed droplets of the poorly soluble material.

³ For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

3.2.10.1 Discussion—

The composition of the WAF depends on the ratio of poorly soluble material to water in the original mixture as well as on the details of the mixing procedure.

3.2.11 *water soluble fraction (WSF), n*—the filtrate or centrifugate of the water accommodated fraction, which includes all parts of the WAF, except the dispersed droplets of the poorly soluble material.

3.3 Definitions of Terms Specific to This Standard:

3.3.1 *dilution water, n*—the water used in the aquatic test under consideration, which may contain organic or inorganic components depending on the test requirements.

3.3.2 *exposure matrix, n*—the water accommodated fraction (WAF), water soluble fraction (WSF), or mechanical dispersion to which test organisms are exposed for toxicity testing.

3.3.3 *test systems, n*—the test organisms plus the exposure matrix and the test chambers themselves.

4. Summary of Practice

4.1 This practice discusses various techniques for the preparation of test material for toxicity testing of poorly water-soluble lubricants or their components under acute or chronic exposure conditions with fish, large invertebrates, or algae.

5. Significance and Use

5.1 This practice gives techniques to use in the preparation of lubricants or lubricant components for acute or chronic aquatic toxicity tests. Most lubricants and lubricant components are difficult to evaluate in toxicity tests because they are mixtures of chemical compounds with varying and usually poor solubility in water. Lubricants or lubricant component mixtures should not be added directly to aquatic systems for toxicity testing because the details of the addition procedure will have a large effect on the results of the toxicity test. Use of the techniques described in this practice will produce well-characterized test systems that will lead to tests with meaningful and reproducible results.

5.2 The toxicity of mixtures of poorly soluble components cannot be expressed in the usual terms of lethal concentration (or the similar terms of effect concentration or inhibition concentration) because the mixtures may not be completely soluble at treat levels that lead to toxic effects. The test material preparation techniques given in this practice lead to test results expressed in terms of loading rate, which is a practical and meaningful concept for expressing the toxicity of this type of material.

5.3 One of the recommended methods of material preparation for lubricants or their components is the mechanical dispersion technique. This particular technique generates turbulence, and thus, it should not be used for poorly swimming organisms.

6. Apparatus

6.1 *Stirrer*, magnetic, size sufficient to accommodate vessel (6.2).

6.2 *Vessel*, glass, bottom drain, capacity 10 L to 20 L.

NOTE 1—The vessels shall be of sufficient size to maintain the vortex intensity and optimize surface area contact between the test material and the water.

NOTE 2—Height to diameter ratio for the WAF preparation at initiation should be between 1:1 and 2:1, and solutions shall not exceed 20 L per individual preparation. If larger volumes of test solution are required for experimental design, separate WAF preparations of the same test load may be combined.

NOTE 3—Aspirator bottles or other vessels with outlet ports at the bottom are preferable for WAF collection, if the test material is less dense than water. If a glass siphon is used to remove the WAF, it shall be in place prior to the introduction of the test material.

NOTE 4—WAF solutions from test materials that are denser than water shall be decanted from the top of the vessel. For these dense fluids, a propeller-driven system that stirs the test solution from the top of the vessel may be preferable to a stir plate that stirs the test solution from the bottom of the vessel.

NOTE 5—In all cases, take precautions to ensure that non-dispersed test material is not collected with the WAF.

7. Reagents

7.1 *Test Material*, lubricant from which water-soluble component(s) is or are to be extracted.

7.2 *Water*, Type II per Specification **D1193**.

8. Preparation of Exposure Matrix

8.1 Experimental design considerations for fish and invertebrates will determine the test species, number of loading rates tested, and number of organisms per treatment. Experimental design considerations for algae will determine the test species, number of loading rates tested, and appropriate initial biomass per treatment.

8.2 An acute or chronic test intended to allow calculation of an LLXX, ILXX, or ELXX will typically consist of a dilution water or test medium control and a series of at least five loading rates of test material.

8.3 Test material shall be added directly to the dilution water to prepare the exposure matrix. Use of a solvent or chemical dispersant is not recommended because these may alter the nature of the exposure to the test material and lead to non-representative ecotoxicity relationships.

8.4 *Preparation of Water-Accommodated Fraction (WAF)*:

8.4.1 Components dissolved in the water phase or entrained as stable droplets in the water phase are evaluated in a WAF study **(6)**. Individual WAFs must be generated for each test exposure loading. Serial dilutions of a single WAF are not appropriate due to differential solubility of constituents at low exposure loads **(6, 7)**. A schematic of the WAF setup is provided in **Fig. 1**.

8.4.2 Fill vessels for WAF preparation with the appropriate volume of dilution water, and prepare them for stirring as described in **6.2**.

8.4.2.1 Slowly add test material to the top of each vessel after dilution water addition.

8.4.2.2 Cap the vessel with foil or a non-reactive covering and stir at the test temperature.

8.4.2.3 If the test temperatures are different than room temperature, house the WAF preparation vessels in water baths or incubators to maintain the desired temperature.

8.4.2.4 If photo-decomposition of the test material is likely, conduct the stirring phase in the dark.

8.4.2.5 Inspect preparation vessels occasionally to ensure an appropriate vortex is maintained.

8.4.3 Determine the amount of test material added to each vessel from the desired nominal exposure load.

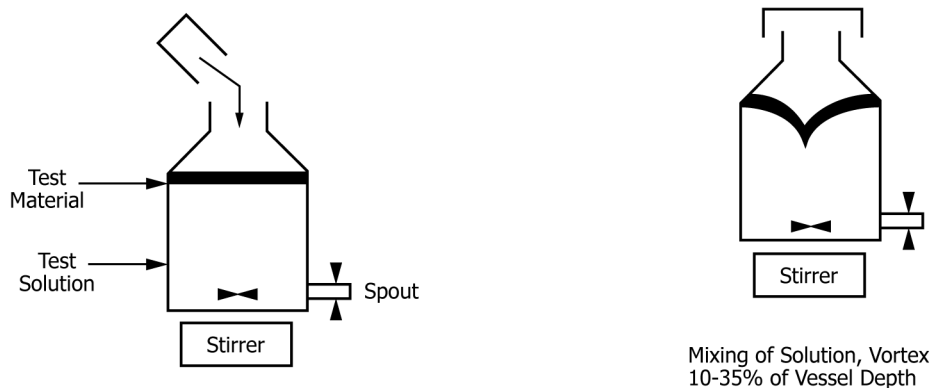
8.4.3.1 Base nominal exposure loads on either direct weight of material added per volume solution (wt/vol) or on the basis of test material density and added on a vol/vol basis.

8.4.4 Operate stir plates at a sufficient speed to ensure a vortex depth of 10 % to 35 % of the test solution height in the WAF preparation vessel.

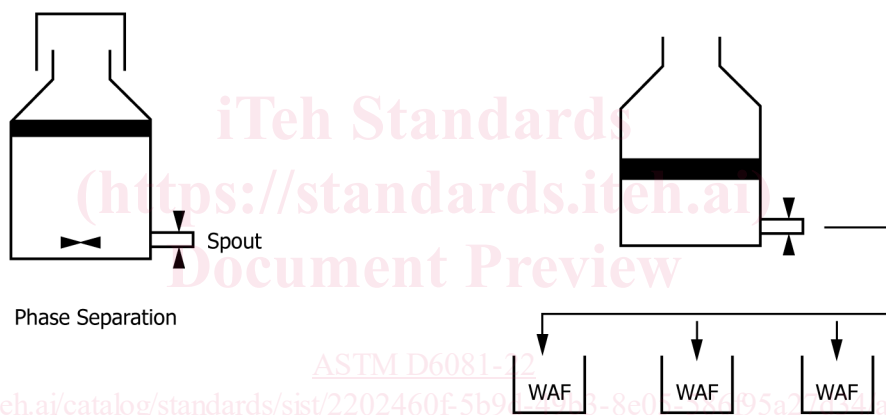
8.4.4.1 Ensure that the vortices in all WAF preparation vessels for any one experiment are the same length (that is, depth from fluid surface).

8.4.4.2 Exercise care to ensure that test material is not pulled down to the bottom of the vessel, and that the rate of stirring is not so vigorous as to promote emulsification.

8.4.5 The duration of WAF preparation will depend on the results of an optional equilibrium/stability study (see **8.7**). In the absence of this study, use a standard 20 h to 24 h mixing and 1 h to 4 h settling period **(6, 8)**.



Slow Addition of Test Material at Required Loading Rate



WAF Decanted for Testing/Chemical Analysis

FIG. 1 Water Accommodated Fraction (WAF) Schematic

8.4.6 Upon completion of the WAF mixing and settling period, drain or decant the aqueous solution from below the surface of each preparation vessel.

8.4.6.1 Discard the first 5 mL to 10 mL of solution decanted from the vessel.

8.4.6.2 Use the remaining solution undiluted in the exposure vessels.

8.5 Preparation of Water-Soluble Fraction (WSF):

8.5.1 Evaluate only components dissolved in the water phase in a WSF study (6).

8.5.1.1 Prepare WSFs by filtering WAFs through a 0.45 µm nominal cellulose-acetate filter or by centrifuging to remove undissolved material.

NOTE 6—Preliminary testing may be necessary to ensure that the filter disc will not remove test material constituents that are dissolved in the water phase.

8.5.1.2 Individual WSFs must be generated for each test exposure load. Serial dilutions of a single WSF are not appropriate due to differential solubility of constituents at low exposure loads. A schematic of the WSF setup is provided in Fig. 2.

8.5.2 The procedures described in 8.4.2 – 8.4.6 for the WAF preparation are the initial steps of the WSF procedure.

8.5.3 Upon completion of the WAF mixing and settling period, decant the aqueous solution from below the surface of each preparation vessel. The first 5 mL to 10 mL of solution decanted from the vessel should be discarded, with the remaining solution used to prepare the WSF. Filtration through a 0.45 µm filter or low speed centrifugation (to remove droplets but not to break an emulsion) is performed on the solution to remove undissolved material. The filtrate or centrifugation supernatant is the WSF exposure matrix and should be used undiluted in the exposure vessels.

8.6 Preparation of a Mechanical Dispersion:

NOTE 7—In the mechanical dispersion technique, a motor-driven propeller assembly is used to continually move test material and dilution water in the test vessel during the study (6, 7, 9).

NOTE 8—This procedure should not be used for small-sized, slowly, or poorly swimming organisms. It is most appropriate for juvenile fish evaluated in acute studies.

8.6.1 Configuration:

8.6.1.1 A schematic of the mechanical dispersion setup is provided in Fig. 3.

8.6.2 Ensure that each test vessel is equipped with a central cylinder equipped with a three-bladed propeller. The cylinder should be of appropriate configuration to allow water and test material to spill into the top of the cylinder and be released from small

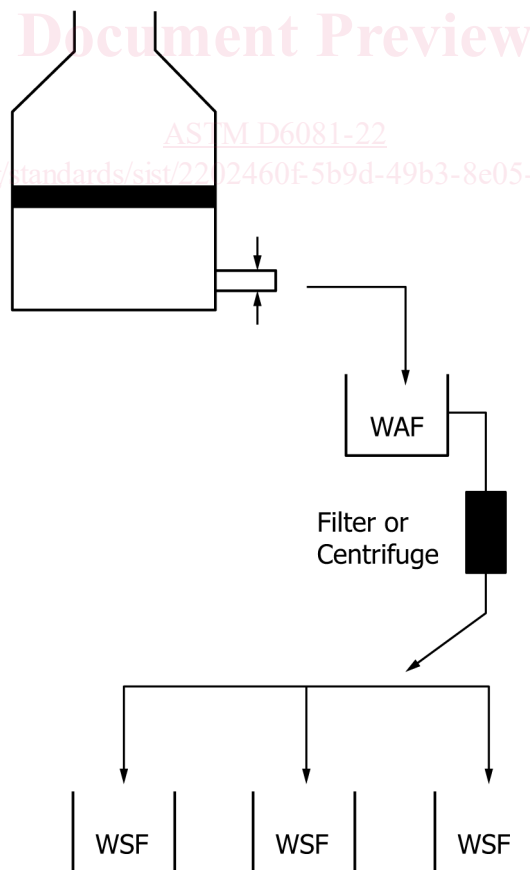


FIG. 2 Water Soluble Fraction (WSF) Schematic

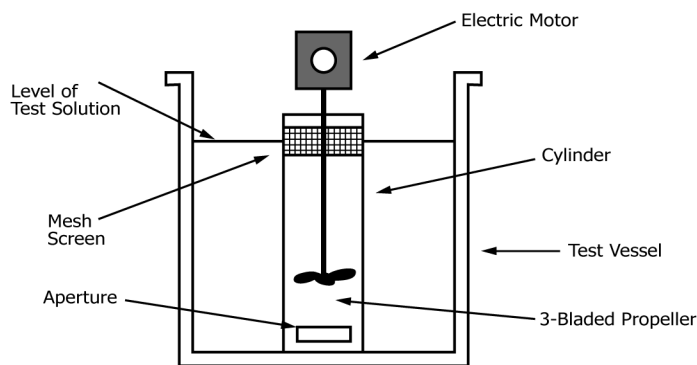


FIG. 3 Mechanical Dispersion Schematic

apertures at the bottom of the cylinder. The pitch of the propeller should be between 30 % to 45 % and should be the same among test vessels. Screening with a non-reactive material should be used at the top and bottom of the cylinder, as necessary, to prevent organism movement into the cylinder.

8.6.3 Ensure that each test vessel is filled with a sufficient volume of dilution water to enable operation of the mechanical dispersion apparatus.

8.6.3.1 Adjust the stirring speed in each vessel from 1000 r/min to 1500 r/min, to create a vortex within the cylinder of 12 mm to 20 mm (5 % to 10 % of cylinder height). Check the motor speed daily and replace evaporative losses with dilution water, as needed, to ensure consistent motor speed.

8.6.3.2 The test vessels should be the same for all test treat rates during a study and should be appropriate for the organism being evaluated.

NOTE 9—The dimensions and configuration of the test vessels have not been specified. Both circular and rectangular vessels have been used with this technique.

8.6.4 Testing:

8.6.4.1 Determine the amount of test material added to each vessel by the desired nominal exposure load.

NOTE 10—Nominal exposure loads may be based on direct weight of material added per volume solution (wt/vol) or calculated on the basis of test material density and added on a vol/vol basis.

8.6.4.2 Add test material to the top of the test vessel slowly.

8.6.4.3 Add non-viscous test material directly into the cylinder.

8.6.4.4 For viscous or semi-solid lubricants or their constituents, transfer test material to petri dishes, glass plates, or TFE-fluorocarbon pads before adding it to the test vessels. This ensures maximum surface area for test material exposure and reproducible test results.

8.7 Equilibrium/Stability Study (Optional):

8.7.1 The stirring and settling periods used to generate individual WAFs or WSFs are material-sensitive and can be optimized (10). Analytical characterization studies can be done to determine the mixing time which results in maximum test material concentration in the matrix and the duration of matrix stability.

8.7.2 To conduct an equilibrium/stability study, prepare at least one WAF or WSF exposure matrix at the highest anticipated loading rate to be used in the toxicity study for each time interval to be evaluated.

8.7.3 During the equilibrium/stability study, use the same dilution water and vessel configuration that will be used during the toxicity study.