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EPA

Protocol for EPA Approval of New Methods or Alternate Test Procedures for Whole Effluent Toxicity

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Engineering and Analysis Division
Office of Science and Technology
Office of Water
U.S. Environmental Protection Agency
Washington, D.C.

Foreword

Within the U.S. Environmental Protection Agency's (EPA) Office of Water (OW), the Office of Science and Technology (OST) has responsibility for administering the Clean Water Act (CWA) Section 304(h) Method Approval Program. The Method Approval Program entails proposal and promulgation of chemical and biological methods at 40 *Code of Federal Regulations* (CFR) part 136 for use in National Pollutant Discharage Elimination System (NPDES) data gathering and compliance monitoring under the CWA. In 1995, program administration was transferred from EPA's Office of Research and Development (ORD)-National Exposure Research Laboratory (NERL) in Cincinnati to OST to centralize the overall responsibility for promulgating effluent guidelines and associated compliance monitoring methods, with the intent of expediting the method approval process. Within OST, the Analytical Methods Staff (AMS) was delegated responsibility for administering the Method Approval Program. This responsibility includes the review and recommendation for approval of new test methods and alternate test procedures (ATPs) within approved methods.

Approval to modify an EPA-approved method can be gained through the ATP process specified at 40 CFR 136.4 and 136.5. To implement the ATP program, EPA developed guidelines for submission and review of ATP applications for chemical methods and selected microbiological methods approved at 40 CFR part 136. In 1995, EPA promulgated a series of whole effluent toxicity (WET) test methods at 40 CFR part 136.3, Table 1A. The EPA-approved WET methods employ standardized aquatic test organisms to directly measure the aggregate toxicity of effluents and receiving waters. This draft protocol provides guidance for validating and submitting an application for approval of a new WET method and approval of an alternate test procedure or modification within an Agency-approved WET method. In preparing this guidance, AMS revised a draft WET ATP document developed by ORD to incorporate literature research on alternate analytical methods, internal EPA comments, critiques by members of EPA's Biological Advisory Committee (BAC), and external expert review comments to develop the current protocol.

This document gives specific instructions to external organizations regarding the validation, submission, and EPA approval of applications for new methods and ATPs that determine whole effluent toxicity. As it applies to modifications to approved WET methods, this document should serve as a supplement to the existing ATP guidance at 40 CFR 136.4 and 136.5. Due to the inherent complexity of the review of new methods, EPA will apply the concepts introduced in this protocol for the review of new WET methods on a case-by-case basis. EPA anticipates that the standardized procedures described herein should expedite the approval of WET methods, encourage the development of innovative technologies for measuring WET, and enhance the overall utility of the EPA methods approved for NPDES compliance monitoring.

This document is not a legal instrument and does not establish or affect legal obligations under Federal regulations. EPA reserves the right to change this protocol without prior notice.

All questions regarding the guidelines presented in this protocol should be directed to:

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1.0 INTRODUCTION AND BACKGROUND

1.1 Regulatory Background and Objectives

The Clean Water Act (CWA) requires the U.S. Environmental Protection Agency (EPA) to promulgate guidelines establishing test procedures for data gathering and compliance monitoring under National Pollution Discharge Elimination System (NPDES) permits. Within EPA, the Office of Water (OW) publishes test procedures for the analysis of wastewater. These test procedures are specified at 40 part 136 of the *Code of Federal Regulations* (CFR) (USGPO, 1996 and FWCA, 1987). On October 16, 1995, EPA promulgated a final regulation approving the use of whole effluent toxicity (WET) test methods to protect aquatic life in NPDES compliance monitoring (60 FR 53529). Whole effluent toxicity is defined as the aggregate toxic effect of an effluent or receiving water measured directly with a toxicity test. The Agency-approved WET test methods are listed at 40 CFR §136.3, Table IA (USEPA, 1994a, 1994b, and 1993a). These WET methods employ standardized aquatic test species to directly measure the acute or short-term chronic adverse effects of effluents and receiving waters in NPDES biomonitoring.

WET is considered a "method-defined analyte," which is an analyte that does not have a specific, known composition, therefore, the analytical result is dependent on the measurement technique. As a result, a change in the analytical technique has the potential to change the numerical value of the sample result. EPA believes that modifications to test procedures that determine method-defined analytes will need to have less flexibility than is allowed for methods that determine individual chemical or biological analytes. Therefore, EPA is restricting the allowable flexibility in methods for method-defined analytes and has established more stringent, standardized requirements for demonstrating the acceptability of new methods and alternate test procedures for EPA approval (USEPA, 1996a).

The objective of this protocol is to provide guidance to external organizations regarding preparation and submission of applications for formal EPA approval of new methods or alternate WET test procedures for use in the NPDES program. This document serves as a supplement to the alternate test procedure (ATP) guidelines published at 40 CFR §136.4 and 136.5. The codified ATP guidelines allow entities to apply for approval to modify an EPA-approved method. Due to the inherent complexity of evaluating new technologies for measuring WET and the fact that guidelines for the approval of new methods are being introduced in this document, EPA will apply the concepts in this protocol for the review of new WET method applications on a case-by-case basis.

Using this protocol, new methods or alternate WET test procedures may be developed and validated using the "tiered" validation procedures delineated in this document. Method validation is the process that establishes the performance of new methods or substantiates the performance of an ATP. Under the tiered system, a proposed WET method is classified based on its intended use, and a method validation study is required that reflects the level of use associated with each of three tier levels. At Tier 1, methods that are proposed for limited use (LU) in a single "matrix type" (e.g., field collected effluent from a single industrial subcategory) or multiple matrix types may be validated through a single-laboratory validation study, without the burden of conducting an interlaboratory (multi-laboratory) study. Methods intended for nationwide use (NW) in a single matrix type (Tier 2) or all matrix types (Tier 3), require interlaboratory testing. The validation data and other supporting documentation must then be submitted to EPA as a part of a formal new method or ATP application to request approval. Based on the

intended level of use, the application must be submitted to the state permitting agency, EPA Region, or EPA's Analytical Methods Staff (AMS) for internal review. The appropriate agency will review the submission and assess the performance and applicability of the proposed WET method for use in NPDES compliance monitoring. Proposed methods submitted for limited use will be formally approved by the Regional Administrator or state agency through a "letter of approval", while nationwide applications are officially approved by the EPA Administrator through rulemaking.

The standardized application and validation procedures described in this protocol are intended to accelerate the method approval process and encourage the development of innovative technologies for measuring whole effluent toxicity. Ideally, proposed WET test methods should include one or more of the following: advances in test species culturing, test procedures, and test concepts; improved data collection and analysis techniques; reduced test complexity; reduced analytical costs; enhanced method performance (sensitivity and/or precision); increased ecological relevance; and heightened protection of aquatic life in receiving waters. EPA believes that submissions that meet the requirements for approval described in this protocol and incorporate any of the above attributes will enhance the utility of the WET methods approved for use in the NPDES program.

1.2 Approved Whole Effluent Toxicity Test Methods

The WET test procedures currently approved by EPA employ a suite of standardized, surrogate freshwater, marine, and estuarine plants, invertebrates, and vertebrates to measure acute and estimate chronic toxicity. Surrogate species are test organisms that can be studied to produce results to estimate toxic responses of other species that are not tested directly. The test species in the EPA-approved WET methods were chosen because as a group they are readily cultured or maintained in the laboratory, they are available in the appropriate life-stages throughout the year from commercial sources in sufficient quantity and quality for testing purposes, they are relatively inexpensive since they may be cultured year-round in the lab, their reliability for use in toxicity testing is better established than for other species, they are better integrated into Toxicity Identification Evaluations (TIEs), and they span the sensitivity range of species of the ecosystems studied (USEPA, 1991a and ASTM, 1998). A list of the EPA WET methods approved at 40 CFR 136.3, Table IA is provided in Table 1. The methods are published in the following documents:

- *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms,* Fourth Edition, EPA-600-4-90-027F, August 1993 (USEPA, 1993a).

- *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to Freshwater Organisms*, Third Edition, EPA-600-4-91-002, July 1994 (USEPA, 1994a).

- *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to Marine and Estuarine Organisms*, Second Edition, EPA-600-4-91-003, July 1994 (USEPA, 1994b).

These documents are referred to collectively throughout this document as the "EPA WET methods manuals".

The approved marine chronic methods do not apply to discharges into marine waters of the Pacific Ocean. EPA intends to propose approval of marine chronic methods applicable to the colder,

Pacific coast waters. However, test species are included in the acute WET methods manual that are applicable to west coast waters.

Table 1: EPA-Approved Whole Effluent Toxicity Methods

Water Matrix	Test Organism	Scientific Name	Test Duration	Endpoint
Acute Toxicity Tests* [USEPA, 1993a]				
Freshwater	Cladoceran	*Ceriodaphnia dubia*	24, 48, optional 96 h	Mortality
		Daphnia magna	24, 48, optional 96 h	Mortality
		Daphnia pulex	24, 48, optional 96 h	Mortality
	Fathead minnow	*Pimephales promelas*	24, 48, 96 h	Mortality
	Rainbow trout	*Oncorhynchus mykiss*	24, 48, 96 h	Mortality
	Brook trout	*Salvelinus fontinalis*	24, 48, 96 h	Mortality
Marine and Estuarine	Mysid shrimp	*Mysidopsis bahia*	24, 48, 96 h	Mortality
		Holmesimysis costata	24, 48, 96 h	Mortality
	Sheepshead minnow	*Cyprinodon variegatus*	24, 48, 96 h	Mortality
	Silverside	*Menidia beryllina*	24, 48, 96 h	Mortality
		Menidia mendia	24, 48, 96 h	Mortality
		Menidia peninsulae	24, 48, 96 h	Mortality
Short-Term Chronic Toxicity Tests - Freshwater [USEPA, 1994b]				
Freshwater	Cladoceran	*Ceriodaphnia dubia*	60% of controls have 3 broods (7 days)	Survival and Reproduction
	Fathead minnow	*Pimephales promelas*	7 days	Larval Survival and Growth
	Fathead minnow	*Pimephales promelas*	7-9 days	Embryo-larval Survival and Teratogenicity
	Green alga	*Selenastrum capricornutum*	96 h	Growth
Short-Term Chronic Toxicity Tests - Estuarine and Marine [USEPA, 1994a]				
Marine and Estuarine	Mysid	*Mysidopsis bahia*	7 days	Survival, Growth, and Fecundity
	Sea urchin	*Arbacia punctulata*	1.5 h	Fertilization
	Sheepshead minnow	*Cyprinodon variegatus*	7 days	Larval Survival and Growth
	Sheepshead minnow	*Cyprinodon variegatus*	7-9 days	Embryo-larval Survival & Teratogenicity
	Inland Silverside	*Menedia beryillina*	7 days	Larval Survival and Growth
	Red Macroalga	*Champia parvula*	7-9 days	Reproduction (Cystocarp production)

* A supplemental list of EPA-approved acute test species is included on pp. 264-266 of USEPA, 1993a.

1.3 Acute and Short-Term Chronic Whole Effluent Toxicity Tests

Both acute and short-term chronic WET tests are approved by EPA. Acute WET tests determine the concentration of water sample (e.g., effluent, receiving water, reference toxicant) that produces an adverse effect on a group of test organisms during either a 24, 48, or 96 hour exposure. Acute test endpoints include the following:

(1) Lethal Concentration or Effect Concentration (LC or EC)- a point estimate of the water sample concentration that causes death or other adverse biological effect in a specified percentage (LC_p or EC_p) of the exposed test species within a selected exposure period. The most common acute value reported is the LC_{50}.

(2) No Observed Adverse Effect Concentration (NOAEC) - the highest concentration of water sample at which survival is not significantly different from control.

(3) Pass/Fail Test - determines whether the survival of test species in the water sample is significantly less than survival in control.

Short-term chronic WET tests estimate chronic toxicity by evaluating, in addition to mortality, the sublethal effects of water samples, such as suppression of growth or length, or impairment of reproduction, fertilization, or larval development. A chronic effect is a stimulus from exposure to a toxicant that lingers or continues for a relatively long period of time. Traditionally, a chronic test is either a full-life cycle test or a shortened test of about 30 days known as an early life stage test. Because of the high cost and time required for these classical chronic toxicity tests, short-term chronic tests that focus on the critical life stage have been developed for use in compliance monitoring. The critical life stage is the period of time in an organism's lifespan in which it is the most susceptible to adverse effects caused by exposure to toxicants. The EPA-approved short-term chronic tests range from 40 minutes to 7 days of exposure. Short-term chronic test endpoints include:

(1) No Observed Effect Concentration (NOEC) - the highest concentration of water sample in which the observed responses are not statistically different from the control response.

(2) Lowest Observed Effect Concentration (LOEC) - the lowest concentration of water sample in which the observed responses are statistically different from the control response.

(3) Lethal Concentration (LC) - defined above.

(4) Effective Concentration (EC) - a point estimate of the water sample concentration that produces an observable effect on an all or nothing response (e.g., death, immobilization).

(5) Inhibition Concentration (IC) - a point estimate of the water sample concentration that produces a given reduction (e.g., 25%, 50%) in survival, fecundity, or growth.

(6) Pass/Fail test - a determination of whether the specified response in a water sample is statistically different from the control response.

A variety of statistical methods are used to generate and interpret the data generated in the tests described above. The LC, EC, and IC WET endpoints are determined using point estimate techniques. Point estimate techniques are statistical analyses that determine the test sample concentration at which a given adverse effect level occurred. These statistical techniques include the Graphical, Spearman-

Karber, Trimmed Spearman-Karber, or Probit Method. The NOAEC, NOEC, LOEC, and pass/fail endpoints are determined using hypothesis testing. Hypothesis testing determines the test sample concentration in which the observed responses are statistically different from the control responses. Additional terminology pertinent to statistical procedures used to generate toxicity endpoints is included in the EPA WET methods manuals and the EPA *Technical Support Document For Water Quality-based Toxics Control* or "TSD" (USEPA, 1994a, 1994b, 1993a, and 1991a).

1.4 Definitions of "New Methods" and "Alternate Test Procedures"

A proposed WET test procedure will be considered either a new method or an ATP, according to the following criteria.

1.4.1 New Methods

A proposed test procedure will be considered a new method if it employs a test species, an endpoint or organism response, or a toxicity test concept that is not represented in the battery of Agency-approved WET methods. Since WET is a method-defined analyte, EPA generally considers the use of new test species, endpoints, or test concepts to be substantial changes, and therefore will be approved as new methods. The applicability and performance (sensitivity and precision) of new methods for NPDES compliance monitoring will be compared to the approved WET tests and considered for promulgation at 40 CFR part 136 on a case-by-case basis. Performance of WET methods is further discussed in Section 1.5 and the selection and use of resident species in WET testing is discussed in Section 1.6. The specific requirements for validating new WET methods are outlined in Section 3.0.

Note: Performance data for the EPA-approved WET methods are included in the EPA WET methods manuals and the TSD (USEPA, 1994a, 1994b, 1993a, and 1991a).

1.4.2 Alternate Test Procedures

A proposed test procedure will be considered an alternate test procedure if it involves a modification to selected attributes (i.e. test conditions) of the Agency-approved method. A proposed method that uses a subspecies, a species that has similar phylogeny (evolutionary history), or a species that occupies the same feeding guild as the Agency-approved species, may be considered for approval as an ATP. As is the case with all method-defined analytes, modifications to WET methods must be carefully considered to guard against changes that will significantly alter the results of the test. Strict review of validation data will ensure that only acceptable ATPs are approved. EPA expects that ATPs may incorporate, but are not limited to, changes to the following aspects of an approved WET method:

- Sample collection, holding time, and handling
- Test type (i.e., static, static-renewal, flow-through)
- Type and size of test chamber and sample delivery system
- Test sample volume and renewal scheme
- Dilution water
- Test species age (life-stage) and loading density in test chambers
- Test species culturing protocols
- Test conditions (i.e, lighting, type and quantity of food)
- Test concentrations, dilution factor, and/or number of replicate chambers/concentration

- Method of data analysis

To be considered for approval, ATPs must meet the physical and chemical parameters and test acceptability criteria of the approved method and demonstrate precision and sensitivity equal to or greater than that demonstrated by the unmodified Agency-approved test procedure in side-by-side tests. Precision and sensitivity in WET methods is further discussed in Section 1.5. The specific requirements for validating a WET ATP are outlined in Section 3.0.

1.5 Precision and Sensitivity in WET Test Data

Sensitivity and precision are attributes of WET test data that allow for the assessment of method performance. In toxicity tests, the "detection limit" is determined by the sensitivity of the test organisms. Sensitivity is defined as the capability of a method to differentiate between different concentrations of an analyte (USEPA, 1996a). In relation to WET testing, sensitivity is the degree of response of a given test species to different types (i.e, salts, metals, organics, effluents, and freshwater, marine, and estuarine receiving waters) and concentrations of test samples. Precision is defined as a measure of mutual agreement among individual measurements or enumerated values of the same property of a sample (USEPA, 1996a). Precision is described by the mean, standard deviation, or relative standard deviation (coefficient of variation) of a given set of data.

The sensitivity of organisms to toxicants is an intrinsic quality, which may vary greatly between species, but also varies somewhat between organisms within the same species. The range of acute and/or chronic response exhibited by the least sensitive and most sensitive species across widely separated taxonomic groups exposed to the same toxicant may differ by several orders of magnitude. In addition, a given species that is relatively insensitive to one compound (e.g. a metals) may be very sensitive to a different compound (e.g. a pesticide). Studies observing test species response to a wide variety of whole effluents and pure compounds have shown that no organism or test method is always the most sensitive (USEPA, 1991a). The sensitivity of test species employed by a given method will depend on the characteristics of the test sample, the age and condition (health) of the organisms, and test conditions such as light quality, intensity, and photoperiod; water temperature, salinity, pH, alkalinity, and hardness; the size of the test chambers, volume of test solution, and number and biomass of test species per chamber (loading); dissolved oxygen concentration; food quality, quantity, and feeding regime; dilution water quality; test duration; genetic make-up; and analyst experience. Because effluents and receiving waters may contain complex mixtures of unknown toxic constituents, knowledge of the range of toxic response exhibited by representative species of aquatic organisms is necessary for the protection of aquatic life in receiving waters. The assessment of toxicity requires the use of several aquatic test species representing different phylogenetic groups that display a wide range of sensitivity.

In WET testing, precision usually is described as the test consistency or repeatability both in a single laboratory (intralaboratory) and among multiple laboratories (interlaboratory) using the same WET test procedure and water sample. Comparison of the toxicity test endpoint determined using the same method, test conditions, and test sample will allow for the calculation of intralaboratory or interlaboratory precision. WET tests must address the inherent variability of toxic response and natural variability of within-species sensitivity exhibited by test organisms. Replicate tests, specified physical and chemical parameters, standardized test procedures, and test acceptability criteria are included in the EPA-approved methods to account for the intrinsic variability of WET tests.

Additional discussion of the sensitivity and precision of WET tests is included in the EPA WET methods manuals and the TSD (USEPA, 1994a, 1994b, 1993a, and 1991a).

1.6 Use of Resident Species

Regulatory agencies may require the use of resident or indigenous species, under the assumption that such tests are needed to assess impact to local biota. Resident species are species that are present at a specified site for some portion of its lifespan. Indigenous species are species that are likely to occur at a selected site for some part of its life as a native species (ASTM, 1998). While the use of species taken from the receiving water may have appeal, EPA considers it unnecessary to conduct WET tests with resident or indigenous species, whether the organisms are collected for direct use in WET tests or for spawning to obtain eggs or larvae for culture, because based on many years of development and use by EPA, states, municipalities, academia, and the regulated community it has been demonstrated that the Agency-approved test species are appropriate for the NPDES program. The following factors may make it impractical to use resident species in toxicity tests (USEPA, 1991a):

- Sensitive organisms may not be present in the local receiving water because they may have acclimated to the ambient environment through prior exposure to toxic effluents, receiving waters, or other pollutants.

- Handling of organisms during field collection and transport may cause excessive mortality or sublethal effects.

- It may be difficult to collect sufficient numbers of feral organisms of the required life-stage and quality from the receiving water throughout the year.

- Culturing organisms from broodstock collected in the field may be difficult and costly.

- Test species collected directly from the receiving water may not be used immediately because of possible parasitic or bacterial disease, physical injury, or collection-induced stress, which may result in early mortality or excessive sensitivity to the test conditions.

- Most states require collection permits, which may be difficult to obtain. Therefore, it is usually more cost-effective to culture them in the lab or obtain them from experienced private or state sources. Fish such as fathead minnows, sheepshead minnows, and silversides, and invertebrates that include daphnids and mysids, are readily reared in the laboratory or obtained from commercial sources.

- Since it is mandatory that the identity of test organisms is known to the species level, it would be necessary to examine each organism caught in the wild to confirm its identity and/or age. Such identification may be impractical or, at the least, stressful to the organisms.

- Unknown reproductive states at the time of collection might produce aberrant results due to interactions between breeding condition and metabolism or toxicity of contaminants.

- Historical quality assurance\quality control (QA/QC) records (control charts) and intralaboratory and interlaboratory performance data indicating the precision and reproducibility of test method may not be available for resident species.

- For nationwide applications, the test species may not be widely available at a fair market price in the appropriate life-stage for testing purposes throughout the year or may require culturing or test conditions, laboratory equipment, or analyst expertise that cannot be duplicated in all qualified WET labs.

EPA generally discourages the use of resident species toxicity testing unless it is required by a state statute or some other legally binding factor, or supporting data has been developed by an EPA Regional or state discharge permit program, permitee, or community member that demonstrates that a unique endemic species would provide a more accurate reflection of effluent compliance and be more protective of the local receiving water than the species in Agency-approved methods (USEPA, 1991a).

In most cases, applicants seeking approval for use of a resident species should follow the method validation and approval process for new WET methods. Further specific guidance on the selection and use of resident species in aquatic toxicity tests is available in the EPA WET methods manuals (USEPA, 1994a, 1994b, 1993a), the TSD (USEPA, 1991a), and a series of guidance documents (USEPA, 1973, 1990, 1993b and ASTM, 1998).

2.0 APPLICATION REQUIREMENTS

Every new method and ATP application shall be made in triplicate and shall include a completed *New Method and ATP Application Form for Whole Effluent Toxicity* (provided in Appendix A) with required attachments.

2.1 Submission Addresses and Approval Authority

A summary of where to submit new methods and ATP applications and the approval authorities for each tier level are provided in Table 2. An application for nationwide (NW) approval (Tier 2 or 3) of a proposed method should be forwarded to the Director, Analytical Methods Staff, Engineering and Analysis Division (4303), USEPA, 401 M Street, SW, Washington, DC 20460. An application for limited use (LU) approval (Tier 1) of a new method or ATP by a Regional EPA laboratory shall be forwarded directly to the appropriate EPA Regional Administrator. An application for limited use (LU) approval (Tier 1) of a proposed method by a state, commercial laboratory, an individual discharger, or permittee shall be forwarded directly to the Director of the state agency authorized to operate an EPA-approved NPDES permit program under Section 402 of CWA. If the state does not operate its own permit program, then the application by a state, commercial laboratory, individual discharger, or permittee shall be forwarded directly to the appropriate EPA Regional Administrator. Applications to the Regional Administrator may be submitted through the Regional ATP Coordinator; an address list for the Regional ATP Coordinators is provided in Appendix B. The Regional Administrator may choose to

forward Tier 1 (LU) applications to the Director of the Analytical Methods Staff (AMS) for an approval recommendation.

Table 2: Submission of Proposed WET Method Applications for Approval

TIER	LEVEL OF USE	APPLICANT	SUBMIT APPLICATION TO	APPROVAL AUTHORITY
Tier 1	Limited Use	USEPA Regional laboratories	EPA Regional Administrator (Regional ATP Coordinator)	EPA Regional Administrator
		States, commercial laboratories, individual dischargers, or permitees in states that do not operate an NPDES permit program under Section 402 of CWA	EPA Regional Administrator (Regional ATP Coordinator)	
		States, commercial laboratories, individual dischargers, or permitees in states that operate an permit program under Section 402 of CWA	Director of state Agency issuing the NPDES permit	
Tier 2	Nationwide Use	All applicants	Director, Analytical Methods Staff, EPA Headquarters	EPA Administrator
Tier 3	Nationwide Use	All applicants	Director, Analytical Methods Staff, EPA Headquarters	EPA Administrator

Upon receipt of the application, the state agency or EPA will assign an identification number to the application. The applicant should use the identification number in all future communications concerning the application.

2.2 Application Information

Information required on the new method or ATP application includes whether the proposed method is a new method or ATP, the name and address of the applicant, the date of submission, the title of the new method or ATP, the title of the EPA-approved method that is being modified in the ATP (ATPs only), the level of use desired (i.e., limited use or nationwide use), the validation tier level, and information about the applicant's NPDES permit if applicable (Tier 1 and 2 validation levels only). The *New Method and ATP Application Form for Whole Effluent Toxicity* is attached as Appendix A.

In addition, the following items must be submitted with the application: a brief summary and rationale for approval of the proposed method; the proposed method, prepared in standard EPA format; a method comparison table that gives a side-by-side comparison of the modified method and the EPA-approved method (ATPs only); the method validation study plan; the method validation study report, including supporting data; and, for nationwide applications that will undergo rulemaking, method information to facilitate preparation of the Preamble and Docket during EPA rulemaking.

Because of the complex nature of assessing the performance of new test methods and ATPs that determine method-defined analytes, it is highly recommended that applicants contact EPA for preliminary guidance prior to performing the validation study and submitting the formal application. All of the above-listed attachments do not need to be submitted with the initial application. The applicant may request that EPA make an initial determination whether or not a modification is allowed within the method-specified flexibility or whether a potential ATP or new method meets the needs of the NPDES biomonitoring program. The minimum information required for EPA to begin reviewing an application is the completed application form, the proposed method in standard EPA format, the method comparison table, and the proposed validation study plan. From this information, EPA can determine whether the applicant should proceed with the full validation.

The elements for a complete application for each tier level are presented in Table 3. The appropriate approval authority must receive all required application information and attachments before the application is considered complete.

Table 3: Application Requirements

Tier	Level of Use	Application Requirements
Tier 1	Limited Use	Completed Application Form Summary and Rationale for Approval Method in EPA Format Method Comparison Table (ATPs only) Validation Study Plan Validation Study Report
Tier 2	Nationwide Use	Completed Application Form Summary and Rationale for Approval Method in EPA Format Method Comparison Table (ATPs only) Validation Study Plan Validation Study Report Method Information for Preamble and Docket
Tier 3		

2.3 Standard EPA Method Format

In accordance with the standard EPA format advocated by EPA's Environmental Monitoring Management Council (EMMC), methods must contain 17 specific topical sections in a designated order. The 17 sections listed in Section 8.0 (Appendix C) of this document are mandatory for all methods. Additional numbered sections, however, may be inserted starting with Section 11.0, Procedure, as appropriate for a particular method. For more detailed information on the format for proposed methods, see EPA's Guidelines and Format document (USEPA, 1996b). If the organization developing the new method or ATP is a voluntary consensus standards organization (e.g., *Standard Methods*, ASTM) or government organization with a standardized format, that format may be used.

2.4 Method Comparison Table

As part of an ATP application, the applicant must perform an in-depth comparison of the proposed ATP with the EPA-approved method that has been modified, and document the comparison in a two-column method comparison table. The two-column method comparison table shall include the number and title of each method, the latest revision date of the proposed ATP, and a detailed discussion of each of the 17 topics (as required by the standard EPA method format described in Section 8.0 of this document). Each topic should be discussed on a separate row in the method comparison table. The applicant should highlight any differences between the proposed ATP and the approved method that has been modified. This comparison is not required for new WET methods.

2.5 Validation Study Plan

Prior to conducting all validation studies, the applicant must prepare a validation study plan. The validation study plan should contain the elements listed below:

- Background
- Objectives
- Study Management
- Technical Approach
- Data Reporting and Evaluation
- Limitations

These elements are further described in Section 3.6.

2.6 Validation Study Report

The applicant must conduct a validation study and provide a comprehensive validation study report with the new method or ATP application. The validation study report must include the following elements:

- Background
- Study Implementation
- Data Reporting and Validation
- Results
- Data Analysis/Discussion
- Conclusions
- Appendix A - The Method
- Appendix B - Validation Study Plan
- Appendix C - Supporting Data (Raw Data and Example Calculations)

These elements are further described in Section 3.7.

2.7 Documentation to Facilitate EPA Rulemaking

For Tier 2 and 3 applications, new methods or ATPs are approved by the EPA Administrator through rulemaking. In these cases, the applicant shall provide information that will aid EPA in preparing the preamble for the proposed rule that will be published in the *Federal Register* and relevant supporting documents used in developing the new method or ATP for EPA inclusion in the rule docket. Specifically, the applicant shall submit information that:

- Defines the purpose and intended use of the method.
- States what the method is based upon, noting any relationship of the method to other existing analytical methods, and indicates whether the method is associated with a sampling method.
- Identifies the matrix types for which the method has been found satisfactory.
- Describes method limitations and indicates any means of recognizing cases where the method may not be applicable.
- Outlines the procedure that is followed to determine the toxicity of the effluent, receiving water, and reference toxicant samples tested. In this description, the basic steps involved in performing the test and data analysis must be identified, but the details that are a necessary part of the complete statement of procedure should be omitted.
- Describes the end-point statistics and gives example calculations.
- Lists options to the method, if applicable.
- Discusses test acceptability criteria development (new methods only).
- Describes and discusses the validation study report, including study design and objectives, study limitations, study management, technical approach, data reporting and validation, results, data analysis discussion, and conclusions.

Previous method rules can serve as examples of the type of information and the appropriate level of detail necessary. Examples of preambles for method rules include 49 FR 43234, October 26, 1984; 56 FR 5090, February 7, 1991; 60 FR 53988, October 18, 1995; and 61 FR 1730, January 23, 1996.

2.8 Proprietary Information in Applications

All information provided to the Federal government is subject to the requirements of the Freedom of Information Act. Therefore, any proprietary information submitted with the proposed WET method application should be marked as confidential. EPA staff will handle such information according to the regulations in subparts A and B of 40 CFR Part 2.

In accordance with 40 CFR §2.203, a business that submits information to EPA may assert a business confidentiality claim covering the information by placing on (or attaching to) the information at the time it is submitted to EPA, a cover sheet, stamped or typed legend, or other suitable form of notice employing language such as *trade secret, proprietary*, or *company confidential.* Allegedly confidential portions of otherwise non-confidential documents should be clearly identified by the business, and may be submitted separately to facilitate identification and handling by EPA. If the business desires confidential treatment only until a certain date or until the occurrence of a certain event, the notice should so state.

If a claim of business confidentiality is not made at the time of submission, EPA will make such efforts as are administratively practicable to associate a late claim with copies of previously submitted

information in EPA files. However, EPA cannot assure that such efforts will be effective in light of the possibility of prior disclosure or widespread prior dissemination of the information.

3.0 METHOD VALIDATION

NOTE: This is a draft document, and the requirements for method validation have not been finalized. Potential new method or ATP applicants should contact EPA during the design phase of validation studies to ensure that the most current requirements are followed.

3.1 Introduction

Method validation is the process that establishes the performance of a new method or substantiates the performance of an ATP. All proposed new methods and ATPs must be validated in single or multiple laboratory studies to demonstrate that they can achieve precision and sensitivity that meets or exceeds that of approved methods. This is necessary to ensure that new methods and ATPs are capable of yielding reliable data for compliance monitoring purposes, and to ensure that the environmental protection provided by the WET program is consistent. Alternate test procedures will be compared to the unaltered approved method, and new test methods will be compared to the suite of approved methods.

To demonstrate precision of the new method or ATP, tests must be conducted on multiple replicates of real-world and reference toxicant samples. For limited use approval, tests may be conducted in a single laboratory, but for nationwide use approval, tests must be conducted in multiple laboratories. Interlaboratory testing of new methods requires a more rigorous nine laboratory design than interlaboratory testing of ATPs, which requires only three laboratories. To demonstrate sensitivity of the new method or ATP, side-by-side testing in a single laboratory must be conducted using the proposed new method or ATP and an approved method.

Proposed new WET methods and ATPs must be validated in a variety of water samples that include real-world matrices and reference toxicants. In relation to WET, a real-world matrix type ("matrix type") is defined as an effluent with common characteristics from a given industrial subcategory or publicly-owned treatment work (POTW) or a field collected freshwater, estuarine, or marine receiving water with common characteristics from a given receiving water source. Tests with effluents and receiving waters are used to evaluate the acceptability of the proposed method using samples collected directly from the environment. There is one effluent representative of each industrial subcategory and POTW facility. The individual industrial subcategories are defined in the regulations at 40 CFR parts 405 - 471 (USGPO, 1996). Examples of appropriate matrix types include the following: primary POTW effluent, C-stage effluent from chlorine bleach mills, effluent from the Continuous Casting subcategory of the Iron and Steel industrial category, Lake Superior water, or Chesapeake Bay water. Validation of proposed methods require testing of one to nine matrix types depending on whether the applicant is seeking limited use or nationwide use approval.

Because the accuracy and sensitivity of test species to detect toxicity cannot be "calibrated" before each WET test, the test must include standard reference toxicants to ensure data integrity. These reference toxicants are chemical compounds prepared in the laboratory that are used to check the performance of the WET method under study. Reference toxicant tests are a part of the routine QA/QC program used in WET testing to evaluate performance of laboratory personnel, equipment, and test species. Reference toxicants, as opposed to effluents or receiving waters, are of a known chemical composition and toxicity. Reference toxicants are used to assess the sensitivity of the organisms being used in the test and the suitability of the test method for compliance monitoring. Salts, metals, and

organic compounds are usually used as reference toxicants in WET tests. Additionally, it is suggested that reference toxicants are selected that display different modes of toxicity (e.g., neurotoxin, teratagen). Validation of proposed methods require testing of at least three reference toxicants. A list of several commonly used reference toxicants are provided in Appendix D.

All validation study results with selected matrix types and reference toxicants must be submitted along with all required documentation to the designated regulatory authority as a part of the formal new method or ATP application. Additionally, the applicant must maintain the completed application and have this documentation available for inspection by permitting authorities, laboratory certification authorities, and regulatory agencies.

Ideally, a proposed WET method should be substantiated through a classical interlaboratory (multi-laboratory) method validation study that resembles those used historically by EPA, ASTM, AOAC-International and other organizations for validating chemical methods (ASTM, 1994, Youden, 1978, and Wernimont, 1985). EPA recognizes, however, that a classical interlaboratory method validation study may be prohibitively costly to implement, especially for small laboratories and regulated entities. Therefore, EPA has developed a three-tiered approach that is applicable to the validation of WET methods. Further discussion of the tiered-validation system and the validation requirements for new methods and ATPs at each tier level are described in Sections 3.2 - 3.4 below.

3.2 Tiered Validation System For Proposed WET Methods

The tiered system classifies the intended use of a method and requires a method validation study that reflects the level of use associated with each tier. The three tiers as they apply to WET methods are summarized in Figure 1 and further discussed in Section 3.2.1- 3.2.3 below.

Figure 1: Tiered Validation System

Tier 1 refers to new methods or ATPs that will be used by a single laboratory (limited use) for one or more matrix type(s). Tier 1 contains two levels of validation, depending on whether the individual laboratory will be applying the new method or ATP to a single matrix type or to multiple matrix types.

Tier 2 refers to new methods or ATPs that will be used by all laboratories (nationwide use) analyzing samples of one matrix type. Validation requirements are for a nine-laboratory validation study for new methods and three-laboratory validation study for ATPs.

Tier 3 refers to new methods or ATPs that will be used by all laboratories (nationwide use) in all matrix types. Validation requirements are for a nine-laboratory validation study for new methods and a three-laboratory validation study for ATPs.

3.2.1 Tier 1 Validation Studies

Tier 1 is expected to be used by commercial laboratories, dischargers, and state and municipal laboratories repetitively testing samples of the same matrix type(s) from the same site(s) on a routine basis. The primary intent of Tier 1 is to allow use of a new WET method or ATP by a single laboratory. Tier 1 can be applied to a single matrix type or multiple matrix types. If a laboratory intends to apply the method to more than one matrix type, the laboratory must validate the method on each matrix type, to a limit of nine matrix types.

For Tier 1 validation studies, all tests must be conducted in a single laboratory on one matrix type and three reference toxicants. New methods and ATPs validated under Tier 1 may be approved for use only at the laboratory at which the Tier 1 validation study was conducted. Applicants may test an effluent or receiving water as the matrix type. At least four complete WET tests (four replicates) must be conducted with separate "aliquots" of each matrix type and reference toxicant included in the study. If a method is validated by a single laboratory using one to eight discrete matrix types, the validation is applicable only to those matrix types tested. However, once a laboratory has validated the method on nine matrix types, the validation is applicable to all other matrix types.

3.2.2 Tier 2 Validation Studies

Tier 2 validation studies are expected to be used by dischargers and state and municipal laboratories repetitively testing samples from the same matrix type from multiple sites on a routine basis. The intent of Tier 2 is to allow all regulated entities and laboratories to apply a new method or ATP to a single matrix type for nationwide use. EPA believes that implementation of Tier 2 will encourage the development and application of new techniques that apply to samples from a given industry , POTW facility, or receiving water source.

Tier 2 validation studies of new methods are performed in a minimum of nine laboratories and the validation of ATPs are conducted in a minimum of three laboratories. Each laboratory must test the same matrix type and the same three reference toxicants. Applicants may test an effluent or receiving water in the Tier 2 study. At least two complete WET tests (two replicates) must be conducted with separate aliquots of each matrix type included in the study, and at least four complete WET tests (four replicates) must be conducted with each reference toxicant included in the study. Following completion of Tier 2 validation, the method will be appropriate for the matrix type in which it was validated. In contrast to Tier 1, once a new method or ATP has been validated under Tier 2, the validation study results can be transferred to other laboratories, and the other laboratories may freely use the method, provided that the method is applied to analysis of samples of the matrix type(s) for which the method has been validated. Additionally, the other laboratories must meet all of the method's test acceptability criteria. If the new method or ATP is to be applied to another matrix type, the proposed method must be validated on that specific matrix type.

3.2.3 Tier 3 Validation Studies

Tier 3 studies are expected to be used by vendors, commercial laboratories, dischargers, and state and municipal laboratories testing a wide variety of matrix types from diverse sites. The primary intent of Tier 3 is to allow nationwide use of a new method or alternate WET test procedure by all regulated

entities and laboratories. For example, Tier 3 should allow commercial vendors to establish that new methods or ATPs for WET produce results that are acceptable for nationwide compliance monitoring purposes and should allow laboratory chains to apply new technologies or alternate techniques throughout their chain of laboratories to a variety of matrix types.

Tier 3 validation studies of new methods are performed in a minimum of nine laboratories using a minimum of nine different matrix types. Each laboratory shall test at least six of the nine matrix types, such that all matrix types are evaluated at a minimum of six laboratories. Tier 3 validation studies of ATPs are performed in a minimum of three laboratories using a minimum of nine different matrix types. For all Tier 3 studies, at least 7 of the nine matrix types must be effluents. Additionally, each laboratory must test the same three reference toxicants. As with Tier 2, at least two complete WET tests (two replicates) must be conducted with separate aliquots of each matrix type included in the study, and at least four complete WET tests (four replicates) must be conducted with each reference toxicant included in the study.

Note: Applicants may choose to employ a "referee laboratory" to conduct preliminary tests and to collect, prepare, and distribute samples to participant laboratories.

3.3 Summary of Tier 1, 2, and 3 Validation Requirements for WET Methods

The requirements for method validation studies for new methods and ATPs are summarized in Table 4 and 5, respectively. Further specific validation study requirements are provided in Section 3.4.

Table 4: Validation Requirements for New Methods

Tier Level	Test Used[1]	Real-World Matrix Types			Reference Toxicants[2]			Total Number of Samples Per Lab	Number of Labs	Total Number of WET Tests
		Number of Real-World Matrix Types[3]	Number of Replicates	Total Number of Real-World Samples Per Lab	Number of Reference Toxicants	Number of Replicates	Total Number of Reference Toxciant Samples Per Lab			
Tier 1 - Single Matrix Type	New Method	1	4	4	3	4	12	16	1	16
	Approved Method	1	4	4	3	4	12	16	1	16
									Total	**32**
Tier 1- Multiple Matrix Types[5]	New Method	1	4	4	3	4	12	16	1	16
	Approved Method	1	4	4	3	4	12	16	1	16
									Total	**32**
Tier 2	New Method	1	2	2	3	4	8[6]	10	9	90
	Approved Method	1	2	2	3	4	12	14	1	14
									Total	**104**
Tier 3	New Method	9	2	12[7]	3	4	8[6]	20	9	180
	Approved Method	9	2	18	3	4	12	30	1	30
									Total	**210**

[1] Side-by-side testing (in one laboratory) must be performed using the proposed new method and an approved test method to demonstrate comparative sensitivity of the new method.
[2] At least one reference toxicant must be included from each of the three different classes: metal, organic, and salt.
[3] For Tier 1 and 2, testing shall use the real-world matrix type for which new method approval is sought. For Tier 3, seven of the nine real-world matrix types must be effluents.
[4] Replicate WET tests must be completed with separate aliquots of each sample.
[5] The requirements apply to the validation of the new method for use with each additional matrix type (8 max.).
[6] Each of nine laboratories must test two of the three reference toxicants, such that each of the three reference toxicant is tested in six laboratories. Hence, the total number of reference toxicant samples tested per lab is eight (2 reference toxicants per lab X 4 replicates) rather than 12 (3 reference toxicants X 4 replicates).
[7] Each of nine laboratories must test six of the nine real-world matrix types, such that each of the real-world matrix types is tested in six laboratories. Hence the number of real-world samples tested per lab is 12 (6 real-world matrix types per lab X 2 replicates) rather than 18 (9 real-world matrix types X 2 replicates).

Table 5: Validation Requirements for Alternate Test Procedures

Tier Level	Test Used[1]	Real-World Matrix Types			Reference Toxicants[2]			Total Number of Samples Per Lab	Number of Labs	Total Number of WET Tests
		Number of Real-World Matrix Types[3]	Number of Replicates[4]	Total Number of Real-World Samples Per Lab	Number of Reference Toxicants	Number of Replicates[4]	Total Number of Reference Toxciant Samples Per Lab			
Tier 1 - Single Matrix Type	ATP	1	4	4	3	4	12	16	1	16
	Approved Method	1	4	4	3	4	12	16	1	16
									Total	**32**
Tier 1- Multiple Matrix Types[5]	ATP	1	4	4	3	4	12	16	1	16
	Approved Method	1	4	4	3	4	12	16	1	16
									Total	**32**
Tier 2	ATP	1	2	2	3	4	12	14	3	42
	Approved Method	1	2	2	3	4	12	14	1	14
									Total	**56**
Tier 3	ATP	9	2	18	3	4	12	30	3	90
	Approved Method	9	2	18	3	4	12	30	1	30
									Total	**120**

[1] Side-by-side testing (in one laboratory) must be performed using the proposed ATP and the approved version of the test method to demonstrate comparative sensitivity of the ATP.
[2] At least one reference toxicant must be included from each of the three different classes: metal, organic, and salt.
[3] For Tier 1 and 2, testing shall use the real-world matrix type for which ATP approval is sought. For Tier 3, seven of the nine real-world matrix types must be effluents.
[4] Replicate WET tests must be completed with separate aliquots of each sample.
[5] The requirements apply to the validation of the ATP for use with each additional matrix type (8 max.).

3.4 Specific Validation Requirements for New Methods and Alternate Test Procedures

The following requirements apply to the validation of new methods and ATPs for WET at all tier levels. Where requirements differ between new methods and ATPs, the individual section is divided into subsections. The information contained in sections that are not subdivided is applicable to the validation of all proposed WET methods.

3.4.1 Distribution of Method and Validation Study Plan

Prior to conducting the validation study, the organization responsible for developing or modifying a method must detail the full method in standard EPA format in accordance with the Guidelines and Format document (USEPA, 1996b). The documented method should be distributed along with the validation study plan and any additional guidance to each laboratory participating in the validation study.

3.4.2 Side-by-Side Tests

Side-by-side testing is required for the validation of new methods and ATPs. Each side-by-side test is referred to as a "test set". For ATPs, side-by-side tests must be conducted with the proposed method and the EPA-approved method that is being modified. For new methods, side-by-side tests must be conducted with the proposed method and at least one of the approved methods. This side-by-side testing requirement is designed to establish the relative sensitivity of the new method or ATP compared to approved methods. Approved methods conducted in the side-by-side testing must be conducted according to the guidelines and procedures in the WET method manuals. For ATPs, each side-by-side test must be conducted with the same batch of test organisms, reference toxicant and/or matrix type, test sample concentrations, number of replicate chambers per concentration, and using the test conditions and procedures specified in the EPA-approved method or specified in the NPDES permits. This list of standardized test parameters for side-by-side tests may be adapted for ATP applications that seek to modify the test sample concentrations, replicate chambers, or test conditions listed in the approved method.

3.4.3 Replicate Testing

At least four complete WET tests (replications or repeat tests) must be conducted with each reference toxicant included in the study. For single laboratory validation studies (Tier 1), four complete WET tests (replications) must also be conducted with aliquots of each real-world matrix sample included in the study. For multiple laboratory validation studies (Tier 2 and 3), at least two complete WET tests (replications) must be conducted with aliquots of each real-world matrix sample. Applicants may include more than the minimum number of replicate tests if they desire. The replicate tests must be conducted with the same tests concentrations, test conditions, and data analysis methods. These replications apply to the entire test and are independent of the "replicate test chambers" required in several approved WET tests. Based on the complexity of a proposed new method or the magnitude of the modification(s) included in a proposed ATP, the required number of replicate tests may be amended on a case-by-case basis.

3.4.4 Matrix Types

Validation of proposed methods requires testing of from one to nine matrix types. All effluent and receiving water samples must be collected using the type of containers, preservation techniques, shipping and handling, and holding times described in the EPA WET methods manuals and outlined in Table II, 40 CFR part 136. In multiple laboratory validation studies (Tier 2 and 3), each real-world matrix sample should be tested in each of the laboratories. However, in validation studies that include multiple matrix types and nine or more laboratories, each laboratory may test six of the nine real-world matrix type samples, such that each of the real-world matrix types is tested in at least six laboratories.

3.4.5 Reference Toxicants

Validation of proposed methods require testing of at least three reference toxicants. Reference toxicants must be included from each of the three different classes: metal, organic, and salt. Reference toxicants also should be selected that display different "modes of toxicity" (e.g., neurotoxin, genotoxin, teratogen, membrane disruptor, enzyme inhibitor, etc.). A list of common reference toxicants is included as Appendix D. In multiple laboratory validation studies (Tier 2 and 3), each laboratory should test each of the same three reference toxicants. However, in validation studies that include nine or more laboratories, each laboratory may test two of the three reference toxicants, such that each of the three reference toxicants is tested in at least six laboratories.

3.4.6 Selection and Use of Test Species

3.4.6.1 New Methods

Applicants seeking nationwide approval (Tier 2 or 3) should ensure that the new test species are widely available at a fair market price in the appropriate life-stage in sufficient quantity and quality for testing purposes throughout the year and do not require unique culturing or test conditions, laboratory equipment, or analyst expertise that cannot be duplicated in all qualified WET labs. The following parameters apply to the selection and use of test species in new methods.

- The taxonomic identity of each test organism must be determined to the species level (or strain for bacteria) by appropriate keys and verified by an appropriate expert. A species profile should be provided that describes the taxonomy, habitat, feeding regime, and other information to characterize the new species.

- If purchased from a commercial supplier, the source of new test species must be reported.

- If cultured in the lab, a protocol for culturing and handling a new test species must be developed and included with the validation study report.

- The appropriate life stage of the proposed species must be available year round for use in testing.
- A new test organism must tolerate handling (or collection) without excessive disruption of its life processes, and should be amenable to culturing, maintenance, and testing under controlled laboratory conditions. An exception to the lab culturing requirement may be considered for the

breeding stock of certain species that cannot be maintained in the lab or a feral organism that has been determined to be the most sensitive.

- The necessary federal or state collection permits, or both, must be acquired prior to collecting feral organisms. Rare or endangered species must not be used in WET tests without prior approval of appropriate regulatory agencies.

- Feral organisms captured by electroshocking must not be used in toxicity testing.

- Test species collected directly from the receiving water should be held for an appropriate time period to observe them for possible parasitic or bacterial disease, physical injury, or collection induced stress which may result in early mortality or excessive sensitivity to the test conditions.

- All individual tests must use a new batch of test species taken from the same in-house culturing facility or outside supplier.

Additional discussion relevant to new test species for WET tests is provided in Sections 1.5 and 1.6.

3.4.6.2 Alternate Test Procedures

Generally, all ATPs submitted for approval will employ approved test species. Proposed methods that use subspecies, species that have similar phylogeny (evolutionary history), or species that occupy the same feeding guild as the Agency-approved species, may be considered for approval as an ATP. Approval of closely related test organisms as ATPs will be evaluated by EPA on a case-by-case basis. Each test set must use a new batch of test species taken from the same in-house culturing facility or outside supplier.

3.4.7 Replicate Chambers Per Concentration

3.4.7.1 New Methods

Tests must employ at least the minimum number of replicate chambers per concentration listed in the new method. If replicate chambers are not applicable to a new test procedure or concept, it is suggested that the applicant include additional test replication (repeat tests).

3.4.7.2 Alternate Test Procedures

The side-by-side tests with the approved and alternate method must be conducted with at least the minimum number of replicate chambers per concentration listed in the approved method, unless the purpose for the ATP is to propose a change in the number of replicate chambers.

3.4.8 Acute Tests - Sample Concentration Series

For each acute test, a series of at least five effluent, receiving water, or reference toxicant sample concentrations based on a dilution factor of 0.5 is suggested. Ideally, the selected dilution series should

provide partial mortalities at two or more concentrations, including at least one concentration resulting in no mortality and at least one concentration resulting in 100% mortality.

3.4.9 Short-Term Chronic Tests - Sample Concentration Series

For short-term chronic tests, a series of at least five effluent, receiving water, or reference toxicant sample concentrations based on a dilution factor of 0.5 is suggested. Ideally, the dilution series chosen should provide at least two concentrations with no statistically significant effect on survival or sublethal endpoints compared to control, and at least two concentrations with a statistically significant effect on survival or sublethal endpoints compared to control.

3.4.10 Preliminary Toxicity Range-Finding Tests

If the appropriate effluent, receiving water, or reference toxicant sample concentration needed to meet the exposure/response requirements set out in Section 3.4.8 and 3.4.9 is unknown, it may be necessary for the laboratory to perform one or more preliminary range-finding tests. While non-toxic samples may be used in the validation study to document false positive rates, samples selected for use in the validation study should generally exhibit a measurable level of toxicity (so that precision measurements can be made). For this reason also, preliminary range-finding tests may be necessary. If blind test samples are employed, a referee laboratory may conduct the preliminary tests and distribute appropriate samples to participant laboratories for use in the study. The procedures for performing a range-finding test are outlined in the EPA WET methods manuals (USEPA 1994a, 1994b, 1993a).

3.4.11 Quality Assurance and Quality Control

3.4.11.1 New Methods

All tests conducted using the approved methods must comply with the general QA/QC criteria provided in the EPA WET methods manuals. For new methods, QA/QC criteria that is at least as rigorous as that for approved WET methods should be developed and detailed in Section 10 of the required EPA formatted method description (see Section 2.3 and Appendix C of this document). All tests conducted using the new method must comply with the QA/QC criteria developed for the method.

3.4.11.2 Alternate Test Procedures

All tests conducted using the approved method and alternate test procedure must comply with the general QA/QC criteria provided in the EPA WET methods manuals.

3.4.12 Data Collection and Recording

All physical, chemical, and biological observations normally required for the Agency-approved methods shall be recorded for proposed and approved methods tested in a validation study report as described in Section 3.7.10. For new methods, alterations to the list of observations may be necessary to include additional parameters important to a new WET technology. Observations must be placed on appropriate bench sheets in the format provided in the EPA WET methods manuals (see sections on physical, chemical, and biological observations during the tests, and test data reporting requirements).

All raw data entries and changes in original entries shall be dated and initialed. Changes to entries must be accompanied by suitable, legible justification. The format and/or identification of the original data sheets shall be such that the date of entry and identity of the observer for each observation is obvious. The original data sheets (e.g., bench sheets, logbook pages) and other required documentation (e.g., sample information, deviations from methods) shall be retained by the performing laboratory, and copies appended to the final validation study report.
Note: All data from the method validation study must be submitted to the approval authority, including tests that were incomplete or results that were not comparable to the EPA-approved method(s).

3.4.13 Physical and Chemical Parameters and Test Acceptability Criteria

3.4.13.1 New Methods

Data assembled in the validation study and previous studies should be used to develop and substantiate optimum physical and chemical test conditions and appropriate test acceptability criteria for the new test method.

3.4.13.2 Alternate Test Procedures

The control data from all side-by-side tests must meet the physical and chemical parameters and test acceptability criteria listed in the approved method.

3.4.14 Raw Data Analysis - Determination of Test Endpoints

Test endpoints for Agency-approved acute and short-term chronic tests performed in validation studies for ATPs shall be determined using the flow charts, guidelines, and statistical methods specified in EPA WET methods manuals. For new methods, a data analysis scheme and flowchart shall be developed in a format comparable to schemes for Agency-approved methods. Computer software used in data analysis must be identified by source, version, and date, and a machine-readable, executable copy provided if the software is accessible to the public. Software specifically recommended and/or provided by the Agency, shall be used where applicable.

3.4.15 Statistical Analysis of Endpoints - Determination of Sensitivity and Precision

The sensitivity and precision of proposed WET methods in selected matrix types and reference toxicants must be determined from the validation data. Method performance will be assessed from test endpoints generated from analysis of four replicates of a minimum of one matrix type and three reference toxicants. Sensitivity will be described as the degree of response of the proposed method, exhibited by the test endpoints, to each matrix type and reference toxicant under study. This will be compared to the response of the approved method in side-by-side testing. For intralaboratory and interlaboratory precision calculations, in cases where the test data are used in Probit Analysis or other point estimate techniques (LC_1, LC_{50} and IC_{25}), precision must be described by the mean, standard deviation, and CV of the calculated endpoints from the replicate tests. When the results are reported in terms of the NOEC, LOEC, or other endpoints derived from hypothesis testing, precision can only be described by listing the NOEC-LOEC interval for each test. The specific method performance requirements for new method and ATP applications are discussed below.

3.4.15.1 New Methods

Determination of sensitivity and intralaboratory precision of the new method is required for validation studies at all tier levels. Assessment of interlaboratory precision of the results is required for Tier 2 and 3 validation studies. The performance of new methods will be compared to the suite of EPA-approved WET tests using appropriate statistical techniques. Precision and sensitivity data for the EPA-approved methods is published in the EPA WET methods manuals and the TSD (USEPA, 1994a, 1994b, 1993a, 1991a).

3.4.15.2 Alternate Test Procedures

Determination of sensitivity and intralaboratory precision of ATPs is required for validation studies at all tier levels. Assessment of interlaboratory precision is required for Tier 2 and 3 validation studies. The performance of ATPs will be compared to the unmodified method using appropriate statistical techniques. In order to be considered for approval, the statistical analysis must demonstrate that the sensitivity and precision of the ATP is equivalent to or greater than that furnished by the approved method in the side-by-side tests.

3.5 Selection of Laboratories to Participate in the Validation Study

It is essential that laboratories selected to participate in a validation study demonstrate that they are qualified to conduct the EPA-approved WET tests. Problems with WET tests are caused most often by the misapplication of the tests, misinterpretation of data, quality of the WET testing laboratory, and lack of training and experience of laboratory personnel. The participants at the *Society of Environmental Toxicology and Chemistry* (SETAC) Whole Effluent Toxicity Workshop in 1995 concluded that the largest source of variability in WET testing is the level of analyst experience and judgement, and test species condition and health. EPA suggests that applicants assess the qualifications of potential laboratories before selecting them to participate in a method validation study. The following list provides some items that applicants may request to assess WET laboratory experience, capability, and proficiency:

- General information on the combination of facilities, equipment, staff, and laboratory capabilities.
- Specific information on sample handling, temperature control, source of water, type of food and source of foods, test equipment used for conduct of each test method, source of organisms, quality control measures for each organism, and resumes of principal laboratory staff.
- Internal Standard Operating Procedures (SOPs) for conducting WET tests and data analysis and preparing cultures, food, and dilution water that have been in use at the laboratory for each method included in the study.
- Historical QA/QC data (cusum charts) with approved methods and standard reference toxicants and an explanation of the derivation of associated control limits.
- Results from DMRQA studies.
- Recommendations from clients for whom they have conducted WET tests.

Applicants may also choose to have potential laboratories perform WET test(s) with a provided "prequalification sample" to demonstrate proficiency with a specific WET method. Additional guidance

and standardized procedures for evaluating potential laboratories is provided in the document titled, *Manual for the Evaluation of Laboratories Performing Aquatic Toxicity Tests* (USEPA, 1991b). Any problems associated with laboratory selection should be discussed with EPA prior to initiation of the validation study.

3.6 Validation Study Plan

Prior to conducting Tier 1, 2, or 3 validation studies, the organization responsible for conducting the study must prepare a validation study plan. A detailed study plan must be provided to the participating laboratories prior to their involvement. As mentioned in the application requirements section, applicants should consult with, or submit a validation study plan to EPA prior to initiation of the validation study. The version of the study plan used in the method validation study must be submitted to EPA as part of the formal application for approval. The validation study plan should contain the elements described in sections 3.6.1 through 3.6.6.

3.6.1 Background

The Background section of the validation study plan must include the following items:

- Identify the method as a new method or ATP.
- Include a method summary.
- If an ATP, cite the method number (given in 40 CFR parts 136, Table 1A) for the approved method.
- If an ATP, describe the reasons for and extent of the modification, the logic behind the technical approach to the modification, and the result of the modification.
- If a new method, describe the rationale for developing the method and identify the matrix types to which the method is believed to be applicable.

3.6.2 Objectives

The Objectives section of the validation study plan should describe overall objectives and data quality objectives of the study.

3.6.3 Study Management

The Study Management section of the validation study plan should include the following information:

- Identify the organization responsible for managing the study.
- Identify laboratories, facilities, and other organizations that will participate in the study.
- Delineate the study schedule.

3.6.4 Technical Approach

The Technical Approach section of the validation study plan should address the following:

- Indicate at which Tier level the study will be performed.
- Describe the approach that will be followed by each organization involved in the study.
- Describe how sample matrices (effluents, receiving water, and reference toxicants) and laboratories will be selected.
- Explain how samples will be collected, prepared, and distributed.

- Specify the numbers and types of analyses to be performed by the participating laboratories.
- Describe how analyses are to be performed.
- Describe the type and timing of monitoring and observations required.

3.6.5 Data Reporting and Evaluation

This section of the validation study plan should explain the procedures that will be followed for reporting and validating study data and should address the statistical analysis of study results.

3.6.6 Limitations

The Limitations section of the validation study plan should explain any limiting factors related to the scope of the study.

3.7 Validation Study Report

Laboratories or other organizations responsible for developing a proposed WET method at Tier 1, 2, or 3 must document the results of the validation study in a formal validation study report that is organized and contains the elements described in this section. The information and supporting data required in the validation study report must be sufficient to enable EPA to evaluate the performance of the new method or support a claim of equivalent performance for an ATP.

Like the validation study plan, the validation study report contains background information and describes the study design. Additionally, the validation study report details the process and results of the study, provides an analysis and discussion of the results, and presents study conclusions. The study plan must be appended to and referenced in the validation study report. The validation study report must identify and discuss any deviations from the study plan that were made when implementing the study. The validation study report must contain the elements described in sections 3.7.1 through 3.7.9.

3.7.1 Background

The Background section of the validation study report must describe the new method or ATP that was validated and must identify the organization responsible for developing the method. This background section must do the following:

- Identify the method as a new method or ATP.
- State if for nationwide or limited use.
- Include a method summary.
- If an ATP, cite the method number for the approved method at 40 CFR part 136, Table 1A.
- If an ATP, describe the reasons for and extent of the modification, the logic behind the technical approach to the modification, and the result of the modification.
- If a new method, describe the rationale for developing the method.
- Identify the matrices, matrix types, and/or media to which the method is believed to be applicable.
- State the purpose of the study.

3.7.2 Study Design and Objectives

The Study Design and Objectives section of the study report must describe the study design, and identify overall objectives and data quality objectives of the study. Any study limitations must be identified.

3.7.3 Study Implementation

The Study Implementation section of the validation study report must describe the methodology and approach undertaken in the study. This section must do the following:

- Identify the organization that was responsible for managing the study.
- Identify the laboratories, facilities, and other organizations that participated in the study; describe how laboratories were selected (prequalification criteria); indicate whether a referee laboratory was employed; and explain the role of each organization involved in the study.
- Indicate the tier level at which the study was performed.
- Clearly describe all samples prepared for the study (e.g., type of samples, number of samples of each type, concentration of reference toxicants in samples).
- Describe the rationale for selection of matrix types and reference toxicants, including a statement of compliance with tier requirements for matrix type selection. Indicate the source of effluent and/or receiving water samples and reference toxicant (chemical supplier).
- Describe how effluent and/or receiving water samples were collected in the field.
- Describe sample handling and storage.
- Provide background information on each matrix type (if available) and reference toxicant (e.g, chemical class, % active ingredients, toxic mode of action), and describe how each was prepared (e.g, compound in which toxicant is dissolved) and distributed (e.g., sample volumes shipped, sample containers, blind samples).
- Describe any preliminary testing conducted prior to the validation study (e.g., physical and chemical, preliminary range-finding toxicity tests).
- Describe the role and specific tasks performed by the referee laboratory (if applicable).
- Provide the source, strain (if appropriate), and species profile of the test species.
- Indicate the dilution water(s) that was used in the study.
- Specify the numbers and types of analyses performed by the laboratories (i.e, test conditions, test procedures, statistical analyses).
- Identify any problems encountered with samples, test species, equipment, etc. and their subsequent resolution.
- Identify any approved deviations from the study plan and their impact on study performance and/or results.

3.7.4 Data Reporting and Validation

This section of the validation study report must describe the procedures that were used to report and validate study data.

3.7.5 Results

This section of the validation study report presents the study results. Raw data and example calculations are required as part of the results and shall be included in an appendix to the validation study report (see section 3.7.10). For ATPs, this section must verify that the test acceptability criteria for the approved method were met for the purposes of assessing equivalency with the EPA-approved method. For new methods, the test acceptability criteria developed from the validation study should be described. A summary table shall be provided that lists the test method, the laboratory number, the sample number and type, individual test results in chronological order, including test endpoints and confidence intervals (where appropriate).

3.7.6 Data Analysis/Calculation

This section of the validation study report must provide a statistical analysis and discussion of the study results. Precision and sensitivity data shall be calculated and summarized in tabular form that includes, the mean, standard deviation, and percent coefficient of variation (CV) for endpoints that are point estimates (LC_{50} and IC_{25}), or ranges for endpoints determined by hypothesis testing (NOEC, LOEC). For ATPs, the discussion must address any discrepancies between the results and test acceptability criteria of the EPA-approved method.

3.7.7 Conclusions

The Conclusions section of the validation study report must describe the conclusions drawn from the study based on the data analysis discussion. The Conclusions section must contain a statement(s) regarding achievement of the study objective(s).

3.7.8 Appendix A - The Method

For new methods, the method, prepared in standard EPA format, must be appended to the validation study report (USEPA, 1996b). For ATPs, the modified portion of the EPA-approved method, prepared in standard EPA format, must be appended to the study report.

3.7.9 Appendix B -Validation Study Plan

The validation study plan must be appended to the validation study report.

3.7.10 Appendix C - Supporting Data

The validation study report must be accompanied by raw data and example calculations that support the results presented in the report. The validation study report must include all raw data so that an independent reviewer familiar with WET testing may verify each analytical determination and calculation performed by the laboratory. The full names, titles, addresses, and telephone numbers of the analysts who performed the analyses, the laboratory contact (lab manager) responsible for the study, and the quality assurance officer who verified the analyses must be provided.

3.7.10.1 Raw Data

The applicant shall provide copies of the original data sheets, bench sheets, or log book pages of sufficient quality that all data entries (and changes, if any) are clearly legible. The raw data may include any of the specific information listed below. Some of the items listed below may have been discussed in the study implementation section of the study report.

(1) Sample Information
- Sample numbers or other identifiers applied to individual samples
- Dates of sample preparation and/or receipt in the laboratory
- Sample temperature when received at laboratory
- Physical and chemical data of sample contents (as required in appropriate method)
- Dilution water
  - Source and time frame water is used or how maintained
  - Collection or preparation date(s), where applicable
  - Pretreatment information
  - Physical and chemical characteristics (pH, hardness, conductivity, salinity, etc.)
- Sample storage information
- Problems with samples and a description of subsequent resolution

(2) Test Procedure and Conditions
- Toxicity test methods used (title, number, source)
- Test duration
- Endpoint(s) to be determined
- Approved deviations/modifications from method(s), if any, and reason(s)
- Date and time test(s) started, date and time samples were prepared and solutions transferred for renewals.
- Date and time test(s) terminated
- Type and volume of test chambers
- Volume of solution used per chamber
- Number of organisms per test chamber
- Number of replicate test chambers per treatment
- Feeding frequency and amount and type of food (be specific with sources, concentrations of foods, and preparation dates)
- Acclimation of test organisms (temperature mean and range and, where applicable, salinity mean and range)
- Test temperature (mean and range)
- Test salinity, where applicable (mean and range)
- Other physical/chemical analyses such as pH, alkalinity, and hardness, and dissolved oxygen, if applicable
- Specify if aeration was needed
- Specify if organisms were dried immediately for weighing or preserved prior to drying
- Specify how food was prepared and sources of food
- Describe how routine chemistries on new solutions were made (in actual test chamber or in beakers after dispensing)
- Describe how randomization was conducted

(3) Test Organisms
- Scientific name of test species, verification of species documented
- Taxonomic key used for species identification
- Age (life stage) of test species, be specific for all species; Age at time of test initiation
- Mean length and weight (where applicable)
- Source and QA/QC test conditions
- Holding conditions
- Diseases and treatment (where applicable)
- For new test species, notation that a voucher specimen was preserved and archived

(4) Results
- Raw data from any preliminary tests (if applicable)
- Table of physical and chemical data taken during study
- Raw toxicity data in tabular form, including daily records of affected organisms in each replicate at each concentration (including controls) and plots of toxicity data or concentration vs. biological response
- Description of data analysis/statistical methods used to calculate endpoints and method performance data summarized in the results section of the validation study report

3.7.10.2 Electronic Data Reporting

Applicants may also choose to submit data in electronic format (Excel spreadsheet, or equivalent) that will allow EPA to create a database of study results. This database will facilitate automated review and statistical analysis of study results. The information included in electronic format may include: laboratory, analyst, method, sample type, sample number, date and time of analysis, test concentration, replicate number, raw toxicity data (e.g., survival counts, dry weight), and calculated toxicity endpoints. The applicant should discuss an appropriate electronic format with EPA prior to data submission.

3.7.10.3 Example Calculations

The validation study report must provide example statistical calculations that will allow the data reviewer to determine how the laboratory used the raw data to arrive at the test acceptability criteria (new methods only), test endpoints, and method performance data.

4.0 EPA REVIEW AND APPROVAL

4.1 EPA Review of New Method and ATP Applications

All requests for approval of proposed WET test methods will undergo review and approval by EPA on an individual basis. The ultimate approval authority for new methods and ATPs is provided in Table 2. New methods and ATPs for limited use (Tier 1) will be approved through an EPA "letter of approval". Proposed methods for nationwide use (Tier 2 and 3) will be approved through rulemaking. Ideally, proposed WET test methodologies prepared under this protocol should demonstrate one or more of the following when compared to the existing EPA-approved WET methods:

- Advances in test organism culturing, toxicity test procedures, and test concepts

- Improved data collection and analysis techniques
- Reduced test complexity
- Reduced time for test completion
- Reduced analytical costs
- Enhanced test sensitivity and/or precision
- Heightened level of protection to aquatic life in receiving water
- Improved ecological relevance of test conditions and/or test endpoints

The Agency believes that applications that meet the submission and validation requirements outlined in this protocol, demonstrate any of the above attributes, and meet general standards of performance and utility, will enhance the value of the Agency-approved methods for use in the NPDES compliance monitoring program.

EPA's Analytical Methods Staff (AMS) at EPA Headquarters will review all nationwide use new methods and ATPs and will review limited use applications if requested by a EPA Regional office or state agency. AMS may be assisted in its technical review by contractor personnel and internal EPA and external expert reviewers. When a formal proposed method application is received, AMS will first check the documentation for completeness. If the documentation is incomplete, AMS will contact the applicant and request missing documentation before proceeding with its review.

At a minimum, an application must include a completed *New Method and ATP Application Form for Whole Effluent Toxicity*, the proposed test procedure in EPA standard format, the method comparison table, and the proposed validation study plan before AMS will review the package. If these elements are present, AMS will assess the application to determine whether or not an ATP is allowed within the method-specified flexibility and whether a potential ATP or new method meets the needs of the NPDES biomonitoring program. AMS will notify the applicant if full validation should be conducted. If all elements of the application are present, including the validation study report and supporting data, AMS will begin an internal review of the new method or ATP for scientific merit, consistency, and appropriateness. The internal EPA review may involve multiple programs and workgroups.

Should any problems or questions arise during the review, EPA or its technical support contractor will communicate with the applicant to resolve outstanding issues. Depending on the circumstances, EPA may return the application to the applicant for revision. Internal review of proposed methods will involve the three steps briefly described below.

The first step of EPA's technical review will evaluate the clarity and completeness of the description of the proposed method and the method comparison table, and assess its applicability for approval at 40 CFR part 136.3, Table IA. In general, the applicability of a proposed WET test will depend on the ease with which the test can be performed on a routine basis, the consistency and availability of test organisms, and whether it meets the needs of the NPDES program. Proposed methods submitted for Tier 2 or 3 approval will be evaluated to confirm that the test species and/or test material are widely available at a fair market price in the appropriate life-stage throughout the year in sufficient quantity and quality for testing purposes and they do not require culturing conditions, test conditions, laboratory equipment, or analyst expertise that cannot be duplicated in all qualified WET labs nationwide. If the proposed method is not applicable to §136.3 and/or the method descriptions are not

acceptable, EPA will recommend rejection of the application. If this information is acceptable, the evaluation will proceed.

In the second step, the performance of the new method or ATP will be evaluated. The performance (sensitivity and precision) and utility of new methods will be compared to the suite of EPA-approved WET tests and considered for inclusion at 40 CFR part 136 on a case-by-case basis. For an ATP, the review will begin with an assessment of the performance of the test results generated by the EPA-approved method. If the data determined with the approved method are sufficient, the sensitivity, precision, compliance with physical and chemical parameters and test acceptability criteria of the results from the side-by-side tests with the ATP and EPA-approved method will be compared. In most cases, data comparisons will be conducted using appropriate statistical techniques described in *ASTM Standards on Precision and Bias for Various Applications* (ASTM, 1997). If method performance is acceptable, the review will continue.

As the third and final step, EPA will perform a detailed audit of the proposed method test data and will verify calculations of test endpoints. The endpoints reported for the tests in the precision and sensitivity validation studies and the descriptive statistics, such as the mean, standard deviation, and percent coefficient of variation of endpoints, will be substantiated.

As required following internal review, EPA may solicit additional evaluation of the proposed new method or ATP application from external expert reviewers.

4.2 Approval Recommendation

After the completeness check and technical review, the AMS Director will prepare EPA's recommendation for approval or rejection of the application. For ATPs, EPA will complete its review and notify the applicant of its approval recommendation within 90 days of receiving a complete application. For new methods, EPA will complete the review and notify the applicant within 120 days of receipt of a complete application. For limited use applications (Tier 1), AMS will notify the applicant of EPA's recommendation, and forward the recommendation to the appropriate EPA Regional or state approval authority (see Table 2) for action. The Regional or state approval authority will issue the formal approval for use of the proposed WET method. For nationwide use applications (Tiers 2 or 3), AMS will notify the applicant of EPA's recommendation, and if the proposed method is recommended for approval, will initiate the rulemaking process through which the new method or ATP is formally approved by the EPA Administrator.

4.3 Rulemaking Process

Using the method information and supporting documents provided with the new method or ATP, EPA will draft the proposed rule to approve the proposed method, prepare the proposed rule docket, pass the draft rule through internal review at EPA, and submit it to the Office of the Federal Register (OFR) for publication. *Preparation, approval, and publication of a proposed rule generally requires a minimum of four months, and may take longer depending on the nature of the method.* When published, the proposed rule requests public comment and allows a specified comment period, generally 30 to 60 days. At the end of the comment period, EPA will forward any significant comments to the method applicant for technical assistance to EPA in drafting responses to comments. All comments that have

scientific or legal merit, or raise substantive issues with the proposed rule, must be answered to complete the rulemaking process.

EPA will review the comment responses provided by the applicant and complete the response-to-comments document for the final rule. EPA will then prepare and submit the final rule to the OFR for publication. The final rule will state the date that the rule becomes effective, typically 30 days after rule publication. As of this effective date, the method is approved by EPA and will be included in the 40 CFR §136.3, Table IA in the next CFR update. *It generally requires a minimum of eight months after the proposed rule is published to receive and respond to comments, prepare and process the final rule through internal EPA review, and publish the final rule in the Federal Register.*

If circumstances merit, EPA may issue a letter of approval to authorize nationwide use of a new method or ATP during the rulemaking period.

5.0 REFERENCES

ASTM, 1994. *Standard Practice for Determination of Precision and Bias of Applicable Methods of Committee D-19 on Water*. Designation D-2777-86 (Reapproved 1994). *Annual Book of ASTM Standards*. Vol. 11.04.

ASTM, 1997. *ASTM Standards on Precision and Bias for Various Applications*. Fifth Edition. American Society of Testing and Materials. West Conshohocken, PA.

ASTM, 1998. *Standard Guide for Selection of Resident Species as Test Organisms for Aquatic and Sediment Toxicity Tests*. Designation E-1850-97. *Annual Book of ASTM Standards*,. Vol. 11.04.

Federal Water Pollution Control Act, amended by the Clean Water Act, 33 U.S.C.A. §§ 1251-1387.

USEPA, 1973. *Biological Field and Laboratory Methods for Measuring the Quality of Surface Waters and Effluents*. U.S. Environmental Protection Agency. Methods Development and Quality Assurance Laboratory, Cincinnati, OH. EPA-600-4-73-001.

USEPA, 1990. *Macroinvertebrate Field and Laboratory Methods for Evaluating the Biological Integrity of Surface Waters*. U.S. Environmental Protection Agency. Environmental Monitoring Systems Laboratory, Cincinnati, OH. EPA-600-4-90-030.

USEPA. 1991a. *Technical Support Document For Water Quality-Based Toxics Control.* Office of Water, U. S. Environmental Protection Agency, Washington, DC. EPA-505-2-90-001.

USEPA, 1991b. *Manual for the Evaluation of Laboratories Performing Aquatic Toxicity Tests*. U.S. Environmental Protection Agency, Washington, DC. EPA-600-4-90-031.

USEPA. 1993a. *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*. Fourth edition. Environmental Monitoring Systems Laboratory, U. S. Environmental Protection Agency, Cincinnati, Ohio. EPA-600-4-90-027F.

USEPA, 1993b. *Field and Laboratory Methods for Evaluating the Biological Integrity of Surface Waters*. U.S. Environmental Protection Agency. Environmental Monitoring Systems Laboratory, Cincinnati, OH. EPA-600-R-92-111.

USEPA. 1994a. *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater* Organisms. Third edition. Environmental Monitoring Systems Laboratory, U. S. Environmental Protection Agency, Cincinnati, Ohio. EPA-600-4-91-002.

USEPA. 1994b. *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms*. Second Edition. Environmental Monitoring Systems Laboratory, U. S. Environmental Protection Agency, Cincinnati, Ohio. EPA-600-4-91-003.

USEPA. 1996a. *Guide to Method Flexibility and Approval of EPA Water Methods*. U.S. Environmental Protection Agency. Office of Water, Engineering and Analysis Division. Washington, DC. EPA-821-D-96-006.

USEPA. 1996b. *Guidelines and Format for Methods to Be Proposed at 40 CFR Part 136 or Part 141.* U.S. Environmental Protection Agency. Office of Water, Engineering and Analysis Division. Washington, DC. EPA-821-B-96-003.

USGPO. 1996. *Code of Federal Regulations*. Guidelines for Establishing Test Procedures for the Analysis of Pollutants. Vol. 40. U.S. Government Printing Office, Washington, DC.

Wernimont, G.T., 1985. *Use of Statistics to Develop and Evaluate Analytical Methods*. AOAC International.

Youden, W.J. and E.H. Steiner, 1975. *Statistical Manual of the AOAC.* AOAC International.

6.0 APPENDIX A - APPLICATION FORM

EPA Office of Water
New Method and ATP Application Form
For Whole Effluent Toxicity

New ☐	ATP: ☐		*EPA Use Only* ATP Case No.
Applicant Name and Address:			
Date Application Submitted:			
New Method or Alternate Test Procedure: *(Method# & Title)*			
EPA-Approved Method for Equivalency Demonstration: *(ATPs only)*			
Level of Use: *(LU or NW)*		Validation Tier: *(1, 2 or 3)*	

FOR TIER 1 AND 2 APPLICATIONS ONLY:

ID number of existing or pending permit:	
Issuing agency:	
Type of permit:	
Discharge serial number:	

ATTACHMENTS:

- ☐ Summary and Rationale
- ☐ Method in Standard EPA Format
- ☐ Method Comparison Table
- ☐ Validation Study Plan
- ☐ Validation Study Report
- ☐ Documentation to Facilitate EPA Rulemaking (Tier 2 and 3 only)
- ☐ Other _____

Submit Application and Attachments in Triplicate

7.0 APPENDIX B - REGIONAL ATP CONTACTS

Region 1
Arthur Clark
USEPA Region 1
EQA
60 Westview Street
Lexington, MA 02173

Region 2
Linda M. Mauel
USEPA Region 2
Division of Science and Monitoring
2890 Woodbridge Avenue (MS-220)
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Region 3
Charles Jones
USEPA Region 3
Environmental Assessment and Protection Division
1650 Arch Street, 3ES-10
Philadelphia, PA 19103-2029

Region 4
Wayne Turnbull
USEPA Region 4
Science & Ecosystems Support Division
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Region 5
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USEPA Region 5
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Chicago, IL 60604

Region 6
Charles Ritchey
USEPA Region 6
1445 Ross Avenue (6MD-HX)
Dallas, TX 75202-2733

Region 7
Doug Brune
USEPA Region 7
726 Minnesota Avenue, ENSV/QA
Kansas City, KS 66101

Region 8
Rick Edmonds
Regional Quality Assurance Officer
USEPA Region 8
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Region 9
Roseanne Sakamoto
USEPA Region 9
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San Francisco, CA 94105

Region 10
Bruce Woods
USEPA Region 10
200 Sixth Avenue, OEA-095
Seattle, WA 98101

8.0 APPENDIX C - STANDARD EPA EMMC FORMAT FOR WET METHODS

1. Scope and Application
   Describe the proposed use of the method in the NPDES Program, and its limitations.

2. Summary of Method
   Describe the essential elements of the test: applicable water matrices, test species, test duration, brief description of test procedure, and test endpoints.

3. Definitions
   Include only specialized terms.

4. Interferences
   Include known or potential interferences.

5. Safety
   Refer to recognized safe laboratory practices, such as the use of hoods, goggles, and/or protective clothing, and appropriate material safety data sheets. Emphasize special procedures.

6. Equipment and Supplies
   In addition to apparatus and equipment include any applicable source documents.

7. Reagents and Standards
   Describe reagent formulations (e.g., dilution waters) and the shelf life of packaged materials.

8. Test Organism Culturing/Maintenance Procedures
   If the proposed test species is not currently described in Agency-approved methods, provide detailed descriptions of the systematics, distribution, ecology, and life history of the test species, and information on the proposed commercial source(s) of test species or detailed methods for their culture and/or laboratory maintenance.

9. Effluent and Receiving Water Collection, Preservation, Shipment, and Storage
   Follow the guidance in Table II, 40 CFR part 136, unless modifications in sampling and sample handling and holding procedures are an essential part of the new method or ATP. Provide full details on procedures used.

10. Quality Control.
    Describe the proposed formal laboratory quality control program; initial and periodic demonstration of performance using common reference toxicants, and maintenance of records, QC charts, etc. Include a summary of test conditions.

11. Calibration and Standardization

12. Toxicity Test Procedure

    Include each procedural step and test condition. Provide a summary table of test conditions similar to those in existing Agency WET methods manuals, including test acceptability criteria.

13. Acceptability of Test Results

    Include test acceptability criteria.

14. Data Analysis

    Describe the raw data to be collected, the data analysis procedures, and statistical methods employed, including tables for recording raw data and calculated test endpoints, Provide data analysis and statistical diagrams and flowcharts.

15. Method Performance

    Indicate the intralaboratory and interlaboratory test precision using appropriate reference toxicants and applicable matrix types.

16. Pollution Prevention

17. Waste Management

18. References

19. Tables, Diagrams, Flowcharts, and Validation Data

9.0 APPENDIX D - LIST OF STANDARD REFERENCE TOXICANTS

Metals:

Cadmium (Cd)
Cadmium chloride ($CdCl_2$)
Copper (Cu)
Copper sulfate ($CuSO_4$)
Hexavalent chromium (Cr^{6+})
Potassium dichromate ($K_2Cr_2O_7$)
Zinc sulfate ($ZnSO_4$)

Salts:

Potassium chloride (KCl)
Sodium chloride (NaCl)

Organics:

Phenol
Sodium dodecyl sulfate (SDS)
Sodium pentachlorophenate (NaPCP)
Sodium pentachlorophenol