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Project Summary

Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms

Second Edition

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This methods manual is a revision of EPA/600/4-85/014, and describes short-term (four- to seven-day) methods for estimating the chronic toxicity of effluents and receiving waters to the fathead minnow (*Pimephales promelas*), a cladoceran (*Ceriodaphnia dubia*), and a green alga (*Selenastrum capricornutum*). Also included are guidelines on laboratory safety, quality assurance, facilities and equipment, dilution water, effluent sampling and holding, data analysis, report preparation, and organism culturing and handling. Supplementary information on statistical techniques for test design and analysis of toxicity test data is provided in the Appendices.

A supplement to the report was published in September 1989 (EPA/600/4-89/001a), to provide an additional method (Linear Interpolation Method) for the analysis of data from the Fathead Minnow Larval Survival and Growth Test and the *Ceriodaphnia* Survival and Reproduction Test. This supplement consists of 42 pages arranged in four parts to facilitate insertion in the appropriate places in the existing report.

This Project Summary was developed by EPA's Environmental Monitoring Systems Laboratory, Cincinnati, OH, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

As a result of the increased awareness of the value of effluent toxicity test data for toxics control in the water quality program and the National Pollutant Discharge Elimination System (NPDES) permit program, which emerged from the extensive effluent toxicity monitoring activities of the regions and states, and the availability of short-term chronic toxicity test methods, the U. S. Environmental Protection Agency (USEPA) issued a national policy statement entitled, "Policy for the Development of Water Quality-Based

Permit Limitations for Toxic Pollutants," in the Federal Register, Vol. 49, No. 48, p. 9016-9019, Friday, March 9, 1984.

This policy proposed the use of toxicity data to assess and control the discharge of toxic substances to the Nation's waters through the NPDES permits program. The policy states that "biological testing of effluents is an important aspect of the water quality-based approach for controlling toxic pollutants. Effluent toxicity data, in conjunction with other data, can be used to establish control priorities, assess compliance with State water quality standards, and set permit limitations to achieve those standards." All states have water quality standards which include narrative statements prohibiting the discharge of toxic materials in toxic amounts.

Objective

The four short-term tests described in this manual are for use in the NPDES Program to estimate one or more of the following: (1) the chronic toxicity of effluents collected at the end of the discharge pipe and tested with a standard dilution water; (2) the chronic toxicity of effluents collected at the end of the discharge pipe and tested with dilution water consisting of non-toxic receiving water collected upstream from or outside the influence of the outfall, or with other uncontaminated surface water or standard dilution water having approximately the same hardness as the receiving water; (3) the toxicity of receiving water downstream from or within the influence of the outfall; and (4) the effects of multiple discharges on the quality of the receiving water. The tests may also be useful in developing site-specific water quality criteria

These methods were developed to provide the most favorable cost-benefit relationship possible, and are intended for use in effluent toxicity tests performed on-site or off-site.

The tests include:

1. A seven-day, sub-chronic, fathead minnow (*Pimephales promelas*), static renewal, larval survival and growth test.
2. A three-brood, seven-day, chronic, cladoceran (*Ceriodaphnia dubia*), static renewal, survival and reproduction test.
3. A seven-day, sub-chronic, fathead minnow (*Pimephales promelas*), static renewal, embryo-larval survival and teratogenicity test.
4. A four-day, chronic, algal, (*Selenastrum capricornutum*), static, growth test.

Short-Term Methods for Estimating Chronic Toxicity

The objective of chronic aquatic toxicity tests with effluents and pure compounds is to estimate the highest "safe" or "no-effect concentration" of these substances. For practical reasons, the parameters observed in these tests are usually limited to hatchability, gross morphological abnormalities, survival, growth, and reproduction, and the results of the tests are usually expressed in terms of the highest toxicant concentration that has no statistically significant observed effect on these parameters, when compared to the controls. The terms currently used to define the endpoints employed in the rapid, chronic and sub-chronic toxicity tests have been derived from the terms previously used for full life-cycle tests. As shorter chronic tests were developed, it became common practice to apply the same terminology to the endpoints. The primary terms in current use are as follows:

Safe Concentration

The highest concentration of toxicant that will permit normal propagation of fish and other aquatic life in receiving waters. The concept of a "safe concentration" is a biological concept, whereas the "no-observed-effect concentration" (below) is a statistically defined concentration.

No-Observed-Effect-Concentration (NOEC)

The highest concentration of toxicant to which organisms are exposed in a full life-cycle or partial life-cycle test, that causes no observable adverse effects on the test organisms (i.e., the highest concentration of toxicant in which the values for the observed parameters are not statistically significantly different from the controls). This value is used, along with other factors, to determine toxicity limits in permits.

Lowest-Observed-Effect-Concentration (LOEC)

The lowest concentration of toxicant to which organisms are exposed in a life-cycle or partial life-cycle test, which causes adverse effects on the organisms (i.e., where the values for observed parameters are statistic significantly different from the controls

Effective Concentration (EC)

A point estimate of the toxicant c centration that would cause observable adverse effect (such as de immobilization, serious incapacitat reduced fecundity, or reduced growth a given percent of the test organis calculated by point estimat techniques. For example, the EC50 f a Probit Analysis is the estimated c centration of toxicant that would ca death, or some other observable qua "all or nothing," response, in 50% of test population. If the observable ef is death (mortality), the term LC - Le Concentration, is used (see below). If observable effect is a non-qua biological measurement, the te Inhibition Concentration (IC), may used (see below). A certain EC, LC IC value might be judged fron biological standpoint to represen threshold concentration, or lowest c centration that would cause an adv effect on the observed parameters.

Lethal Concentration (LC)

Identical to EC when the observ adverse effect is death or mortality.

Inhibition Concentration (IC)

A point estimate of the toxicant c centration that would cause a gi percent reduction in a non-qua biological measurement such fecundity or growth. For example, IC25 would be the estimated concer tion of toxicant that would cause a reduction in mean young per femal some other non-quantal biolog measurement.

If the objective of chronic aqu toxicity tests with effluents and compounds is to estimate the hig "safe or no-effect concentration" of th substances, it is imperative to unders how the statistical endpoint of these t is related to the "safe" or "no-eff concentration. NOECs and LOECs determined by hypothesis testing, LCs, ECs, and ICs are determined point estimation techniques. There inherent differences between the us an NOEC, LOEC, or other estin derived from hypothesis testing estimate a "safe" concentration, and use of a LC, EC, IC, or other estimate derived from curve fit interpolation, etc.

Most point estimates, such as the LC, 'C, or IC are derived from a athematical model that assumes a ontinuous dose-response relationship. 3y definition, any LC, EC, or IC value is in estimate of some amount of adverse ffect. Thus the assessment of a safe oncentration must be made from a iological standpoint. In this instance, ne biologist must determine some mount of adverse effect that is deemed o be "safe," in the sense that it will not om a practical biological viewpoint, ffect the normal propagation of fish and ther aquatic life in receiving waters. hus, to use a point estimate such as an C, EC, IC to determine a "safe" concen- ation requires a biological judgment of hat constitutes an acceptable level of dverse effect.

The use of NOECs and LOECs, on the ther hand, assumes either (1) a ontinuous dose-response relationship, or 2) a noncontinuous threshold model of ne dose-response relationship.

In the first case, it is also assumed that dverse effects that are not "statistically bservable" are also not significant from biological standpoint, since they are not ronounced enough to test statistically ignificant against some measure of the atural variability of responses.

In the second case, it is assumed that ere exists a true threshold, or concen- ation below which there is no adverse ffect on aquatic life, and above which nere is an adverse effect. The purpose f the statistical analysis in this case is to stimate as closely as possible where nat threshold lies.

In either case, it is important to realize nat the amount of the adverse effect that statistically observable (LOEC) or not bservable (NOEC) is highly dependent n all aspects of the experimental design. hese aspects include the choice of tatistical analysis, the choice of an alpha evel, and the amount of variability etween responses at a given concentra- ion. The sensitivity of the test, which is elated to the magnitude of the adverse ffect that is statistically observable, can e controlled by the experimental design nd by controlling the amount of ariability between responses at the given concentration.

In the first case, where the assumption f a continuous dose-response relation- hip is made, clearly the NOEC estimate s an estimate of some amount of adverse effect that is dependent on the experimental design. In the second case, the NOEC may be an estimate of a fe" or "no-effect" concentration but y if the amount of adverse effect that appears at the threshold is great enough to test as statistically significantly different from the controls in the face of all aspects of the experimental design mentioned above. The NOEC in that case would indeed be an estimate of a "safe" or "no-effect" concentration. If, however, the amount of adverse effect were not great enough to test as statistically different, then the NOEC might well be an estimate that again represents some amount of adverse effect which is assumed safe because it did not test as statistically significant. In any case, the estimate of the NOEC with hypothesis testing is always dependent on the aspects of the experimental design mentioned above. For this reason, the reporting and examination of some measure of the sensitivity of the test (either the minimum significant difference or the percent change from the control that this minimum difference represents) is extremely important.

In summary, the assessment of a "safe" or "no-effect" concentration cannot be made from the results of statistical analysis alone, unless (1) the assumptions of a strict threshold model are accepted, and (2) it is assumed that the amount of adverse effect present at the threshold is statistically detectable by hypothesis testing. In this case, estimates obtained from a statistical analysis are indeed estimates of a "no-effect" concentration. If the assumptions are not deemed tenable, then estimates from a statistical analysis can only be used in conjunction with an assessment from a biological standpoint of what magnitude of adverse effect constitutes a "safe" concentration. In this instance, a "safe" concentration is not necessarily a "no-effect" concentration, but rather a concentration at which the effects are judged to be of no biological significance.

Health and Safety

Collection and use of effluents in toxicity tests may involve significant risks to personal safety and health. Personnel collecting effluent samples and conducting toxicity tests should take all safety precautions necessary for the prevention of bodily injury and illness which might result from ingestion or invasion of infectious agents, inhalation or absorption of corrosive or toxic substances through skin contact, and asphyxiation due to lack of oxygen or presence of noxious gases.

Prior to sample collection and laboratory work, personnel will determine that all necessary safety equipment and materials have been obtained and are in good condition.

Quality Assurance

Quality Assurance (QA) practices for effluent toxicity tests consist of all aspects of the test that affect data quality, such as: (1) effluent sampling and handling; (2) the source and condition of the test organisms; (3) condition of equipment; (4) test conditions; (5) instrument calibration; (6) replication; (7) use of reference toxicants; (8) record keeping; and (9) data evaluation.

Dilution Water

The source of dilution water used in effluent toxicity tests will depend largely on the objectives of the study:

1. If the objective of the test is to estimate the inherent chronic toxicity of the effluent, which is the primary objective of NPDES permit-related toxicity testing, a standard dilution water (moderately hard water) is used.
2. If the objective of the test is to estimate the chronic toxicity of the effluent in uncontaminated receiving water, the test may be conducted using dilution water consisting of a single grab sample of receiving water (if non-toxic), collected upstream and outside the influence of the the outfall, or with other uncontaminated surface water or standard dilution water having approximately the same characteristics (pH, hardness, alkalinity, conductivity, and total suspended solids) as the receiving water. Seasonal variations in the quality of surface waters may affect effluent toxicity. Therefore, the pH, alkalinity, hardness, and conductivity of receiving water samples should be determined before each use.
3. If the objective of the test is to determine the additive effects of the discharge on already contaminated receiving water, the test is performed using dilution water consisting of receiving water collected upstream from the outfall.

Effluent and Receiving Water Sampling and Handling

The effluent sampling point usually should be the same as that specified in

the NPDES discharge permit. Conditions for exception would be: (1) better access to a sampling point between the final treatment and the discharge outfall; (2) if the processed waste is chlorinated prior to discharge to the receiving waters, it may also be desirable to take samples prior to contact with the chlorine to determine toxicity of the unchlorinated effluent; or (3) in the event there is a desire to evaluate the toxicity of the influent to municipal waste treatment plants or separate wastewater streams in industrial facilities prior to their being combined with other wastewater streams or non-contact cooling water, additional sampling points may be chosen.

The decision on whether to collect grab or composite samples is based on the objectives of the test and an understanding of the short and long-term operations and schedules of the discharger. If the effluent quality varies considerably with time, which can occur where holding times are short, grab samples may seem preferable because of the ease of collection and the potential of observing peaks (spikes) in toxicity. However, the sampling duration of a grab sample is so short that full characterization of an effluent over a 24-h period would require a prohibitive number of separate samples and tests. Collection of a 24-h composite sample, however, may dilute toxicity spikes, and average the quality of the effluent over the sampling period. Sampling recommendations are provided for grab and composite samples.

Sample Handling and Preservation and Shipping

If the data from the samples are to be acceptable for use in the NPDES Program, the lapsed time from collection of a grab or composite sample and its first use for initiation of a test, or for test solution renewal, should not exceed 36 h. Composite samples should be chilled during collection, where possible, and maintained at 4°C until used. Samples collected for on-site tests should be used within 24 h. Samples collected for off-site toxicity testing are to be chilled to 4°C when collected, shipped iced to the central laboratory, and there transferred to a refrigerator (4°C) until used. Every effort must be made to initiate the test with an effluent sample on the day of arrival in the laboratory.

Samples may be shipped in 4-L (1-gal) CUBITAINERS® or new plastic "milk" jugs. All sample containers should be rinsed with source water before being filled with sample. After use, CUBITAINERS® and plastic jugs are punctured to prevent reuse. Several sample shipping options are available, including Express Mail, air express, bus, and courier service. Express Mail is delivered seven days a week. Shipping and receiving schedules of private carriers on weekends vary with the carrier.

Sample Preparation

With the *Ceriodaphnia* and fathead minnow tests, effluents and surface waters must be filtered through a 60-µm plankton net to remove indigenous organisms that may attack or be confused with the test organisms (see *Ceriodaphnia* test method for details). Surface waters used in algal toxicity tests must be filtered through a 0.45-µm pore diameter filter before use. It may be necessary to first coarse-filter the dilution and/or waste water through a nylon sieve having 2- to 4-mm holes to remove debris and/or break up large floating or suspended solids. Caution: filtration may remove toxicity.

The DO concentration in the dilution water should be near saturation prior to use. Aeration will bring the DO and other gases into equilibrium with air, minimize oxygen demand, and stabilize the pH.

If the dilution water and effluent must be warmed to bring them to the prescribed test temperature, supersaturation of the dissolved gases may become a problem. To prevent this problem, the effluent and dilution water are checked for dissolved oxygen (DO) with a probe after heating to 25°C. If the DO is greater than 100% saturation or lower than 40% saturation, the solutions are aerated moderately with a pipet tip for a few minutes until the DO is within the prescribed range.

Data Analysis

The choice of a statistical method to analyze toxicity test data and the interpretation of the results of the analysis of the data from any of the toxicity tests described in this manual can become problematic because of the inherent variability and sometimes unavoidable anomalies in biological data. Analysts who are not proficient in statistics are strongly advised to seek the assistance of a statistician before selecting the method of analysis and using any of the results.

The recommended statistical methods presented in the manual are not the only possible methods of statistical analysis. Many other methods have be proposed and considered. Amo alternative hypothesis tests some, l Williams' Test, require additional a sumptions, while others, like the bo strap methods, require computer- tensive computations. Alternative pc estimation approaches most probal would require the services of statistician to determine the a propriateness of the model (goodness fit), higher order linear or nonlin models, confidence intervals estimates generated by inver regression, etc. In addition, po estimation or regression approacl would require the specification biologists or toxicologists of some l level of adverse effect that would deemed acceptable or safe. Certai there are other reasonable and defensil methods of statistical analysis of this k of toxicity data. The methods contair in this manual have been chosen, amc other reasons, because they are (1) w tested and well-documented, applicable to most different toxicity t data sets for which they a recommended, but still powerful, hopefully "easily" understood by n statisticians, and (4) amenable to u without a computer, if necessary.

The data should be plotted, both a preliminary step to help detect proble and unsuspected trends or patterns in responses, and as an aid in interpretat of the results. Further discussion a plotted sets of data are included in methods and the Append Transformations of the data, e.g., arc s square root and logs, are used wh necessary to meet assumptions of proposed analyses, such as t requirement for normally distributed da

Statistical independence amo observations is a critical assumption the statistical analysis of toxicity da One of the best ways to ensu independence is to properly foll rigorous randomization procedur Randomization techniques should employed at the start of the te including the randomization of the pla ment of test organisms in the test cha bers and randomization of the test cha ber location within the array of chambe A discussion of statistical independen outliers and randomization, and a sam randomization scheme, are included Appendix A.

The number of replicates employed each toxicant concentration is important factor in determining sensitivity of chronic toxicity tests. T sensitivity generally increases as

number of replicates is increased, but the point of diminishing returns in sensitivity may be reached rather quickly. The level of sensitivity required by a hypothesis test or the confidence interval for a point estimate will determine the number of replicates, and should be based on the objectives for obtaining the toxicity data.

In a statistical analysis of toxicity data, the choice of a particular analysis and the ability to detect departures from the assumptions of the analysis, such as the normal distribution of the data and homogeneity of variance, is also dependent on the number of replicates. More than the minimum number of replicates may be required in situations where it is imperative to obtain optimal statistical results, such as with tests used in enforcement cases or when it is not possible to repeat the tests. For example, when the data are analyzed by hypothesis testing, the nonparametric alternatives cannot be used unless there are at least four replicates at each toxicant concentration. If there are only two replicates, Dunnett's Procedure may be used, but it is not possible to check the assumptions of the test.

The recommended statistical analysis of most data from chronic toxicity tests with aquatic organisms follows a decision process illustrated in a flow chart. An initial decision is made to use point estimation techniques and/or to use hypothesis testing. If hypothesis testing is chosen, subsequent decisions are made on the appropriate hypothesis testing procedure for a given set of data, as illustrated in the flow chart. If point estimation is chosen, the equivalent of an NOEC can be calculated. A specific flow chart is included in the analysis section for each test.

Since a single chronic toxicity test might yield information on more than one parameter (such as survival, growth, and reproduction), the lowest estimate of a "no-observed-effect concentration" for any of the parameters would be used as the "no-observed-effect concentration" for each test. It follows logically that in the statistical analysis of the data, concentrations that had a significant toxic effect on one of the observed parameters would not be subsequently tested for an effect on some other parameter. This is one reason for excluding concentrations that have shown a statistically significant reduction in survival from a subsequent statistical analysis for effects on another parameter such as reproduction. A second reason is that the exclusion of such concentrations usually results in a more powerful and appropriate statistical analysis.

Analysis of Growth and Reproduction Data

Growth data from the fathead minnow larval survival and growth test are analyzed using hypothesis testing or point estimation techniques. (Note that the nonparametric hypothesis tests can be used only if at least four replicates were used at each toxicant concentration.)

Reproduction data from the *Ceriodaphnia* survival and reproduction test, after eliminating data from concentrations with a significant mortality effect as determined by Fisher's Exact Test, are analyzed using hypothesis testing or point estimation techniques. (Note that the nonparametric hypothesis tests can be used only if at least four replicates were used at each toxicant concentration).

Analysis of Algal Growth Response Data

The growth response data from the algal toxicity test, after an appropriate transformation if necessary to meet the assumptions of normality and homogeneity of variance, may be analyzed by hypothesis testing. Point estimates, such as the EC1, EC5, EC10, or EC50, would also be appropriate in analyzing algal growth data.

Analysis of Mortality Data

Mortality data from the fathead minnow larval survival and growth test and the fathead minnow embryo-larval survival and teratogenicity test are analyzed by Probit Analysis, if appropriate. The mortality data can also be analyzed by hypothesis testing, after an arc sine transformation.

Mortality data from the *Ceriodaphnia* survival and reproduction test are analyzed by Fisher's Exact Test prior to the analysis of the reproduction data. The mortality data may also be analyzed by Probit Analysis, if appropriate.

Dunnett's Procedure

Dunnett's Procedure consists of an analysis of variance (ANOVA) to determine the error term, which is then used in a multiple comparison method for comparing each of the treatment means with the control mean, in a series of paired tests. Use of Dunnett's Procedure requires at least two replicates per treatment and an equal number of data points (replicates) for each concentration. However, as stated above, it is not possible to check the assumptions of the test. In cases where the number of data points for each concentration are not equal, a t test may be performed with Bonferroni's adjustment for multiple comparisons, instead of using Dunnett's Procedure.

The assumptions upon which the use of Dunnett's Procedure is contingent are that the observations within treatments are independent and normally distributed, with homogeneity of variance. Before analyzing the data, the assumptions must be verified using the procedures provided in Appendix B.

Some indication of the sensitivity of the analysis should be provided by calculating: (1) the minimum difference between means that can be detected as statistically significant, and (2) the percent change from the control mean that this minimum difference represents for a given test.

The estimate of the safe concentration derived from this test is reported in terms of the NOEC. A step-by-step example of Dunnett's Procedure is provided in the Appendix.

If, after suitable transformations have been carried out, the normality assumptions have not been met, Steel's Many-One Rank Test should be used if there are four or more data points per toxicant concentration. If the numbers of data points (replicates) for each toxicant concentration are not equal, the Wilcoxon Rank Sum Test with Bonferroni's adjustment should be used.

Bonferroni's T-Test

Bonferroni's T-test is used as an alternative to Dunnett's Procedure when the number of replicates is not the same for all concentrations. This test sets an upper bound of alpha on the overall error rate, in contrast to Dunnett's Procedure, for which the overall error rate is fixed at alpha. Thus Dunnett's Procedure is a more powerful test.

Steel's Many-One Rank Test

Steel's Many-One Rank Test is a multiple comparison method for comparing several treatments with a control. This method is similar to Dunnett's Procedure, except that it is not necessary to meet the assumption for normality. The data are ranked, and the analysis is performed on the ranks rather than on the data themselves. If the data are normally or nearly normally distributed, Dunnett's Procedure would

be more sensitive (would detect smaller differences between the treatments and control). For data that are not normally distributed, Steel's Many-One Rank Test can be much more efficient. It is necessary to have at least four replicates per toxicant concentration to use Steel's test. The sensitivity of this test cannot be stated in terms of the minimum difference between treatment means and the control mean.

The estimate of the safe concentration is reported as the NOEC. A step-by-step example of Steel's Many-One Rank Test is provided in the Appendix.

Wilcoxon Rank Sum Test

The Wilcoxon Rank Sum Test is a nonparametric test for comparing a treatment with a control. The data are ranked and the analysis proceeds exactly as in Steel's Test except that Bonferroni's adjustment for multiple comparisons is used instead of Steel's tables. When Steel's test can be used (i. e., when there are equal numbers of data points per toxicant concentration), it will be more powerful (able to detect smaller differences as statistically significant) than the Wilcoxon Rank Sum Test with Bonferroni's adjustment.

The estimate of the safe concentration is reported as the NOEC. A step-by-step example of the use of the Wilcoxon Rank Sum Test is provided in the Appendix.

Interpolation Approach

Chronic toxicity test data can be analyzed by an interpolation approach. Precision estimates can be calculated using this approach. The round robin data show that the endpoints estimated by this approach are much less variable than those estimated by hypothesis testing.

Probit Analysis

Probit Analysis is used to analyze percentage data from concentration-response tests. The analysis can provide an estimate of the concentration of toxicant affecting a given percent of the test organisms and provide a confidence interval for the estimate. Probit Analysis assumes a normal distribution of log tolerances and independence of the individual responses. To use Probit Analysis, at least two partial mortalities must be obtained. If a test results in 100% survival and 100% mortality in adjacent treatments (all or nothing effect), an LC50 may be estimated using the graphical method, and the LC50 and confidence interval may be estimated by the moving average angle, Spearman-Karber, or other methods.

It is important to check the results of Probit Analysis to determine if the analysis is appropriate. The chi-square test for heterogeneity provides one good test of appropriateness of the analysis. In cases where there is a significant chi-square statistic, where there appears to be systematic deviation from the model, or where there are few data in the neighborhood of the point to be estimated, Probit results should be used with extreme caution.

The natural rate of occurrence of a measured response, such as mortality in the test organisms (referred to as the natural spontaneous response), may be used to adjust the results of the Probit Analysis if such a rate is judged to be different from zero. If a reliable, consistent estimate of the natural spontaneous response can be determined from historical data, the historical occurrence rate may be used to make the adjustment. In cases where historical data are lacking, the spontaneous occurrence rate should optimally be estimated from all the data as part of the maximum likelihood procedure. However, this can require sophisticated computer software. An acceptable alternative is to estimate the natural occurrence rate from the occurrence rate in the controls. In this instance, greater than normal replication in the controls would be beneficial.

A discussion of Probit Analysis and the natural occurrence rate, along with a computer program for performing the Probit Analysis, are included in Appendix I.

Summary of Test Methods

1. Fathead minnows, *Pimephales promelas*, larvae are exposed in a static renewal system for seven days to different concentrations of effluent or to receiving water. Test results are based on the survival and growth (increase in weight) of the larvae, compared to the controls.
2. Fathead minnows, *Pimephales promelas*, embryos and larvae are exposed to different concentrations of effluent or to receiving water in a static renewal system for seven days, starting shortly after fertilization of the eggs. Test results are based on the total frequency of both mortality and gross morphological deformities (terata), compared to the controls.
3. Cladocera, *Ceriodaphnia dubia* are exposed in a static renewal system to different concentrations of effluent, or to receiving water, until 60% of surviving control organisms have three broods of offspring. Test results are based on survival and reproduction. If the test is conducted as described, the control organisms should produce three broods of young during a seven-day period compared to the controls.
4. The fresh water alga *Selenastrum capricornutum*, is exposed in a static system to a series of concentrations of effluent, or to receiving water, for 96 h. The response of the population is measured in terms of changes in cell density (cell counts per mL), biomass, chlorophyll content, or absorbance, compared to the controls.

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The complete report, entitled "Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms—Second Edition," (Order No. PB 89-207 013/AS; Cost: $31.00, subject to change); and a supplement to the report (Order No. PB 90-145 764/AS; Cost $15.00) will be available only from:

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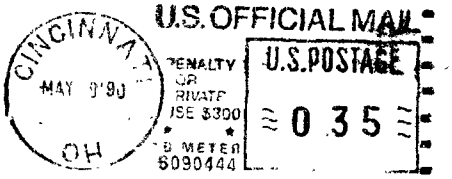

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