Germination Growth Test: Oxic Conditions

Standard Operating Procedure for Test Methods using Wild Rice, *Zizania palustris*

1.1 Scope and Application

1.1.1 This method describes procedures to perform a toxicity test using wild rice in exposures of solutions containing elevated sulfate or cation concentrations under aerobic conditions.

1.1.2 This method consists of a test using a dilution series of at least four concentrations of a test chemical and a control.

1.2 Summary of Method

1.2.1 Seeds of the aquatic macrophyte *Zizania palustris* are conditioned for germination and exposed in a static- renewal system to a dilution series of concentrations of sulfate or cations. The exposure duration is 10 days. The response of the germinating seeds is measured in terms of changes to germination rate and growth in control plants vs. treatment.

1.3 Quality Control Considerations

1.3.1 Toxic substances may be introduced by contaminants in dilution water, sampling hardware, or testing equipment.

1.3.2 Adverse effects of pH changes and cationic constituents in test media may augment or mask adverse effects of toxic substances.

1.3.3 Improper sampling of test solutions may adversely affect test results (see section 1.5 on Standards and Reagents and section 1.6 on Toxicity Test Procedures)

1.3.4 Additional details are found in the document titled: "Hydroponic Experiment on Response of Wild Rice to Sulfate - Quality Assurance Project Plan"

1.4 Necessary Apparatus and Materials

1.4.1 Seeds of *Zizania palustris* are prepared in the laboratory for germination (see section 1.7 on wild rice seed preparation). To initiate exposures, sufficient numbers of conditioned seed must be available. Each exposure jar contains 50 conditioned seeds.

1.4.2 Environmental Growth Chamber: Temperature control range of 15° C to 30° C ± 1°C). Germination growth tests are performed in the dark.

1.4.3 Test chambers: One pint (470 mL) glass jars with lids.

1.4.4 Meter: pH for routine physical measurements.

1.4.5 Volumetric flasks and graduated cylinders: class A, 10 – 2000 mL borosilicate glass for preparation of test solutions.

1.4.6 Volumetric pipets

1.4.7 Pipet bulbs and fillers

1.4.8 Balance: analytical, capable of accurately weighing 0.1 mg.

1.4.9 Magnetic stirrer and stir bars: for mixing test and growth media solutions.

1.4.10 Filtering apparatus: for membrane and /or glass fiber filters.

1.4.11 Tape: for labeling test chambers and containers for solutions.

1.4.12 Water purification system: deionized water or equivalent.

1.5 Standards and Reagents

1.5.1 Reagent-grade chemicals are used to prepare hydroponic growth media.

1.5.2 25 liters of a modified 1/5 strength Hoagland's stock solution (Table 1) is prepared using a ½ strength stock solution daily or more often as needed from 1.0 M stock solutions.

1.5.3 Stock SO_4 solution (3.200 g/L) is prepared daily as needed by adding 4.73 g anhydrous Na_2SO_4 (Fisher S421) or 8.22 g $MgSO_4*7H_2O$ (Fisher M63) to 800 mL deionized water and filling to 1 liter. Mixtures of Na_2SO_4 and $MgSO_4$ are determined by solving 2 equations with known Mg:Na ratios and known SO_4 final concentration.

1.5.4 Reagent water: defined as deionized water that does not contain substances that are toxic to the test organisms.

1.5.5 Appropriate amounts of each test solution (one pint (approximately 400 mL) times number of replicates plus extra for analysis sample, i.e. 1400 mL for 3 replicates and ~200 mL sample) are made up immediately before use. Pre-determined amounts of 1/5 strength Hoagland's, PIPES buffer (Piperazine-N,N'-bis(2-ethanesulfonic acid) sesquisodium salt, Fisher Scientific/Acros Organics # AC32778-5000) , N, and P stock solution, and SO_4 stock solution are mixed and made to volume. The pH is adjusted to 6.8 +/- 0.2 with 1 M HCl.

Table 1. Composition of 1/5 Hoagland's Solution

Compound	Molar concentration in 1/5th strength growth solution
MgCl	0.4 mM
$CaCl_2 \cdot 2\ H_2O$	2 .0 mM
KCl	1.0 mM
NH_4Cl	0.08 mM
NaNO3	0.08 mM
KH_2PO_4	0.026 mM
H_3BO_3	22.5 µM
$MnCl \cdot 4\ H_2O$	4.5 µM
$ZnSO_4 \cdot 7\ H_2O$	0.5 µM
$CuSO_4 \cdot 5\ H_2O$	0.15 µM
MoO_3	0.07µM
Fe-EDTA	45.0 µM
$Na_2SiO_3*9H_2O$	1.5 mM
PIPES buffer	5.0 mM

1.6 Toxicity Test Procedures: Toxicant Exposures

1.6.1 Each toxicity test will consist of at least four test concentrations of the toxicant (e.g., sodium sulfate) and a control (hydroponics medium).

1.6.2 Each test concentration and control exposure solution is replicated using three - one pint glass jars.

1.6.3 Conditioned wild rice seed as described in section 1.7, Wild Rice Seed Preparation, are used to initiate the toxicity test.

1.6.4 Each jar is labeled with tape using a unique descriptor for the particular concentration of test solution and replicate for that jar. Each jar also is numbered from 1 to 18 and a table of these integers (1 – 18) randomized is prepared.

1.6.5 Each labeled jar is filled to close to the top with the particular solution as identified on its label.

1.6.6 Conditioned seeds (50) are removed from the pool of initial seeds (section 1.8, Test Organisms) using a light forceps and put into the jar corresponding to the first integer read from the random integer table. This is done for all jars prepared for testing.

1.6.7. The jars (solution and seed) are placed onto a tray.

1.6.8 Screw caps are placed loosely on the jars.

1.6.9 The tray of jars is placed in the growth chamber and covered with aluminum foil to exclude light.

1.6.10 Test solutions in the jars are renewed every two (2) days.

1.6.11 Solution renewals are accomplished by gently decanting or siphoning off the old solution leaving approximately one vertical cm of solution in the jar bottom.

1.6.12 New solutions are added by gently pouring into the jar until it reaches the top of the jar. The screw cap is then replaced on the jar.

1.6.13 Old solutions are retained for chemistry as described in the section 1.12, Analytical Chemistry.

1.6.14 Duration of the exposure is 10 days.

1.7 Wild Rice Seed Preparation

Wild rice seed must undergo a conditioning phase following its harvest from the field. In the wild, wild rice drops into the water after the seed has ripened, and sinks to the sediment. This seed, if left undisturbed, stays on or just below the surface of the sediment over the winter. This cold phase serves to condition the seed to enable it to germinate once water temperatures increase in the spring.

1.7.1 The following is a procedure that describes the method and handling of wild rice seed from initial harvest to its use in the germination growth toxicity tests.

1.7.2 Freshly harvested seed should be kept cool and moist and be placed into storage as soon as possible after field collection.

1.7.3 Harvested seed prepared for storage can be kept a) in air tight bags in a cooler set at just above freezing (4° C), or b) submerged in water just above freezing in the dark. Seed stored in either manner can have satisfactory germination rates for one to two years.

1.7.4 To begin the seed conditioning for germination, an aliquot of seed (approximately 2000 seeds) is removed from this ‘dry’ cold storage (as described in option (a) in 1.7.3) and placed into a container with water kept submerged at near freezing temperatures for at least one month. Following this time period, seed is ready (or conditioned) for germination for at least several months. For purposes of use in laboratory testing, seed set in this conditioning phase is kept for up to two months before a fresh aliquot of seed is brought into the conditioning phase. Use of storage option (b) keeps the seed in this wet, cold conditioned phase until needed for testing.

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1.3.4 Additional details are found in the document titled: "Hydroponic Experiment on Response of Wild Rice to Sulfate - Quality Assurance Project Plan"

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1.4.4 Meter: pH for routine physical measurements.

1.11 Acceptability of Test Results

1.11.1 At least 90% of germinated seeds in control jars are living at test termination.

1.11.2 Mesocotyl length of germinated seeds from control exposures will be at least 2.0 cm at the end of the 10 d duration of growth.

1.1.3 Control germinated seeds should not indicate any visible phytotoxic or developmental symptoms at any time during the test.

1.12 Analytical Chemistry

1.12.1 Sampling and analysis of chemical solutions used for initiating and renewing test exposures will use the following procedures.

1.12.2 New test solutions –Immediately after adding the new test solution into the jars an aliquot (approximately 250 ml) of the remaining unused portion is poured directly into a pre-labeled sample bottle.

1.12.3 Old test solutions – When exchanging solution or before decanting the final solution the jar is swirled to mix the solution and is poured directly into a pre-labeled sample bottle.

1.12.4 Sulfate concentration is measured following the method titled, "Determination of Sulfate by Flow Injection Analysis" found in Appendix C of the document titled: "Hydroponic Experiment on Response of Wild Rice to Sulfate - Quality Assurance Project Plan"

References

U.S. EPA. 2012. Ecological Effects Test Guidelines. OCSPP 850.4230: Early Seedling Growth Toxicity Test. EPA 712-C-010.

U.S. EPA. 2012. Ecological Effects Test Guidelines. OCSPP 850.4100: Seedling Emergence and Seedling Growth. EPA 712-C-012.

U.S. EPA 2012. Ecological Effects Test Guidelines. OCSPP 850.4400: Aquatic Plant Toxicity Test Using *Lemna* spp. EPA 712-C-008.

U.S. EPA. 2002. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th ed. EPA-821-R-02-013.

Image 1. Examples of conditioned seeds used for initiating germination growth tests.

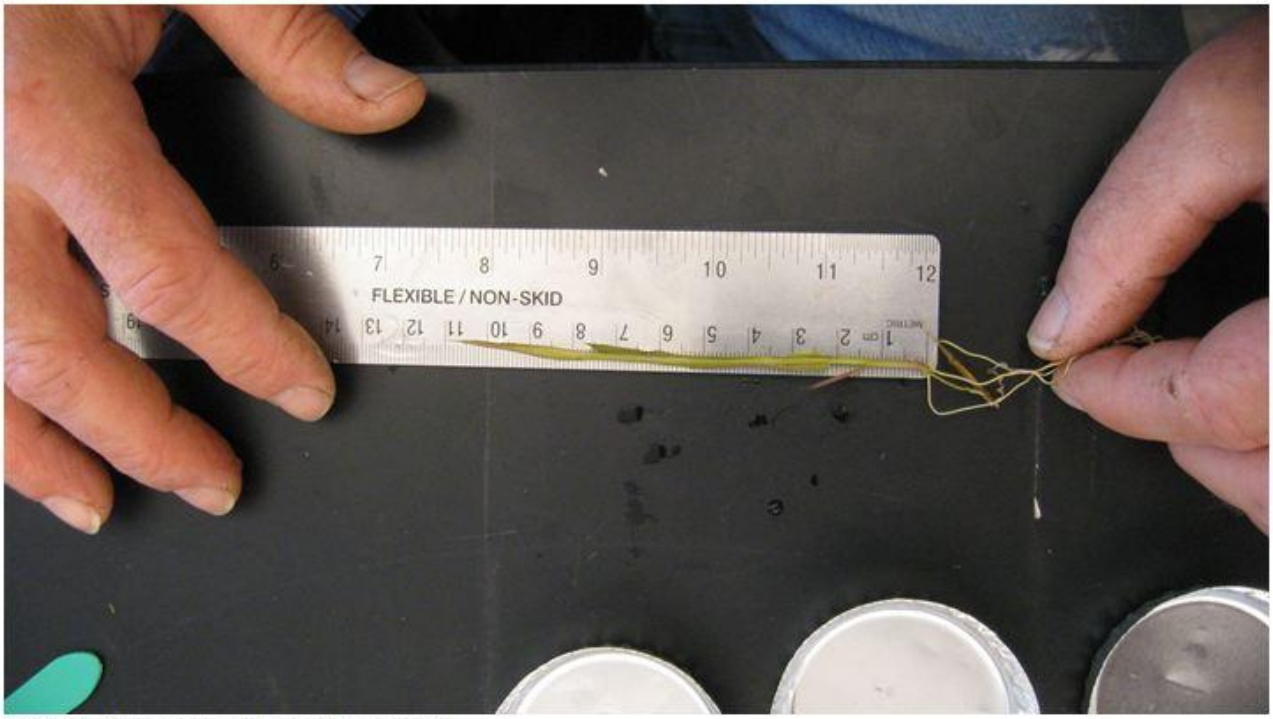

Image 2. Measurement of seedling length.