OECD 201 Freshwater Alga Growth Inhibition Test

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Tradename: PhytoCide Lichen

Code: M16008

CAS #: 513-85-9 & 92346-91-3

Test Request Form #: 9620

Lot #: N220506K

Sponsor: *Active Micro Technologies, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

Test Performed:
OECD 201
Freshwater Alga Growth Inhibition Test

Introduction

The purpose of the present study is to determine the toxicity of **PhytoCide Lichen** by exposing the exponentially growing test organism *Pseudokirchneriella subcapitata* to the test substance for 72 hours and measuring the growth and growth inhibition through cell counting against the control. The response is evaluated as a function of the exposure concentration in comparison with the average growth of replicate, unexposed control cultures.

OECD Guideline 201 on "Fresh Alga and Cyanobacteria, Growth Inhibition Test", adopted in 1984, extended the guideline to include additional species and update it to meet the requirements for hazard assessment and classification of chemicals in 2006.

Assay Principle

Pseudokirchneriella subcapitata, are exposed to the test substance at a range of concentrations for a period of 72 hours. The cultures are allowed unrestricted exponential growth under nutrient sufficient conditions and continuous light for a sufficient period of time to measure reduction of the specific growth rate. Growth and growth inhibition are quantified from measurements of the algal biomass as a function of time. The test endpoint is inhibition of growth, expressed as the logarithmic increase in biomass during the 72 hour exposure period. The results are analyzed in order to calculate the EC_{10} and EC_{20} at 72 hours. The response is evaluated as a function of the exposure concentration in comparison with the average growth of replicate, unexposed control cultures.

A reliable analytical method for the quantification of the substance in the test solutions with reported recovery efficiency and limit of determination should be available. A reference substance may be tested for EC_{50} as a means of assuring that the test conditions are reliable.

Analysis of the concentration of the test substance at the start and end of the test of a low and high test concentration around the expected EC_{50} may be sufficient where it is likely that exposures concentrations will vary less than 20% from the nominal values during the test.

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Materials

- Glass Flasks with air-permeable stopper
- Automated Pipette
- pH Meter
- Temperature Control Apparatus
- Microscope with counting chamber
- *Pseudokirchneriella subcapitata* (ATCC 22662)
- Gorham's medium for algae (ATCC MD-0625)

Methods

Test Conditions

- Incoculum Culture
  - Inoculum culture is incubated under the same conditions as the test cultures for 2-4 days allowing for exponential growth to prevail before the start of the test. This is done to ensure that growth is within the normal range for the test strain under the culturing conditions.
- Initial Biomass
  - The initial biomass in the test cultures must be the same in all test cultures and sufficiently low to allow exponential growth throughout the incubation period without risk of nutrient depletion. The initial biomass should not exceed 0.5 mg/L as dry weight.
- Exposure Period
  - 72 hours
- Number of Test Organisms
  - *Pseudokirchneriella subcapitata* 5 x $10^{3\text{-}4}$ cells/ml
- Test Concentration
  - Adopt a concentration range of at least 5 concentrations, causing a range of 5-75% inhibition of algal growth rate expressed as E_rC_x
- Culture Method
  - Illumination: Continuous uniform fluorescent illumination
  - Temperature: The temperature is between 21°C to 24°C
  - pH: pH of the control medium should not increase be more than 1.5 units during test

Measurement of Test Substance Concentrations

- Measurement of biomass is done by manual cell counting by microscope.
- Algal biomass in each flask is determined daily during test period.
- At the beginning and end of exposure, measure test substance concentrations at the lowest and highest test concentration groups.
  - For volatile or adsorptive substances, additional measurements are recommended at 24 hours intervals during exposure period.

Observation

- Microscopic observation should be performed to verify a normal and healthy appearance of the inoculum culture and to observe any abnormal appearance of the algae at the end of the test.

Test Condition Measurements

- Measure pH in the control and at the highest test concentration at the beginning and end of the exposure period.
- Water temperature should be measured at the beginning and end of the exposure period.

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Data and Reporting

I. Data
   a. Tabulate the estimated biomass concentration in test cultures and controls together with the concentrations of test materials and the times of measurement, recorded with a resolution of at least whole hours, to produce plots of growth curves.
   b. For each response variable to be analyzed, use the concentration-response relationship to calculate point estimates of EC_x values. Recent scientific developments have led to a recommendation of abandoning the concept of NOEC and replacing it with regression based point estimates EC_x, specifically EC_{10} and EC_{20}.

II. Test Report
   a. The test report must include the following:
      i. Test substance:
         1. Physical nature and relevant physical-chemical properties
         2. Chemical identification data, including purity
      ii. Test species:
         1. Source and species of *Pseudokirchneriella subcapitata*, supplier of source (if known), and the culture conditions (including source, kind and amount of food, feeding frequency)
      iii. Test conditions:
         1. Description of test vessels: type and volume of vessels, volume of solution, density of *Pseudokirchneriella subcapitata* per test vessel, number of test vessels (replicates) per concentration
         2. Methods of preparation of stock and test solutions including the use of any solvent or dispersants, concentrations used
         3. Details of dilution water: source and water quality characteristics (pH, hardness, Ca/Mg ratio, Na/K ratio, alkalinity, conductivity, etc); composition of reconstituted water if used
         4. Incubation conditions: temperature, light intensity and periodicity, pH, etc.
      iv. Results:
         1. The nominal test concentrations and the result of all analyses to determine the concentration of the test substance in the test vessels; the recovery efficiency of the method and the limit of determination should also be reported
         2. All physical-chemical measurements of temperature and pH made during the test
         3. The EC_{10} and EC_{20} at 72 hours for percent inhibition with confidence intervals and graphs of the fitted model used for calculation, the slopes of the dose-response curves and their standard error; statistical procedures used for determination of EC_{10} and EC_{20}.

$$Percent\ (\%)Inhibition = \frac{\mu c - \mu T}{\mu c} \times 100$$

μ_c: mean value for average specific growth rate (μ) in the control group
μ_T: average specific growth rate for the treatment replicate

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Results

General Information:

Name of new chemical substance	PhytoCide Lichen		
INCI Nomenclature	2,3-Butanediol & Cladonia Rangiferina Extract		
CAS number	513-85-9 & 92346-91-3		
Formulation Method	Milling & Extraction		
Molecular weight	375 Da		
Purity of the new chemical substance used for the test (%)	100%		
Lot number of the new chemical substance used for the test	N220506K		
Names and contents of impurities	n/a		
Solubility in water	Soluble		
Properties at room temperature	Clear to Slightly Hazy Light Yellow to Amber Liquid, Characteristic Odor		
Stability	Stable Under Normal Conditions		
Solubility in solvents, etc.	**Solvent**	**Solubility**	**Stability in solvent**
	N/A	N/A	N/A

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ACTIVE MICRO TECHNOLOGIES

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Test Materials and Methods:

Items			**Contents**
Test Organisms	Species		*Pseudokirchneriella subcapitata*
	Source		ATCC
	Reference substance (EC_{50})		3,5-dichlorophenol
Culture	Kind of Medium		Gorham's Medium for Algae
	Conditions (Temperature)		22°C ± 2°C
Test Conditions	Test Vessel		Glass
	Material Water	Kind	Deionized
		Hardness	250 mg/L
		pH	7.4
	Date of Exposure		05/16/2022
	Test Concentrations		200, 89.4, 42.3, 19.2, 7.8 mg/L
	Number of organisms		5 x 103-4 cells/ml
	Number of Replicates	Exposure Group	4
		Control Group	4
	Test Solution Volume		5 mL
	Vehicle	Use or Not	N/A
		Kind	N/A
		Concentration	N/A
		Number of Replicates	N/A
	Photoperiod		Continuous

Test Results:

Items		**Contents**
Toxicity Value	Percent Inhibition EC_{10} and EC_{20}	106.99 mg/L and 197.50 mg/L
Exposure Concentrations Used for Calculation	Nominal Values	200, 89.4, 42.3, 19.2, 7.8 mg/L
Remarks		Not harmful to aquatic organisms

Discussion

After 72 hours, the percent inhibition for **PhytoCide Lichen** was determined to be 106.99 mg/L EC_{10} and 197.50 mg/L EC_{20}. The conditions of OECD guideline 201 for the validity of the test were adhered to, this product is not classified and therefore not harmful to aquatic organisms.

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