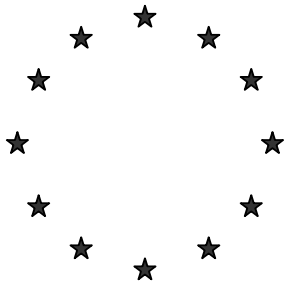
# COMPETENT AUTHORITY REPORT



# 1,2-Benzisothiazol-3-(2*H*)-one (BIT)

# (PT 6)

# Document III-A

## Active Substance

Rapporteur Member State: Spain

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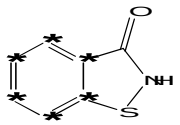
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.1** | **Fate and Behaviour in Water** |
| **Subsection A7.1.1** | **Degradation, initial studies** |
| **Subsection A7.1.1.1** | **Abiotic** |
| **Subsection A7.1.1.1.1** | **Hydrolysis** |
| **Subsection A7.1.1.1.1/01** | **HYDROLYSIS AS A FUNCTION OF PH AND IDENTIFICATION OF BREAKDOWN PRODUCTS** |
| **Annex Point IIA7.6.2.1** | |

| | | | Official use only |
|---|---|---|---|
| | | **1. REFERENCE** | |
| **1.1** | **Reference** | **A7.1.1.1.1/01** [redacted] Rohm and Haas Company, Technical Report N° GLP 2007-003, 17 May 2007 | |
| **1.2** | **Data protection** | Yes | |
| 1.2.1 | Data owner | Rohm and Haas Company | |
| 1.2.2 | Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into Annex I/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | | **2. GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **Guideline study** | Yes. OECD Guideline 111, Hydrolysis as a Function of pH (April 2004) and US EPA OPPTS 835.2110, Hydrolysis as a Function of pH (January 1998) | |
| **2.2** | **GLP** | Yes | |
| **2.3** | **Deviations** | None | |
| | | **3. MATERIALS AND METHODS** | |
| **3.1** | **Test material** | $^{14}$C-BIT<br>  <br>* site of $^{14}$C label | |
| 3.1.1 | Lot/Batch number | Lot No. 1069.00, sublot 1069.0005 | |

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| | **1. REFERENCE** | **Official use only** |
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| 3.1.2 Specification | As specified in the study guidelines, $^{14}$C-BIT was employed. Specifications for the $^{14}$C-materials are listed elsewhere. | |
| 3.1.3 Purity | Radiopurity of the $^{14}$C-test material was 98.3% | |
| 3.1.4 Specific Activity | Specific activity of the $^{14}$C-test material was 53.57 mCi/g | |
| 3.1.5 Further relevant properties | • Water solubility is greater than 0.7 g/L. | |
| **3.2 Reference substance** | No reference substances were employed to validate the hydrolysis study. The following compound was used as chromatography reference standards.<br>• $^{12}$C-BIT, lot MJB3787. Purity 99.8% | |
| 3.2.1 Initial concentration of reference substance | Not applicable | |
| **3.3 Test solution** | A treatment solution of $^{14}$C-BIT was prepared by dissolving 20.018 mg in 16.85 mL of acetonitrile. Actual concentrations of the test solutions, determined from the Time 0 samples, are tabulated below.<br><br>**Dosing Concentration (µg/g)**<br>pH 4 / pH 7 / pH 9<br>9.73 / 9.56 / 9.75<br><br>A non-radiolabeled treatment solution was prepared by dissolving 5.794 mg of $^{12}$C-BIT in 4.8 mL of acetonitrile. This solution was used for dosing samples that were used for sterility and pH examinations. | |

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| **1. REFERENCE** | | | **Official use only** |
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| **3.4** | **Testing procedure** | | |
| 3.4.1 | Test system | The guidelines employed for this study, OECD 111 and OPPTS 835.2110, are designed as a tiered approach. The first tier is to measure the stability of the test material at pH 4, 7, and 9 for 5 days at 50°C. If the compound is stable at elevated temperatures, no additional testing is required. BIT was stable so the only testing performed was tier 1.<br><br>pH 4, 7, and 9 buffers were prepared as outlined in Table A7.1.1.1.1-1. The buffers were degassed by sonication and then purged with nitrogen to exclude dissolved oxygen.<br><br>Thirty-six vials were prepared, twelve for each pH. To each vial, 3 ml of the appropriate buffer solution was added, the headspace purged with nitrogen and the vial sealed with crimped PTFE-lined septa. The vials were then sterilized by autoclaving. Prior to dosing the vials were placed in a water bath maintained in the dark at 50 ± 0.2°C. For each pH, the 12 vials were dosed and employed as described in the table below. | |

| Number of Vials | $^{14}$C-BIT (µl) | $^{12}$C-BIT (µl) | Use |
|---|---|---|---|
| 2 | 25 | | 0 hour samples |
| 2 | 25 | | 5 day samples |
| 2 | 25 | | Spare samples |
| 2 | | | Pre-application pH determination |
| 2 | | 25 | Post-application sterility determination |
| 2 | | 25 | Post-application pH determination |

The study was initiated by injecting $^{14}$C-BIT into the buffered solution through the septa.

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| | | Samples dosed with $^{14}$C-BIT were removed for analysis immediately (Time 0) and at Day 5. The samples were placed in ice water and aliquots removed for radioassay. Additional aliquots were transferred to vials for HPLC analysis.<br>The pH was determined in duplicate samples from each pH after sterilization and prior to dosing with BIT. The pH was again measured in two additional vials dosed with $^{12}$C-BIT on Day 5.<br>Two samples from each pH dosed with $^{12}$C-BIT were remove on Day 5 and the their sterility examined by counting colony forming units on agar plates incubated at 35°C for 2 days.<br>Prior to study initiation, it was found that BIT did not adsorb to the glass walls of the vials used. | |
| 3.4.2 | Temperature | The temperature of the water bath used was 50 ± 0.2°C. | |
| 3.4.3 | pH | pH 4.0 ± 0.2<br>pH 7.0 ± 0.2<br>pH 9.0 ± 0.2 | |
| 3.4.4 | Duration of the test | The duration of the test at pH 4, 7, and 9 was 5 days. | |
| 3.4.5 | Number of replicates | Duplicate vials were removed at Time 0 and Day 5. | |
| 3.4.6 | Sampling | Sampling intervals were:<br>pH 4: 0 and 5 days<br>pH 7: 0 and 5 days<br>pH 9: 0 and 5 days<br>Aliquots were removed immediately after sampling for radioassay. Additional aliquots were taken for chromatographic analysis. | |
| 3.4.7 | Analytical methods | Radioassay was performed using Packard liquid scintillation counters.<br>Thin layer chromatography (TLC) was performed on 250 µm | |

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| | thick silica gel plates (Whaman). The development solvent was ethyl acetate:methanol:acetonitrile:acetic acid (90:5:5:1). Solutions were cochromatographed with non-radiolabeled BIT. Radiolabeled compounds were detected using a phophorimager while non-labeled compounds visualized with a UV lamp (254 nm).<br><br>Aliquots were analyzed by HPLC using a modified C-18 column and a binary gradient composed of 0.5% aqueous formic acid and 0.5% methanolic formic acid. Detection employed a $^{14}$C-flow through monitor and/or UV detector (254 nm).<br><br>Representative samples at each pH were analyzed by LC-MS to confirm the presence of parent. Analysis employed a modified C-18 column and a binary gradient composed of 0.5% aqueous formic acid and 0.5% methanolic formic acid. The LC effluent was introduced in to the MS via an API interface and positive ionization was employed. | |
| | **4. RESULTS** | |
| **4.1 pH, storage, and sterility stability** | After 5 days of incubation the pH of the buffer solutions were stable; 4.0, 7.0, and 9.0.<br><br>Overnight storage at room temperature of the acetonitrile dosing solution resulted in no degradation of BIT.<br><br>Examination of the buffer solutions after 5 days incubation showed they were still sterile (no detectable colony forming units). | |
| **4.2 Material Balance** | The material balance was determined by radioassaying the hydrolysis solutions at Day 0 and 5 and the results expressed as a percent of applied radioactivity in Table A7.1.1.1.1-2. Recovery was greater than 97% with the average being 98.6 ± 1.7%. | |
| **4.3 Quantitation of parent and hydrolytic products** | Table A7.1.1.1.1-3 contains the replicate average data for the quantitation, as a percent of applied, of parent compound and total hydrolytic degradates at the three pH's. Quantitation in µg/g is presented in Table A7.1.1.1.1-4. These results show that parent compound is stable at pH 4, 7 and 9 since BIT comprises over 97% of the applied radioactivity. Thus there is essentially no | |

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| | | degradation OIT observed at pH 4, 7 and 9. | |
| **4.1** | **Hydrolysis rate constant ($k_h$)** | There is no rate constant since BIT did not hydrolyze under the test conditions. Thus no higher tier testing is required. | |
| **4.2** | **Dissipation time** | Since BIT did not hydrolyze, the dissipation time ($DT_{50}$) cannot be determined. | |
| **4.3** | **Specification of the transformation products** | The transformation products were insignificant since BIT did not hydrolyze under the test conditions. | |
| | | **5. APPLICANT'S SUMMARY AND CONCLUSION** | |
| **5.1** | **Materials and methods** | The Guidelines followed were OECD 111, Hydrolysis as a Function of pH and US EPA OPPTS 835.2110, Hydrolysis as a Function of pH. The tier one test examined the stability of the test compound at pH 4, 7, and 9 for 5 days at 50°C. If the compound is stable, no further testing is required.<br>Sterile and degassed pH 4, 7, and 9 buffers were prepared and dosed at nominal 10 ppm with $^{14}$C-BIT. The buffered aliquots were incubated in the dark at 50 ± 0.2°C and duplicate samples removed on Day 0 and Day 5. Solutions were radioassayed and chromatographed to quantitate parent. | |
| **5.2** | **Results and discussion** | In pH 4, 7, and 9 buffers no significant hydrolysis of BIT was observed after 5 days of incubation at 50°C. As a result, the compound is considered hydrolytically stable and no additional tiered testing is required. Over 97% of the applied radioactivity was recovered as BIT after the 5 day incubation. | |
| 5.2.1 | $k_H$ | Not determined since BIT was stable at pH 4, 7, and 9. | |
| 5.2.2 | $DT_{50}$ | Not determined since BIT was stable at pH 4, 7, and 9. | |
| 5.2.3 | $r^2$ | Not determined since BIT was stable at pH 4, 7, and 9. | |
| **5.3** | **Conclusion** | Following the tier 1 guidelines, BIT was found to be hydrolytically | |

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| | | stable at an elevated temperature and thus no additional testing is required.<br>This study fulfils the requirement for determining the effect of aqueous hydrolysis on the fate of BIT in the environment. As discussed further in Document III-A sections A7.1.1.1.2, BIT rapidly photodegrades. Additionally, BIT rapidly biodegrades (7.1.1.2.1). Therefore, hydrolysis will have minimal, if any influence on the fate of MI and on its risk assessment. | |
| 5.3.1 | Reliability | 1, valid without restrictions. | |
| 5.3.2 | Deficiencies | No significant deficiencies that will affect the results and conclusions. | |
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| **Evaluation by Competent Authorities** | |
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| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *November 2010* |
| **Materials and Methods** | *Applicant´s version is accepted* |
| **Results and discussion** | *Applicant´s version is accepted* |
| **Conclusion** | *BIT was found to be hydrolytically stable.*<br>*This study fulfils the requirement for determining the effect of aqueous hydrolysis on the fate of BIT in the environment.* |
| **Reliability** | *2* |
| **Acceptability** | *Acceptable* |

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| **Subsection A7.1.1.1.1** | **Hydrolysis** |
| **Subsection A7.1.1.1.1/01**<br>**Annex Point IIA7.6.2.1** | **HYDROLYSIS AS A FUNCTION OF PH AND IDENTIFICATION OF BREAKDOWN PRODUCTS** |

| **1. REFERENCE** | **Official use only** |
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| **Remarks** | |

**Table A7.1.1.1.1-1: Type and composition of buffer solutions**

| pH | Type of buffer (final molarity) | Composition |
|---|---|---|
| 4 | 0.05 M Phthalate | 5.108 g potassium hydrogen phthalate made up to 500 mL with water. The pH was 4.03 |
| 7 | 0.05 M Phosphate | 3.0407 g $KH_2PO_4$ made up to 500 mL with water. The pH was adjusted with 0.05 NaOH to 6.95. |
| 9 | 0.01 M Sodium Tetraborate-HCl | 4.768 g of $Na_2B_4O_7 \cdot 10H_2O$ made up to 500 mL with water. The pH was 9.01. |
| The pH of the bulk buffer solutions were adjusted to 4.0 ± 0.2, 7.0 ± 0.2, and 9.0 ± 0.2. | | |

**Table A7.1.1.1.1-2: Recovery of Applied $^{14}C$-Activity**

| pH | Material Balance as a Percent of Applied Radioactivity (%)[1] | |
|---|---|---|
| | Day 0 | Day 5 |
| 4 | 99.4 | 98.6 |
| 7 | 97.2 | 99.0 |
| 9 | 98.9 | 98.5 |

[1] Average of duplicate samples

**Table A7.1.1.1.1-3: Percent of Parent and Hydrolytic Products**

| pH | Sampling Day | Percent of Applied Activity (%)[1] | | |
|---|---|---|---|---|
| | | BIT | Other | Total |
| 4 | 0 | 98.3 | 1.1 | 99.4 |
| | 5 | 97.7 | 0.8 | 98.6 |
| 7 | 0 | 96.6 | 0.6 | 97.2 |
| | 5 | 98.5 | 0.5 | 99.0 |
| 9 | 0 | 98.4 | 0.4 | 98.9 |
| | 5 | 97.2 | 1.2 | 98.5 |

[1] Average of duplicate samples.

**Table A7.1.1.1.1-4: Concentration of Parent and Hydrolytic Products**

| pH | Sampling Day | Percent of Applied Activity (%)[1] | | |
|---|---|---|---|---|
| | | BIT | Other | Total |
| 4 | 0 | 9.73 | 0.11 | 9.84 |
| | 5 | 9.67 | 0.08 | 9.76 |
| 7 | 0 | 9.56 | 0.05 | 9.62 |
| | 5 | 9.75 | 0.05 | 9.80 |
| 9 | 0 | 9.75 | 0.04 | 9.79 |
| | 5 | 9.63 | 0.12 | 9.75 |

[1] Average of duplicate samples.

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| **Subsection A7.1.1.1.2** | **Phototransformation in water** |

| | | | Official use only |
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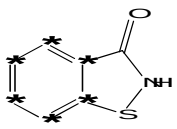
| | | **1. REFERENCE** | |
| **1.1** | **Reference** | **A7.1.1.1.2/01** [redacted] (2007). [$^{14}$C]-BIT: Photodegradation in Sterile Aqueous Solution; [redacted] Rohm and Haas Technical Report N° TR-07-019, June 2007 Unpublished. | |
| **1.2** | **Data protection** | Yes | |
| 1.2.1. | Data owner | Rohm and Haas Company | |
| 1.2.2. | Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into AnnexI/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | | **2. GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **Guideline study** | OECD Draft Guideline: Phototransformation of Chemicals in Water – Direct and Indirect Photolysis (August 2000) | |
| **2.2** | **GLP** | Yes | |
| **2.3** | **Deviations** | None | |
| | | **3. MATERIALS AND METHODS** | |
| **3.1** | **Test material** | $^{14}$C-BIT<br>  <br>* site of $^{14}$C label | |
| 3.1.1 | Lot/Batch number | Lot 1069.00, Sublot 1069.0005 | |
| 3.1.2 | Specification | As specified in the study guidelines, $^{14}$C-material was employed. Specifications for the $^{14}$C-material are listed below. | |

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| **Subsection A7.1.1.1.2** | **Phototransformation in water** |
| 3.1.3 Radiopurity | Radiopurity was 98.3% |
| 3.1.4 Specific Activity | 53.57 mCi/g |
| 3.1.5 UV/VIS absorption spectra | The absorption spectra for BIT at pH 5, 7, and 9 are presented in Figures A7.1.1.1.2-1, A7.1.1.1.2-2, and A7.1.1.1.2-3, respectively. |
| 3.1.6 Further relevant properties | • Water solubility is greater 0.7 g/L.<br>• The Henry's Law constant is $1.45 \times 10^{-5}$ Pa $m^3$ $mol^{-1}$<br>• The compound is hydrolytically stable at pH 5, 7, 9 |
| **3.2 Reference substances** | No reference substances were employed to validate the photolysis study. The compounds listed in Table A7.1.1.1.2-1 were used as chromatography and mass spectral reference standards. |
| **3.3 Test solution** | A $^{14}$C-BIT stock solution was prepared by dissolving 20.018 mg with 16.85 mL of acetonitrile.<br>A $^{12}$C-BIT stock solution was prepared by dissolving 5.794 mg with 4.8 mL acetonitrile<br>The pH 5, 7, and 9 buffers were prepared as follows.<br>• pH4: 1.544g of ammonium acetate was dissolved in 1L of water and the pH adjusted with 0.05M NaOH<br>• pH 7: 2.727g of $KH_2PO_4$ was dissolved in 1L of water and the pH adjusted using either 0.05M NaOH or 0.05M HCl<br>• pH 9: 3.819g of $Na_2B_40_7 \cdot 10H_2O$ was dissolved in 1L of water and the pH adusted with either 0.05M NaOH or 0.05M HCl<br>Aliquots (25 mL) of the buffer solutions were transferred to glass vessels and sterilized in an autoclave. |
| **3.4 Testing procedure** | |
| 3.4.1 Tier 1 Screen | The UV-VIS spectrum of BIT was recorded over the wavelength range of 295-800 at pH 5, 7, and 9 (Figures A7.1.1.1.2-1, A7.1.1.1.2-2, and A7.1.1.1.2-3, respectively). The loss of compound is calculated as follows:<br>$Percent\ loss = 100[e^{KaT}]$ Eq 1<br>Where T = 30 days |

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$Ka = \sum_{\lambda 297.5}^{\lambda 800} \varepsilon_\lambda L_\lambda$ Eq 2

$\varepsilon_\lambda$ is the molar adsorption coefficient

$L_\lambda$ is the solar irradiance

3.4.2 Test System for Tier 2 and 4

Test Vessels

The glass test vessels had an inlet and an outlet as well as an injection port. Their height was approximately 41 mm and the diameter approximately 24 mm (yielding an exposure surface of 452.6 $mm^2$). 25 ml buffer portions were added to each vessel. Vessels to be irradiated were fitted with quartz lids while the dark control vessels were sealed with a crimped PTFE-lined cap. Bacterial air filters were attached to the inlet and outlet prior to autoclaving the unit.

Properties of the light source

A Hanau Suntest Xenon lamp was used as the light source. Radiation below 290 nm was removed with a filter. The spectral properties and intensity was measured using an LI-1800 spectroradiometer.

Traps

To each irradiation unit four traps were attached to capture evolved volatiles. The traps contained ethanediol (25 g) to collect polar organic volatiles, 2% paraffin in xylene (25 g) to collect non-polar organic volatiles, and two 2M NaOH (25 g) to collect $CO_2$.

Temperature

The vessels were placed into a cooling block and the temperature maintained at 20 ± 3°C by circulating temperature controlled water through the block and thus around the vessels. The temperature on the dark control samples were maintained at 20 ± 3°C in a similar manner to the irradiated.

3.4.3 Tier 2 (preliminary kinetics)

For each pH, 6 vessels containing 25 mL of the buffer solution were prepared and dosed with either 0.1 µg/mL $^{14}$C-BIT or 10 µg/mL $^{14}$C-BIT. The system is described below.

| Sample Type | $^{14}$C BIT µg/ml | Irradiated | Number of Samples |
|---|---|---|---|
| Time 0 | 0.1 | NA | 1 |
| Day 1, 2, 7 | 0.1 | Yes | 1 |
| Time 0 | 10 | NA | 1 |
| Day 1, 2, 7 | 10 | Yes | 1 |

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|---|---|---|---|
| Dark Control | 0.1 | No | 1 |
| Dark Control | 10 | No | 1 |

NA = Not Applicable

The experiment was initiated by injecting $^{14}$C-BIT into the glass test vessel through the septum on the injection port. After application of BIT the Time 0 samples were removed, radioassayed and chromatographed. The samples to be irradiated were placed under the xenon lamp while the controls were placed in a dark chamber. On Days 1, 2, and 7 aliquots were removed from the irradiated samples, radioassayed, and chromatographed. The dark controls were only analyzed on Day 7. Selected samples were analyzed by LC-MS to confirm the presence of parent. The rate constant was determined by non-linear regression and the loss determined by equation 1 (section 3.4.1 above).

The irradiation intensity was 42 Watts/m$^2$ (between 300 and 400 nm) resulting in the samples receiving the equivalent of 12 days of natural sunlight (30°N-50°N latitude) in the 7 days of xenon lamp exposure.

3.4.4 Tier 4 (Definitive test)

For each pH, 22 glass vessels containing 25 mL of buffer solution were sterilized. To 16 sterile vessels a nominal 10 µg/mL $^{14}$C-BIT was added through the injection port septum and the vessels gently swirled. Fourteen vessels were placed under the xenon lamp and the volatile traps connected. Humidified air was pulled through the system to remove volatiles from the test vessel. The remaining two dosed vessels were analyzed immediately as Time 0 samples. In addition the following samples were prepared; duplicate dark controls containing 10 µg/mL $^{14}$C-BIT, duplicate samples without BIT to check the pH at Time 0, duplicate irradiated samples containing $^{12}$C-BIT to check the pH and solution sterility at the end of the exposure period, and duplicate dark control samples containing $^{12}$C-BIT to check the pH and solution sterility at the end of the exposure period.

At various intervals, duplicate vessels were removed for analysis. Aliquots of solution were radioassayed and chromatographed (HPLC). In addition representative samples were analyzed by LC-MS to confirm the presence of parent and for identification of photodegradtes.

The volatiles traps and a polyurethane bung placed between the glass vessel and the traps were radioassayed when their respective glass vessel was removed for analysis. The bung was soaked in acetonitrile and the extract radioassayed. The presence of $CO_2$ was confirmed in selected samples of the NaOH traps by precipitation

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| | with $BaCl_2$.<br>The irradiation intensity was 25 Watts/$m^2$ (between 300 and 400 nm) resulting in the samples receiving the equivalent to 1 day of natural sunlight (30°N-50°N latitude) for every day of exposure under the xenon lamp. |
| 3.4.5 Duration of the test | The duration of the Tier 2 test was 7 days (equivalent to 12 days of natural sunlight)<br>The duration of the Tier 4 test was 30 days (equivalent to 30 days of natural sunlight) |
| 3.4.6 Number of replicates | In the Tier 2 test single samples were employed<br>In the Tier 4 test duplicate samples were employed |
| 3.4.7 Sampling | In the Tier 1 test, irradiated samples were taken on Days 0, 1, 2 and 7. The dark control was analyzed on Day 7.<br>In the Tier 4 test, the following schedule was employed for irradiated samples.<br>• pH 5: 0, 2, 4, 8 hours and 1, 15 and 30 days<br>• pH 7: 0, 0.5, 1, 2 hours and 1, 15, 30 days<br>• pH 9: 0, 0.5, 1, 2 hours and 1, 15, 30 days<br>The dark controls dosed with $^{14}$C-BIT were analyzed on Day 30. Sterility and pH samples were analyzed at the start of the exposure period and on Day 30. |
| 3.4.8 Analytical methods | Radioassay was performed using Packard liquid scintillation counter.<br>Radiopurity and aliquots from the buffer solutions were analyzed by HPLC using a modified C-18 column and a binary gradient composed of 0.5% aqueous formic acid and 0.5% methanolic formic acid. Detection employed a $^{14}$C-flow through monitor and/or UV detector (254 nm).<br>Thin layer chromatography (TLC) was used for radiopurity determination. Silica gel plates (250 µm thick) were developed with ethyl acetate:methanol:acetonitrile:acetic acid (90:5:5:1). Solutions were cochromatographed with non-radiolabeled BIT. Radiolabeled compounds were detected using a phophorimager while non-labeled compounds visualized with a UV lamp (254 nm).<br>Representative samples were analyzed by LC-MS (ion trap) to confirm the presence of parent. Analysis employed a modified C-18 |

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| | column and a binary gradient composed of 0.5% aqueous formic acid and 0.5% methanolic formic acid. Detection was by a radioactivity flow monitor and the mass spectrometer. The LC effluent was split between the two detectors and introduction in to the MS via an API interface and positive and negative ionization were employed.<br><br>For metabolite identification, accurate masses were obtained using an LC-Fourier Transform MS. A modified C-18 column was employed with a gradient consisting of 0.5% aqueous formic acid and 0.5% formic acid in acetonitrile. The LC effluent was introduced into the MS via an API interface and both positive and negative ionization was employed. |
| **3.5 Transformation products** | |
| 3.5.1 Method of analysis for transformation products | Transformation products were quantitated by HPLC and identified by LC-MS. |
| | **4. RESULTS** |
| **4.1 Tier 1** | The UV/VIS spectra for BIT at pH 5, 7, and 9 are presented in Figures A7.1.1.1.2-1, A7.1.1.1.2-2, and A7.1.1.1.2-3, respectively. The maximum possible rate constants determined for BIT at pH 5, 7, and 9 are 994 $day^{-1}$, 953 $day^{-1}$, and 965 $day^{-1}$, respectively. These rate constants predicte that photolysis could account for 100% loss of BIT over a 30 day period at all three pH's. Therefore additional testing is necessary. |
| **4.2 Tier 2 (preliminary kinetics test)** | The distribution and recovery of $^{14}C$-activity from Tier 2 testing is presented in Table A7.1.1.1.2-2. Over 94% of the applied activity remained in the buffer solution with less than 1% being found in volatile organic traps and less than 10% as evolved $^{14}CO_2$.<br><br>Quantitation of BIT at Day 0, 1, 2, and 7 is presented in Table A7.1.1.1.2-3. The results demonstrate that photolysis could account for 100% loss of BIT within 30 days. Therefore additional testing, Tier 4, is required. |
| **4.3 Tier 4 (definitive test)** | |

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4.3.1 Distribution and recovery

The results from the distribution and recovery of applied activity are presented in Table A7.1.1.1.2-4. The results are similar to that observed in the Tier 2 test.

For pH 5 irradiated samples, over 93% of the applied activity was detected in the buffer solution. Less than 0.3% was found in the volatile organic traps and less than 4% in the $CO_2$ trap. For the dark control sample, 99.6% was detected in the buffer solution and no volatiles were detected. The mean recovery of $^{14}C$-activity was 98.8 ± 2.2%.

The results for the pH 7 irradiated samples were similar to pH 5. Over 86% of the applied activity was detected in the buffer solution. Less than 0.5% was in the volatile organic traps. By Day 30, 9.1% of the applied activity was present as $CO_2$. For the dark control, 99.8% was detected in the buffer solution with no volatiles detected. The mean recovery of $^{14}C$ was 98.5 ± 1.7%.

Over 89% of the applied activity from the irradiated pH 9 samples was detected in the buffer solution. Less than 0.7% was detected in the volatile organic traps. On Day 30, $CO_2$ accounted for 6.9% of the applied activity. For the dark controls, 98.9% was detected in the buffer solution with no volatiles detected. The mean recovery of $^{14}C$-activity was 97.8 ± 2.1%

4.3.2 Quantitation of BIT and photoproducts

The quantitation of BIT and its photoproducts at various sampling intervals is presented in Tables A7.1.1.1.2-5, A7.1.1.1.2-6, and A7.1.1.1.2-7 for pH 5, 7, and 9, respectively. BIT rapidly photolyzed so that by Day 15 there was no parent remaining at any pH tested. At pH 5, after two hours of irradiation there remained 85.7% of the applied activity as BIT while at pH 7 and 9, 13.1% and 20.1% remained.

There were seven major photodegradates detected. One cochromatographed with the standard, 2-sulfobenzoic acid (2-SBAH). At pH 5 the major degradates were 2-SBAH, Unknown A and Unknown B. At pH 7 the major degradates were 2-SBAH, Unknown B, and Unknown E. At pH 9 the major degradates were 2-SBAH, Unknown B, Unknown C, Unknown E, and Unknown M. At all pH's Unknown B is transient probably serving as a precursor for a subsequent product.

No apparent degradation was observed in the dark control samples. On Day 30 BIT comprised 97.8%, 98.2% and 95.6% of the applied activity at pH 5, 7, and 9, respectively.

4.3.3 Kinetics

The kinetic results are tabulated below.

| Parameter | pH 5 | pH 7 | pH 9 |
|---|---|---|---|

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| | | | |
|---|---|---|---|
| k ($day^{-1}$) | 1.813 | 22.879 | 23.833 |
| $DT_{50}$ (h) | 9 | 0.7 | 0.7 |
| $DT_{75}$ (h) | 18 | 1.4 | 1.4 |
| $DT_{90}$ (h) | 30 | 2.4 | 2.4 |
| $R^2$ | 0.992445 | 0.996478 | 0.988083 |

Figure A7.1.1.1-4 provides a graphical representation of the natural log decline of BIT at pH 5, 7, and 9.

4.3.4 Confirmation of BIT and Identification of the Degradation Products

Using LC-MS, the presence of BIT in selected samples was confirmed.

Identification of the photodegradation products was undertaken using LC-MS. A summary of the results is presented in Table A7.1.1.1.2-8 providing the structures, names, and maximum percentage of each photodegradate. One photodegradate was initially identified as 2-sulfobenzoic acid (2-SBAH) based on cochromatography with a standard. However LC-MS analysis demonstrated that 2-SBAH was a minor component of this fraction with 2-sulfobenzamide being the major component.

Unknown D has two possible structures; dihydroxylated BIT (hydroxylation of the benzene ring) and the benzene ring mono-hydroxylated sulfoxide. Fragmentation, even from daughter ions (MS/MS), was not sufficient to assign the absolute structure and both photoproducts have the same exact mass. Thus it was not possible to differentiate between these two possibilities.

It was not possible to assign absolute structures to Unknown E and Unknown M. LC-MS did demonstrate that they contained multiple components and probably no single component was greater than 10% of the applied activity.

4.3.5 Photolytic pathway

A photolytic pathway is presented in Figure A7.1.1.1.2-4.

4.3.6 pH and sterility

The solution pH was measured pre and post-irradiation and is provided below.

| Interval | Mean Solution pH | | |
|---|---|---|---|
| | pH 4 | pH 7 | pH 9 |
| Pre-irradiation | 4.96 | 7.03 | 9.02 |
| Post-irradiation | 5.08 | 7.04 | 8.72 |
| Dark control: post-irradiation | 5.32 | 7.04 | 9.04 |

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Aliquots of the Day 30 irradiated and dark control solutions were checked for sterility on nutrient agar plates. No colony forming units were detected in any solutions.

**5. APPLICANT'S SUMMARY AND CONCLUSION**

**5.1 Materials and methods**

The test guidelines employed were OECD Draft Guideline: Phototransformation of Chemicals in Water – Direct and Indirect Photolsysis (August 2000).

An initial screen involving an analysis of the UV/VIS spectrum showed that BIT could substantially photodegrade so additional testing was performed. A preliminary kinetic test was performed by adding sterile pH 5, 7, or 9 buffer to a test vessel, dosing at 0.1 µg/mL and 10 µg/mL BIT, and irradiating the sample using a xenon lamp. The solution was analyzed on Days 0, 1, 2, and 7. The results showed that additional testing was warranted.

A definitive photolysis study was undertaken by preparing photolysis vessels with either sterile pH 5, 7, or 9 buffer. The vessels were dosed at 10 µg/mL, a series of traps designed to capture volatile organic and evolved $CO_2$ were attached to each vessel, a stream of sterile moistened air was pulled through the system, and the vessels irradiated with a xenon lamp. pH 5 samples were removed at 0, 2, 4, and 8 hours and 1, 15, and 30 days. pH 7 and 9 samples were removed at 0, 0.5, 1, and 2 hours and 1, 15, and 30 days. Samples and their traps were radioassayed. Aliquots of the buffer solutions were chromatographed (HPLC) to quantitate parent and photodegradates. Photodegradates were identified by LC-MS

**5.2 Results and discussion**

BIT rapidly photodegrades and the rate is dependent on pH. The photolytic half-life in pH 5 buffer was 9 hours while in pH 7 and 9, 0.7 hours. Organic volatiles were less than 1% of the applied activity and evolved $CO_2$ less than 10%. On average, the recovery of applied radioactivity in the definitive study was over 98%. The major photoproducts were:

- 2-sulfobenzamide (small quantities of 2-sulfobenzoic acid cochromatographed)
- 1,2-benzthiazolin-2-one
- hydroxy-1,2-benzisothiazolin-3-one
- Saccharin (1,2-benzisothiazolin-3-one-1,1-dioxide)
- Dihydroxy-1,2-benzisothiazolin-3-one or hydroxy-1,2-benzisothiazolin-3-one-1-oxide
- Unknown E: unable to assign a structure but it contained

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| | multiple components<br>• Unknown M: unable to assign a structure but it contained multiple components |
| **5.3 Conclusion** | This study fulfils the requirement for determining the effect of aqueous photolysis on the fate of BIT in the environment. The half-life at pH 5 is 9 hours and at pH 7 and 9, 0.7 hours. Photodegradation of BIT involves cleavage of the isothaizolone ring, hydroxylation of the benzene ring, and/or oxidation of the sulfur. |
| 5.3.1 Reliability | 1- valid without restrictions |
| 5.3.2 Deficiencies | None |

| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *November 2010* |

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| **Materials and Methods** | *Applicant's version is accepted with the following remarks:*<br>*Samples are duplicated only in Tier 4. In Tier 2, two substance concentrations were tested, but only one replicate for each concentration was analyzed.*<br>*Test solution: pH 4 should be pH 5*<br>*At the pH and sterility section, Table should read pH 5 and not pH4.*<br>*Testing procedure: Tier 1 screen, Eq 1 is not correct.*<br>*Only an aliquot of 1ml was removed for sampling at day 1 and day 3, instead of using an entire irradiated photolysis cell at each sampling interval. In addition, dark control was only analyzed in day 7, instead of being analyzed at each sampling interval.*<br>*Transformation products are identified and quantified, but there is no information about the degradation rate of these products.*<br>*The following sentence should be added in "Testing Procedure-Tier 1 Screen" section:*<br>*"The extent of overlap between the absorption bands of the substance and the spectral distribution of the incident sunlight gave and indication of the potential for photolysis. The result showed that photolysis could account for 100% loss of thest substance over the equivalent of 30 days, so further testing was performed.* |
| **Results and discussion** | *Accepted* |
| **Conclusion** | *This study fulfils the requirement for determining the effect of aqueous photolysis on the fate of BIT in the environment. The half-life at pH 5 is 9 hours and at pH 7 and 9, 0.7 hours. Photodegradation of BIT involves cleavage of the isothaizolone ring, hydroxylation of the benzene ring, and/or oxidation of the sulfur.* |
| **Reliability** | *2* |
| **Acceptability** | *Acceptable* |
| **Remarks** | |

**Table A7.1.1.1.2-1: Chromatographic Reference Standards**

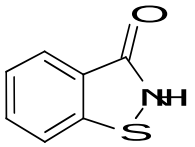
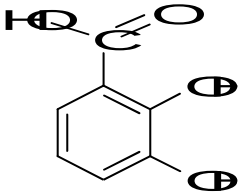
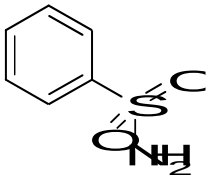
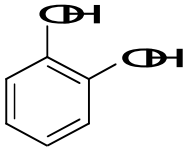
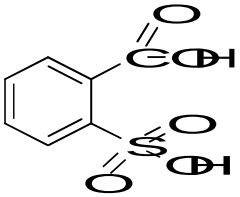
| Chemical name | Abbreviation used | Sponsor lot number | Purity (%) | Expiry date | Structure |
|---|---|---|---|---|---|
| 1,2-Benzisothiazolin-3-one | BIT | MJB3787 | 99.8 | 18 April 2012 |  |
| 2,3-Dihydroxybenzoic acid | 2,3-DHBA | 09026KB | 99.9 | 16 Nov 2008 |  |
| Benzene sulphonamide | BS | 14024BB | 99.0 | 30 Nov 2008 |  |
| Catechol | NA | 03812AD | 99.2 | 29 Nov 2008 |  |
| 2-Sulfobenzoic acid hydrate | 2-SBAH | 151001MB | 75.4† | 16 Feb 2009 |  |

**Table A7.1.1.1.2-2: Distribution and Percent Recovery from Tier 2 (Preliminary Kinetics) Test**

| Conditions | Sample Day | Percent of Applied Activity | | | |
|---|---|---|---|---|---|
| | | Solution[1] | Volatile Organic Traps[2] | NaOH | Recovery |
| **pH 5** | | | | | |
| Light | 0 | 103.1 | NA[3] | NA | 103.1 |
| | 1 | 101.6 | ND[3] | MD | 101.6 |
| | 2 | 99.1 | ND | 0.1 | 99.2 |
| | 7 | 101.3 | 0.1 | 0.8 | 102.1 |
| Dark | 7 | 102.3 | NA | ND | 102.2 |
| **pH 7** | | | | | |
| Light | 0 | 102.3 | NA | NA | 102.3 |
| | 1 | 99.1 | ND | 0.1 | 99.2 |
| | 2 | 97.1 | ND | 0.9 | 97.9 |
| | 7 | 94.7 | 0.2 | 5.4 | 100.3 |
| Dark | 7 | 101.0 | NA | NA | 100.9 |
| **pH 9** | | | | | |
| Light | 0 | 101.6 | NA | NA | 101.5 |
| | 1 | 101.3 | ND | 7.3 | 116.9[4] |
| | 2 | 96.9 | ND | 7.4 | 113.7[4] |
| | 7 | 99.7 | ND | 8.9 | 124.4[4] |
| Dark | 7 | 99.0 | NA | NA | 106.0 |

[1] Buffer solution plus rinse of glass vessel
[2] Combined results of the Ethanediol trap + Paraffin/Xylene trap + polyurethane bung
[3] NA = Not Applicable; ND= Not Detected
[4] The high values may be due to contamination of the first sodium hydroxide trap.

**Table A7.1.1.1.2-3: Quantitation of BIT in Tier 2 (Preliminary Kinetic) Test**

| Conditions | Sample Day | Percent BIT at Dose Rate | |
|---|---|---|---|
| | | 0.1 µg BIT/mL | 10 µg BIT/mL |
| **pH 5** | | | |
| Light | 0 | 103.4 | 100.6 |
| | 1 | 6.9 | 5.8 |
| | 2 | 2.1 | ND |
| | 7 | ND | ND |
| Dark | 7 | 102.6 | 99.6 |
| **pH 7** | | | |
| Light | 0 | 102.9 | 99.5 |
| | 1 | 3.5 | 0.4 |
| | 2 | 2.4 | 0.7 |
| | 7 | ND | ND |
| Dark | 7 | 100.6 | 97.7 |
| **pH5** | | | |
| Light | 0 | 96.5 | 100.0 |
| | 1 | 8.4 | ND |
| | 2 | 1.8 | 0.3 |
| | 7 | 2.6 | ND |
| Dark | 7 | 99.5 | 93.1 |

**Table A7.1.1.1.2-4: Distribution and Percent Recovery from Tier 4 (Advanced) Test**

| Conditions | Sample Interval | Percent of Applied Activity[1] | | | |
|---|---|---|---|---|---|
| | | Solution[2] | Volatile Organic Traps[3] | NaOH | Recovery |
| **pH 5** | | | | | |
| Light | 0 | 100.0 | NA[4] | NA | 99.9 |
| | 2 hr | 98.3 | 0.2 | ND | 98.5 |
| | 4 hr | 99.8 | 0.1 | ND | 99.9 |
| | 8 hr | 100.4 | 0.1 | ND | 100.5 |
| | 1 day | 98.6 | ND[4] | ND | 98.6 |
| | 15 days | 93.2 | 0.1 | 2.4 | 95.5 |
| | 30 days | 94.2 | 0.1 | 3.5 | 97.7 |
| Dark | 30 days | 99.6 | NA | | 99.6 |
| **pH 7** | | | | | |
| Light | 0 | 100.2 | NA | NA | 100.2 |
| | 0.5 hr | 98.9 | 0.2 | ND | 98.8 |
| | 1 hr | 99.0 | 0.4 | ND | 99.4 |
| | 2 hr | 98.7 | ND | ND | 98.7 |
| | 1 day | 98.5 | ND | 0.1 | 98.6 |
| | 15 days | 90.3 | 0.1 | 6.7 | 97.1 |
| | 30 days | 86.6 | 0.1 | 9.1 | 95.7 |
| Dark | 30 days | 99.8 | NA | NA | 99.8 |
| **pH 9** | | | | | |
| Light | 0 | 99.6 | NA | NA | 99.6 |
| | 0.5 hr | 98.6 | 0.2 | ND | 98.7 |
| | 1 hr | 96.8 | 0.6 | ND | 97.4 |
| | 2 hr | 98.7 | ND | ND | 98.6 |
| | 1 day | 98.8 | 0.1 | ND | 98.9 |
| | 15 days | 90.9 | ND | 3.3 | 94.1 |
| | 30 days | 89.6 | ND | 6.9 | 96.5 |
| Dark | 30 days | 98.9 | NA | NA | 98.9 |

[1] Average of duplicate samples
[2] Buffer solution plus rinse of glass vessel
[3] Combined results of the Ethanediol trap + Paraffin/Xylene trap + polyurethane bung
[4] NA = Not Applicable; ND= Not Detected

**Table A7.1.1.1.2-5: Quantitation of BIT and its Photodegradates—pH 5**

| Conditions | Sample Interval | Quantitation of BIT and Photodegradates as a Percent of Applied Activity[1] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | BIT | 2-SBAH | Unknown A | Unknown B | Unknown C | Unknown D | Unknown E | Unknown M | Other[2] | Total |
| Light | 0 | 98.7 | ND[3] | ND | ND | ND | ND | ND | ND | 0.7 | 99.5 |
| | 2 hrs | 85.7 | ND | 4.9 | 6.3 | ND | ND | ND | ND | 0.6 | 97.6 |
| | 4 hrs | 76.8 | 0.6 | 10.1 | 11.4 | ND | ND | ND | ND | 0.5 | 99.4 |
| | 8 hrs | 55.1 | 2.5 | 19.6 | 21.7 | 0.2 | ND | 0.1 | ND | 0.7 | 99.9 |
| | 1 day | 14.0 | 7.8 | 39.9 | 34.3 | ND | ND | ND | 1.1 | 1.0 | 98.2 |
| | 15 days | ND | 17.1 | 46.7 | 19.4 | 2.4 | 1.0 | 2.5 | 2.3 | 1.1 | 92.7 |
| | 30 days | ND | 22.7 | 49.8 | 9.1 | 2.5 | 1.1 | 4.1 | 3.3 | 1.3 | 93.9 |
| Dark | 30 days | 97.8 | ND | ND | ND | ND | ND | ND | ND | 1.3 | 99.1 |

[1] Average of duplicate samples
[2] Other = Total Other Unknowns and Unresolved Background
[3] ND = Not Detected

**Table A7.1.1.1.2-6: Quantitation of BIT and its Photodegradates—pH 7**

| Conditions | Sample Interval | Quantitation of BIT and Photodegradates as a Percent of Applied Activity[1] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | BIT | 2-SBAH | Unknown A | Unknown B | Unknown C | Unknown D | Unknown E | Unknown M | Other[2] | Total |
| Light | 0 | 98.7 | ND | ND | ND | ND | ND | ND | ND | 1.1 | 99.8 |
| | 0.5 hrs | 62.2 | 2.6 | 0.8 | 31.3 | 0.3 | ND | ND | 0.3 | 0.7 | 98.3 |
| | 1 hrs | 38.9 | 4.8 | 1.6 | 49.9 | 0.7 | ND | 0.8 | 0.7 | 1.3 | 98.6 |
| | 2 hrs | 13.1 | 12.0 | 2.6 | 65.4 | 1.5 | 0.6 | ND | 1.8 | 1.1 | 98.1 |
| | 1 day | 0.7 | 25.2 | 3.5 | 51.0 | 2.8 | 2.8 | 2.7 | 6.9 | 2.3 | 98.0 |
| | 15days | ND | 56.4 | 4.6 | ND | 3.0 | 6.1 | 11.9 | 6.8 | 1.1 | 89.9 |
| | 30 days | ND | 53.0 | 3.7 | ND | 1.8 | 5.8 | 13.8 | 6.1 | 1.9 | 86.2 |
| Dark | 30 days | 98.2 | ND | ND | ND | ND | ND | ND | ND | 1.2 | 99.4 |

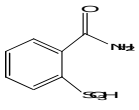
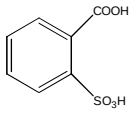
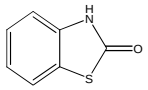
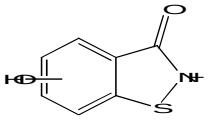
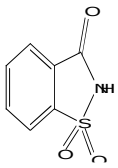
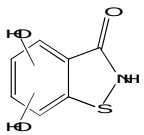
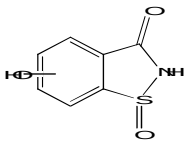
[1] Average of duplicate samples
[2] Other = Total Other Unknowns and Unresolved Background
[3] ND = Not Detected

**Table A7.1.1.1.2-7: Quantitation of BIT and its Photodegradates—pH 9**

| Conditions | Sample Interval | Quantitation of BIT and Photodegradates as a Percent of Applied Activity[1] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | BIT | 2-SBAH | Unknown A | Unknown B | Unknown C | Unknown D | Unknown E | Unknown M | Other[2] | Total |
| Light | 0 | 97.7 | ND | ND | 0.4 | ND | ND | ND | ND | 1.1 | 99.1 |
| | 0.5 hrs | 54.0 | 4.6 | ND | 34.8 | 1.0 | 0.7 | 1.1 | 1.1 | 0.7 | 98.1 |
| | 1 hrs | 33.5 | 7.5 | ND | 48.4 | 1.4 | 1.3 | 1.8 | 1.3 | 1.2 | 96.4 |
| | 2 hrs | 20.1 | 10.7 | ND | 59.0 | 2.2 | 2.5 | 2.0 | 0.9 | 0.5 | 98.0 |
| | 1 day | 0.2 | 27.2 | ND | 41.4 | 9.6 | 4.8 | 3.0 | 10.0 | 2.1 | 98.4 |
| | 15days | ND | 39.5 | ND | ND | 12.1 | 8.7 | 15.7 | 10.4 | 2.9 | 89.2 |
| | 30 days | ND | 36.9 | ND | ND | 13.2 | 7.8 | 26.1 | 4.6 | 0.7 | 89.3 |
| Dark | 30 days | 95.6 | ND | ND | ND | ND | ND | ND | ND | 2.8 | 98.4 |

[1] Average of duplicate samples
[2] Other = Total Other Unknowns and Unresolved Background
[3] ND = Not Detected

**Table A7.1.1.1.2-8: Major Photodegradates Detected, Their Structures, and Maximum Percentage Detected**

| Designation | Structure | | Maximum Mean Percent | | |
|---|---|---|---|---|---|
| | | | pH 5 | pH 7 | pH 9 |
| 2-SBAH | major component  | minor component  | 22.7 (30 days) | 56.4 (15 days) | 39.5 (15 days) |
| | 2-sulfobenzamide | 2-sulfobenzoic acid | | | |
| Unknown A |  1,2-benzthiazolin-2-one | | 49.8 (30 days) | 4.6 (15 days) | ND |
| Unknown B |  hydroxy-1,2-benzisothiazolin-3-one | | 34.3 (1 day) | 65.4 (2 hours) | 59.0 (2 hours) |
| Unknown C |  Saccharin (1,2-benzisothiazolin-3-one-1,1-dioxide) | | 2.5 (30 days) | 3.0 (15 days) | 13.2 (30 days) |
| Unknown D |  | or  | 1.1 (30 days) | 6.1 (15 days) | 8.7 (15 days) |

| Designation | Structure | | | Maximum Mean Percent | | |
|---|---|---|---|---|---|---|
| | | | | pH 5 | pH 7 | pH 9 |
| | dihydroxy-1,2-benzisothiazolin-3-one | | hydroxy-1-2-benzisothiazolin-3-one-1-oxide | | | |
| Unknown E | Multiple components that are chromatographically very polar | | | 4.1 (30 days) | 13.8 (30 days) | 26.1 (30 days) |
| Unknown M | Unable to assign structures despite having exact mass information | | | 3.3 (30 days) | 6.9 (1 day) | 10.4 (15 days) |

**Figure A7.1.1.1.2-1: UV Absorption Spectrum of BIT in pH 5 Buffer Solution**

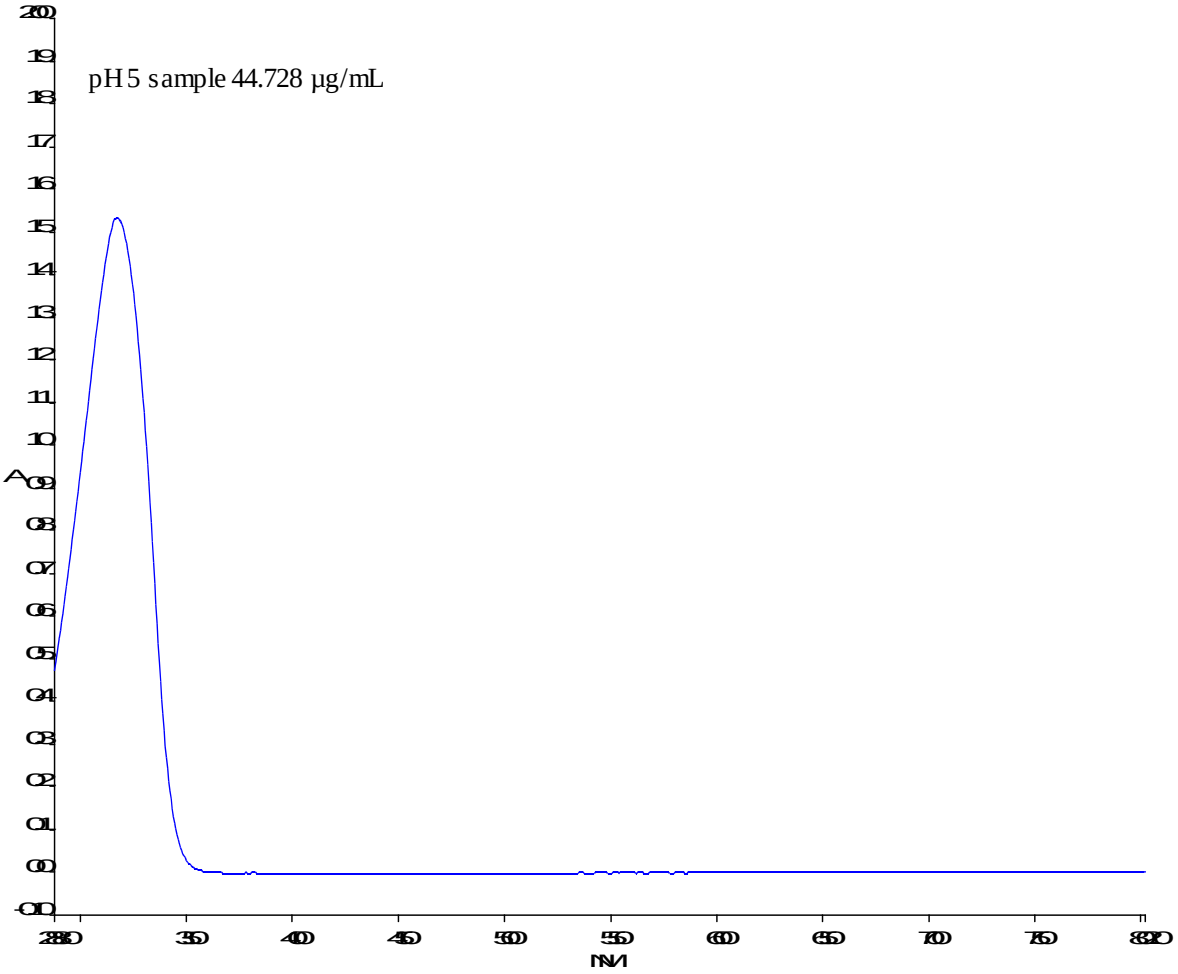

pH 5 sample 44.728 µg/mL
AU
NM

**Figure A7.1.1.1.2-2: UV Absorption Spectrum of BIT in pH 7 Buffer Solution**

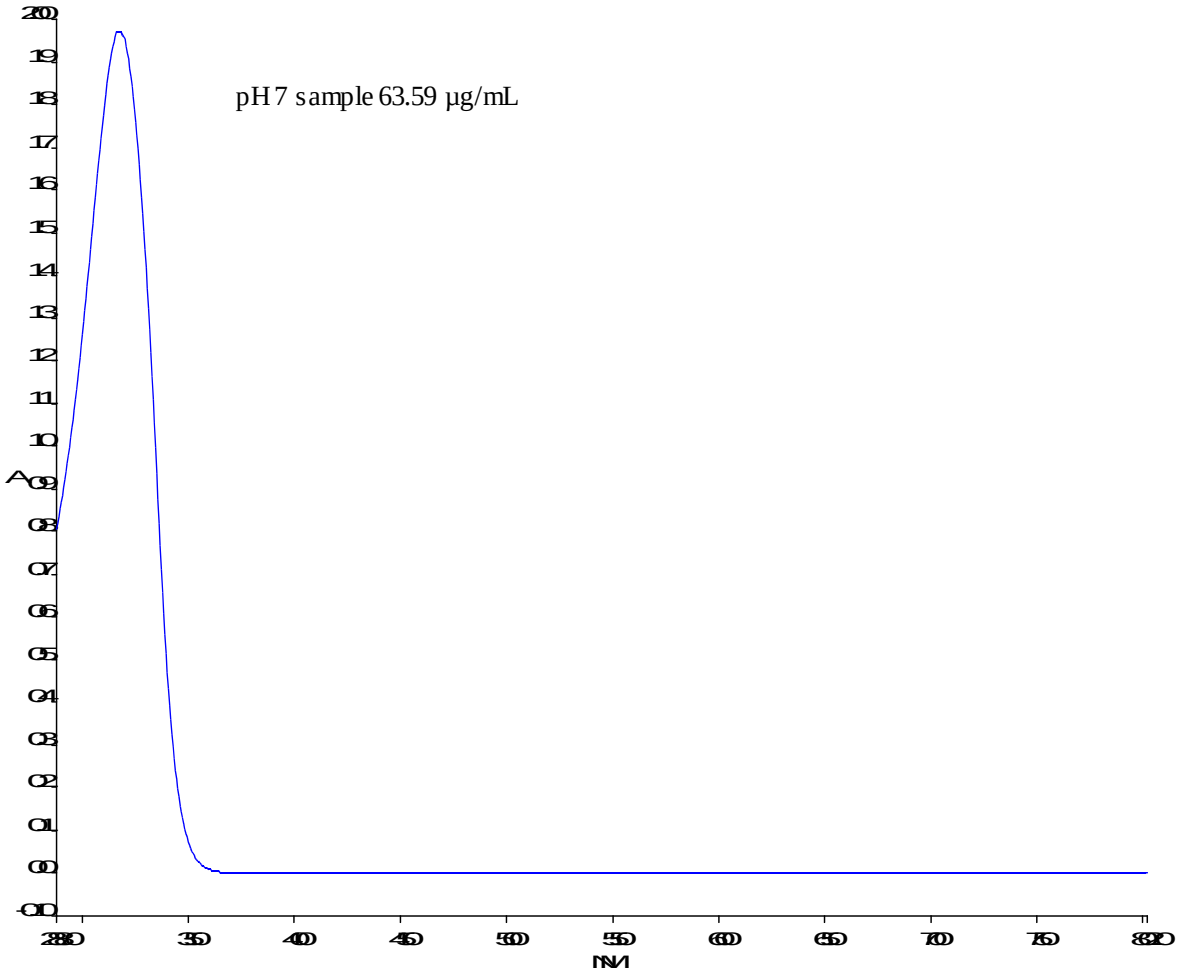


**Figure A7.1.1.1.2-3: UV Absorption Spectrum of BIT in pH 9 Buffer Solution**

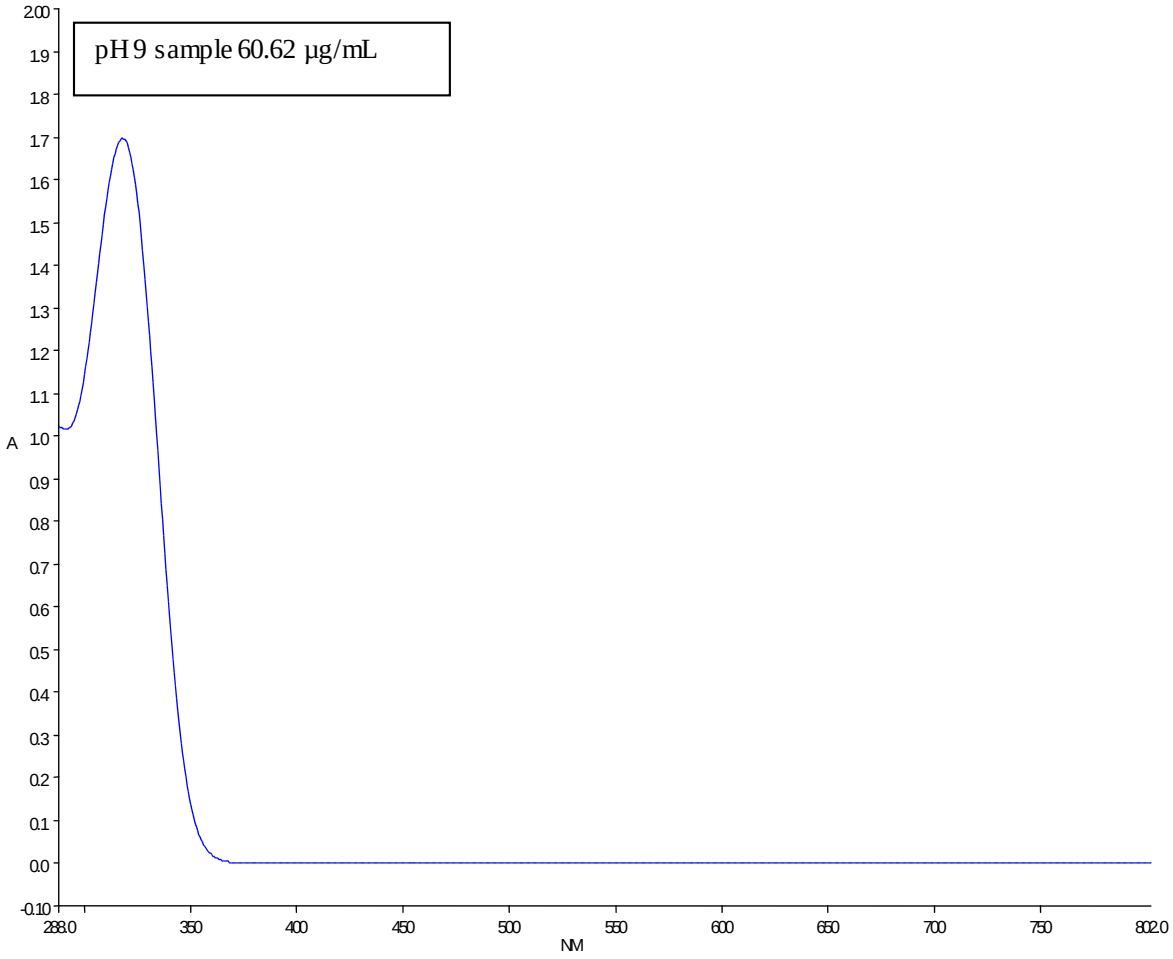


**Figure A7.1.1.1.2-4: Dissipation of Parent Compound**

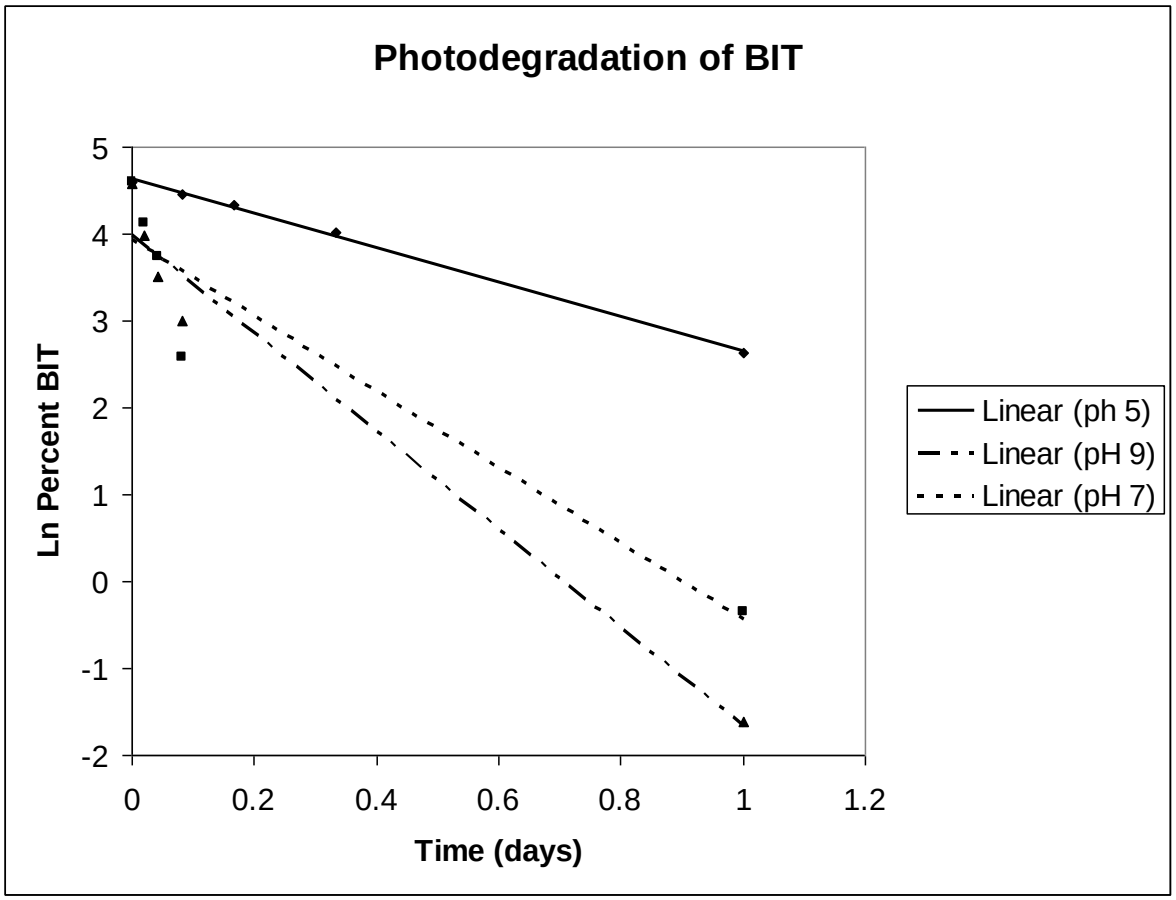
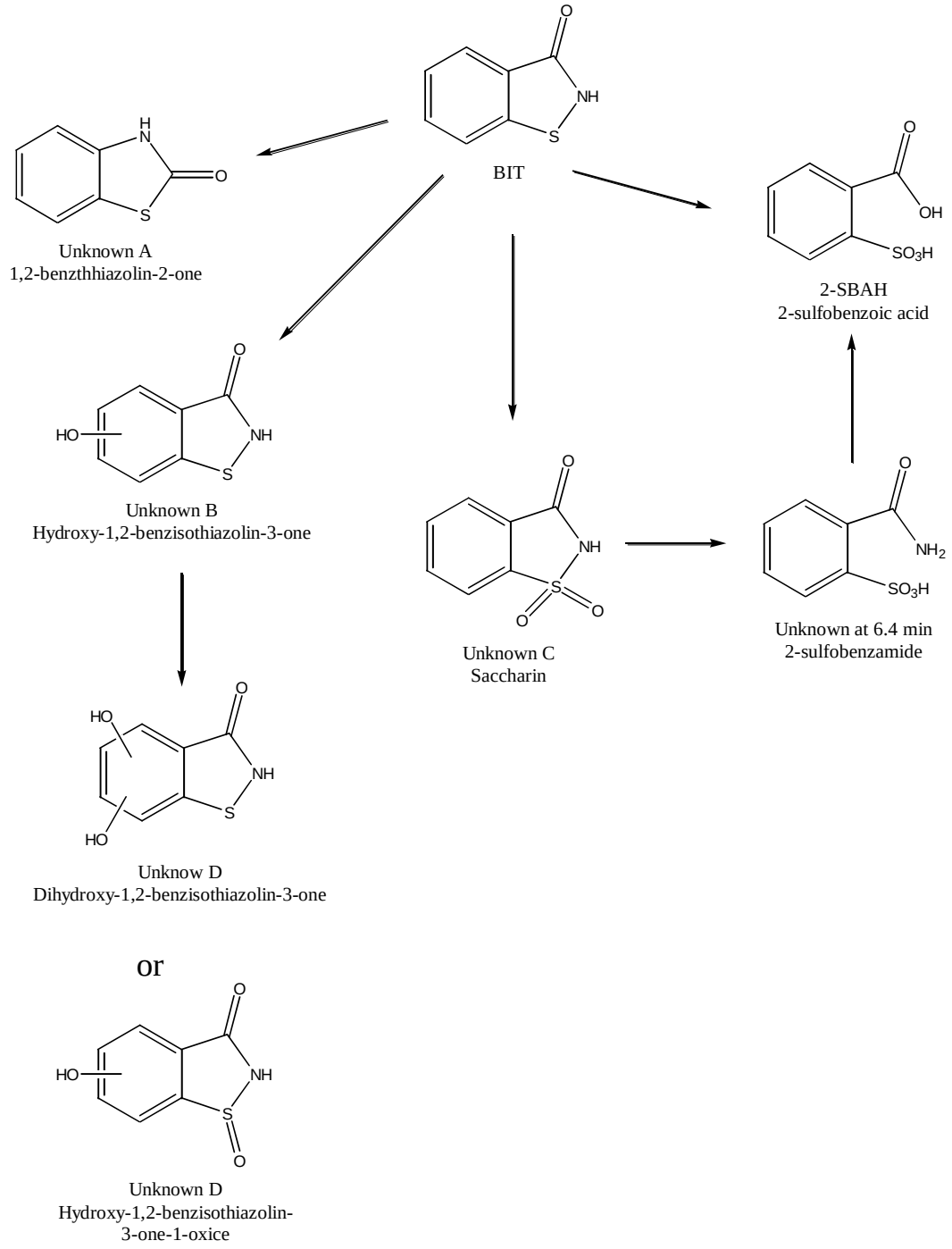


**Figure A7.1.1.1.2-5: Aqueous Photolytic Degradation Pathway of BIT**



**Section A7** **Ecotoxicological Profile Including Environmental Fate and Behaviour**

**Subsection A7.1** **Fate and Behaviour in Water**

**Subsection A7.1.1** **Degradation, initial studies**

**Subsection A.7.1.1.1** **Abiotic**

**Subsection A.7.1.1.2.1/01** **READY BIODEGRADABILITY**

**IIA VII 7.6.1.2**

**Official use only**

## 1 REFERENCE

**1.1 Reference**

**A7.1.1.2.1/01:** [redacted] (2006) 1,2-Benzisothiazolin-3-one: Ready Biodegradability in a $CO_2$ Evolution (Modified Sturm) Test; [redacted] Rohm and Haas Report N° GLP-2006-008 (April 24, 2006), unpublished.

**1.2 Data protection** Yes

1.2.1 Data owner Rohm and Haas Company

1.2.2 Criteria for data protection Data on existing a.s. submitted for the first time in support of the first inclusion into Annex I/IA.

Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive.

## 2 GUIDELINES AND QUALITY ASSURANCE

**2.1 Guideline study** Yes.

OECD No. 301B Ready Biodegradability: $CO_2$ Evolution (Modified Sturm Test), 1992; EU Commission Directive 92/69 EEC, Part C.4-C, Carbon Dioxide ($CO_2$) Evolution (Modified Sturm Test), 1992.

**2.2 GLP** Yes.

**2.3 Deviations** No.

## 3 MATERIALS AND METHODS

**3.1 Test material** 1,2-Benzisothiazolin-3-one (BIT)

3.1.1 Lot/Batch number 220904

3.1.2 Specification As given in section 2.

| | |
|---|---|
| 3.1.3 Purity | Purity : 100 % |
| 3.1.4 Further relevant properties | Solubility in water : > 0.7 g/L<br>Vapor pressure : 2.3 x $10^{-4}$ Pa at 25°C |
| 3.1.5 Composition of Product | Not applicable. |
| 3.1.6 TS inhibitory to microorganisms | In an activated sludge respiration inhibition test (OECD 209), BIT had an NOEC of 1-3 mg/L (see section A7.4.1.4). BIT is a biocidal active substance and as such, inhibitory to microorganisms (see section A5). |
| 3.1.7 Specific chemical analysis | Total inorganic carbon was quantitated by a TOC analyzer (Shimadzu TOC-5000A) equipped with an autosampler. |
| **3.2 Reference substance** | Yes. Sodium Benzoate. |
| 3.2.1 Initial concentration of reference substance | 25.7 mg/L |
| **3.3 Testing procedure** | |
| 3.3.1 Inoculum/ test species | Aerobic activated sludge was obtained from a wastewater treatment facility (ARA Ergolz II, Füllinsdorf, Switzerland) treating primarily domestic wastewater (Table A7.1.1.2.1-1). The sludge was washed twice via centrifugation with tap water and the liquid supernatant phase was decanted. A homogenized aliquot of the final sludge suspension was weighed, thereafter dried and the ratio of wet to dry weight was calculated. Sludge was used at a final concentration of 30 mg dry material per liter. |
| 3.3.1 Test system | The test system is described in Table A7.1.1.2.1-2. |
| 3.3.2 Test conditions | Table A7.1.1.2.1-3 describes the test conditions including the composition of the aqueous mineral salts medium, temperature, pH, and aeration.<br>To each of nine 5 L flasks, approximately 2400 mL of test water containing mineral salts ($KH_2PO_4$, $K_2HPO_4$, $Na_2HPO_4$, $NH_4Cl$, $MgSO_4$, $CaCl_2$, and $FeCl_3$) plus 90 mL of activated sludge inoculum were added. The flasks were aerated overnight with $CO_2$-free air to purge the system of $CO_2$. The morning after purging, 17.9-18.2 mg/L of the test item, BIT (10.0-10.1 mg TOC/L), was added to four flasks. To one of these flask, 10 mg/L of $HgCl_2$ was added (Abiotic control) while to another flask 25.7 mg/L (15 mg OC/L) of the reference item, sodium benzoate, was added (Toxicity control). To 2 procedure control flasks, only sodium benzoate (25.7 mg/L) was added while to 2 additional flasks neither the test substance nor the reference substance was added (Inoculum control). The final |

| | |
|---|---|
| | flask contained only $HgCl_2$ (10 mg/L) (Abiotic control blank). The flasks were made up to a volume of three liters with test water. Inoculum was not added to the abiotic control and the abiotic control blank.<br><br>The test vessels were incubated in a dark room at 20-22 °C. pH of the test flasks solutions was measured on day 0 and again on day 28. The pH measured on Day 0 was between 7.6 and 7.7 and on Day 28 (end of exposure) between 7.6 and 7.8. |
| 3.3.3 Initial TS concentration | 17.9 – 18.2 mg/L (10.0 – 10.1 mg total organic carbon/L) |
| 3.3.4 Duration of test | 28 days (exposure period). |
| 3.3.5 Analytical parameter | $CO_2$ produced from degradation of test substance measured by TOC analyzer. |
| 3.3.6 Sampling | On Days 2, 6, 9, 12, 14, 19, 23, 27, 28, and 29 a five mL sample was withdrawn from each of the first NaOH absorber in series. Additionally on Days 14 and 28 samples were drawn from the second NaOH absorber to correct for any carryover $CO_2$. Total inorganic carbon was quantitated by a TOC analyzer. After sampling on Day 28, 1ml of concentrated HCl was added to each flask and the flask aerated<br><br>overnight to drive off any residual $CO_2$ into absorber allowing for quantitation of dissolved $CO_2$. |
| 3.3.7 Intermediates/ degradation products | Not identified |
| 3.3.8 Nitrate/nitrite measurement | No. |
| 3.3.9 Controls | Toxicity control: 18.2 mg/L BIT (Test item) and 25.7 mg/L Sodium Benzoate (Reference item).<br><br>Procedure control: 25.7 mg/L Sodium Benzoate (Reference item)<br><br>Abiotic control: 18.2 mg/L BIT (test item) poisoned with 10 mg/L $HgCl_2$<br><br>Inoculum control: neither test item nor reference item<br><br>Abiotic control blank: neither test item nor reference item added. Flasks were poisoned with 10 mg/L $HgCl_2$ |
| 3.3.10 Calculations/Statistics | IC content in absorber flask :<br><br>mg IC[1] = IC in absorber x Volume of absorber<br><br>IC removed in analytical samples :<br><br>mg IC in sample = IC in absorber x Volume of sample |

IC produced by Test flask :

mg IC produced = mg IC + ∑mg IC in sample

$$\%deg = \frac{mgICproducedintestflask - mgICproducedinblank}{mgTOC} x100$$

[1] *IC= inorganic carbon*

## 4 RESULTS

### 4.1 Degradation of test substance

4.1.1 Graph

The percent biodegradation for flasks containing BIT (2 replicate flask), sodium benzoate (2 replicate flask), BIT + sodium benzoate, and BIT + $HgCl_2$ is presented in Table A7.1.1.2.1-4 and Figure A7.1.1.2-1.

The percent biodegradation of the test item was calculated based on a total carbon content (TOC) of 0.56 mg C/mg BIT. The $CO_2$ produced in flask containing only BIT was slightly less than that of the inoculum controls (no additions). Consequently BIT was not ready biodegradable under the test conditions within 28 days.

In the abiotic control (BIT + $HgCl_2$) no significant degradation was observed at the end of the 28 day test period (i.e. <10% of the TOC).

The percent biodegradation of the reference item was based on total carbon content of 0.58 mg C/mg sodium benzoate. The reference item was degraded by an average of extent of 78% by day 14 thus confirming the suitability of the activated sludge (>60% by Day 14). By Day 28 the sodium benzoate was biodegraded to an average extent of 85%.

The extent of biodegradation of sodium benzoate in the presence of BIT was slightly delayed over the course of the experiment compared to sodium benzoate alone.

4.1.2 Degradation

% degradation = $\frac{\text{mg } IC_{prod} \text{ in the test flask - mg } IC_{prod} \text{ in blank}}{\text{mg TOC}} \times 100$

| Flask Description | % degradation at the end of incubation (mean) |
|---|---|
| Test item[1] | -19.0 |
| Procedure control (Sodium Benzoate) [1] | 85.4 |
| Toxicity control[1] | 35.8 |
| Abiotic control[2] | 2.4 |

[1] Corrected for the inoculum controls

[2] Corrected for the abiotic blank

4.1.3 Degradation of TS in abiotic control

Degradation of BIT in abiotic control corresponds to approximately 3 %.

4.1.4 Degradation of reference substance

See Figure A7.1.1.2-1.

4.1.5 Intermediates/ degradation products

Not applicable.

## 5 APPLICANT'S SUMMARY AND CONCLUSION

**5.1 Materials and methods**

BIT was investigated for its ready biodegradability in a 28-day $CO_2$ Evolution (Modified Sturm) test according to EU Commission Directive 92/69/EEC C.4-C (1992) and OECD Guideline for testing of Chemicals N° 301 B: Ready Biodegradation: $CO_2$ Evolution (Modified Sturm Test), 1992.

To each of nine 5 L flasks, 2400 to 3000 ml of test water containing mineral salts ($KH_2PO_4$, $K_2HPO_4$, $Na_2HPO_4$, $NH_4Cl$, $MgSO_4$, $CaCl_2$, and $FeCl_3$) plus 90 ml of activated sludge inoculum were added. The flasks were aerated overnight with $CO_2$-free air to purge the system of $CO_2$. The morning after purging, 17.9-18.2 mg/L of the test item, BIT, was added to four flasks. To one of these flask, 10 mg/L of $HgCl_2$ was added (abiotic control) while to another flask 25.7 mg/L of the reference item, sodium benzoate, was added. To 2 procedure control flasks, only sodium benzoate (25.7 mg/L) was added while to 2 additional flasks neither the test substance nor the reference substance was added. The final flask contained only $HgCl_2$ (10 mg/L). The flasks were made up to a volume of three liters. Two 0.05 M NaOH traps were connected in series to the exit air line of each test flask. The flasks were incubated in the dark at 20-22°C.

On Days 2, 6, 9, 12, 14, 19, 23, 27, 28, and 29 a five ml sample was

| | | |
|---|---|---|
| | | withdrawn from each of the first NaOH absorber in series. Additionally on Days 14 and 28 samples were drawn from the second NaOH absorber to correct for any carryover $CO_2$. Total inorganic carbon was quantitated by a TOC analyzer. After sampling on Day 28, 1 mL of concentrated HCl was added to each flask and the flask aerated overnight to drive residual $CO_2$ into absorber allowing for quantitation of dissolved $CO_2$. |
| **5.2** | **Results and discussion** | The test item, BIT, was found to be not ready biodegradable under the test conditions within 28 days.<br>In the abiotic control containing BIT and $HgCl_2$, no significant degradation was noted at the end of the 28-day exposure period (<10 %). In the toxicity control containing both BIT and the reference item sodium benzoate, biodegradation was slightly delayed over the course of the experiment compared to sodium benzoate alone.<br>In the procedure controls, sodium benzoate was degraded to an average extent of 78 % by exposure day 14, confirming suitability of the activated sludge. By the end of the test, the reference item was degraded 85%. |
| **5.3** | **Conclusion** | BIT was found to be not biodegradable under the tests conditions within 28 days. However testing biocides for ready biodegredability may not be relevant since biocides which are toxic to the inoculum may give false negative test results which may lead to requirements for further tests. |
| 5.3.1 | Reliability | 1-valid without restrictions. |
| 5.3.2 | Deficiencies | No. |

| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *November 2010* |
| **Materials and Methods** | *3.3. Testing procedure*<br>*3.3.1. Inoculum test/species: heading Table A7.1.2.3./01-1 should be A7.1.1.2.1-1*<br>*3.3.2. Test system: heading Table A7.1.2.3./01-2 should be A7.1.1.2.1-2*<br>*3.3.3. Test conditions: heading Table A7.1.2.3./01-3 should be A7.1.1.2.1-3* |

| | |
|---|---|
| **Results and discussion** | *Applicant's version is accepted, but with the following comments:*<br>*The percentage of biodegradation shows a negative biodegradation rate, compared to the inoculum control.* |
| **Conclusion** | *BIT was found to be not biodegradable under the tests conditions within 28 days.*<br>*BIT at the concentration used to fulfill the requirements of test OECD 301B seems to be toxic to the inoculum.*<br>*In the toxicity control, containing both 1,2-Benzisothiazolin-3-one and the reference item sodium benzoate, no inhibitory effect on the biodegradation of the reference item was determined. Thus 1,2-Benzisothiazolin-3-one had no inhibitory effect on the activity of activated sludge microorganisms at the tested concentration of 18 mg/l.* |
| **Reliability** | *2* |
| **Acceptability** | *Acceptable* |
| **Remarks** | |

**Table A7.1.2.3/01-1: Inoculum**

| Criteria | Details |
|---|---|
| Nature | Activated sludge |
| Source | Wastewater treatment plant treating predominantly domestic wastewater |
| Sampling site | ARA Ergolz II, Füllinsdorf, Switzerland |
| Preparation of inoculum | Sludge was washed twice with tap water by centrifugation and the supernatant liquid phase decanted. |
| Pretreatment | Sludge was added to mineral salt solution and aerated with $CO_2$ free air overnight prior to addition of test compound |
| Concentration | 30 mg of washed sludge on a dry weight basis/L |

**Table 7.1.2.3/01-2: Test System**

| Criteria | Details |
|---|---|
| Number and Nature of Culture Flask | Nine 5L flask were dosed as below.<br>Identification / mg/L Test Item / mg/L Reference Item / mg/L $HgCl_2$ / Inoculum Added<br>Test Flask / 18.0 / / / +<br>Test Flask / 17.9 / / / +<br>Abiotic Control / 18.2 / / 10 / -<br>Toxicity Control / 18.2 / 25.7 / / +<br>Ref. Control / / 25.7 / / +<br>Ref. Control / / 25.7 / / +<br>Inoculum Control / / / / +<br>Inoculum Control / / / / +<br>Abiotic Blank / / / 10 / - |
| Aeration Device | $CO_2$-free air is passed through the 5 liter flask and into traps at a rate of 30-100 mL/min. |
| Measuring equipment | TOC analyzer (Shimadzu TOC-5000A) |
| Trapping System | From the exit line of each flask, two 0.05 M NaOH traps were placed in series to capture evolved $CO_2$. At sampling, 5 ml aliquots were taken from the first trap for assaying. On Day 15 and 28 a 5 mL aliquot was also taken from the second NaOH trap to correct for carry-over. |
| Test performed in closed vessels due to significant volatility of test substance | No |

**Table A7.1.2.3/01-3: Test Conditions**

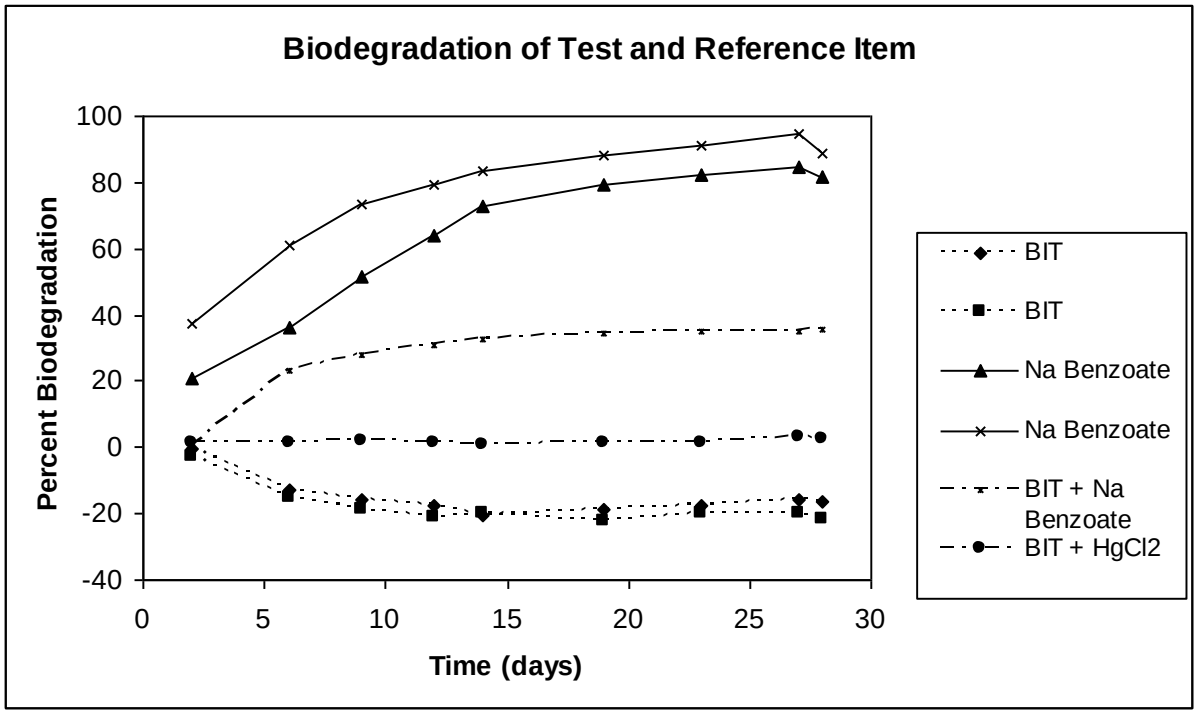
| Criteria | Details |
|---|---|
| Composition of test medium | Stock solutions using analytical grade salts<br>a) $KH_2PO_4$: 8.50 g/L<br>$K_2HPO_4$: 21.75 g/L<br>$Na_2HPO_4 \cdot 2H_2O$ 33.40 g/L<br>$NH_4Cl$: 0.50 g/L<br>b) $MgSO_4 \cdot 7H_2O$: 22.50 g/L<br>c) $CaCl_2 \cdot 2H_2O$: 36.40 g/L<br>d) $FeCl_3 \cdot 6H_2O$: 0.25 g/L<br>**One drop of concentrated HCl was added to solution d) as a preservative.**<br>The final testing solution was prepared by adding 10 mL of solution a) and 1 ml of solutions b), c), and d) to 800 ml of purified water. The solution was then made up to 1000 mL with purified water and the pH adjusted to 7.4 with dilute HCl. |
| Inoculum | The day before the addition of BIT, 90 mL of activated sludge inoculum was added to between 2400-3000 mL of the mineral salt test medium. |
| Additional substrates | No |
| Test temperature | 20-22°C (temperature controlled room) |
| pH | At the start the pH in the test samples ranged from 7.6-7.7. At termination, the pH ranged from 7.6-7.8 |
| Aeration of dilution water | The test solutions were aerated through out the study using $CO_2$-free air |

**Table A7.1.2.3/01-4: Biodegradation of 1,2-Benzisothiazolin-3-one (BIT, Test Compound) and Sodium Benzoate (Reference Compound)**

| Time (days) | Percent Biodegradation[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Test Flask (BIT) | | | Reference Flask (Sodium Benzoate) | | | Toxicity Control[b] | Abiotic Control[c] |
| | 1 | 2 | Mean | 1 | 2 | Mean | | |
| 2 | -0.5 | -2.8 | -1.7 | 20.7 | 37.1 | 28.9 | 0.0 | 1.2 |
| 6 | -12.9 | -14.9 | -13.9 | 36.2 | 61.1 | 48.6 | 23.4 | 1.2 |
| 9 | -15.5 | -18.8 | -17.1 | 51.6 | 73.2 | 62.4 | 27.8 | 1.7 |
| 12 | -17.6 | -21.3 | -19.5 | 64.0 | 79.5 | 71.8 | 30.6 | 1.1 |
| 14 | -20.4 | -20.0 | -20.2 | 73.0 | 83.3 | 78.2 | 32.6 | 0.6 |
| 19 | -19.0 | -22.5 | -20.7 | 79.4 | 88.0 | 83.7 | 34.2 | 1.3 |
| 23 | -17.5 | -20.0 | -18.7 | 82.4 | 91.0 | 86.7 | 35.3 | 1.4 |
| 27 | -16.0 | -19.9 | -17.9 | 84.4 | 94.5 | 89.5 | 34.9 | 3.1 |
| 28 | -16.4 | -21.5 | -19.0 | 81.5 | 88.9 | 85.4 | 35.8 | 2.4 |

[a] Values corrected for inoculum control or abiotic blank as appropriate
[b] Toxicity control contains BIT and sodium benzoate.
[c] Abiotic control contains BIT and $HgCl_2$.

**Figure A7.1.1.2.1-1: Biodegradation of the test item and the reference item during incubation period**



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**Subsection A.7.1.1.1** **Abiotic**

**Section A7.1.1.2.1/02** **Ready Biodegradability**

**Annex Point IIA7.6.1.1**

| | | | Official use only |
|---|---|---|---|
| | | **1 REFERENCE** | |
| **1.1** | **REFERENCE** | **A7.1.1.2.1/02** [redacted] (2007) $^{14}$C-BIT: Assessment of ultimate biodegradation at a non-biocidal concentration under the conditions of a "ready" biodegradation test, [redacted] Rohm and Haas Technical Report N° TR-07-018 (19 July 2007), Unpublished. | |
| **1.2** | **DATA PROTECTION** | Yes | |
| 1.2.1 | Data owner | Rohm and Haas Company | |
| 1.2.2 | Companies with letter of access | | |
| 1.2.3 | Criteria for data protection | Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into AnnexI/IA.<br>Data protection claimed in accordance with Article 12.1(c)(ii), as data generated after the entry into force of the Directive. | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **GUIDELINE STUDY** | Yes. OECD No. 301B, Ready Biodegradability, $CO_2$ Evolution (Modified Sturm Test) | |
| **2.2** | **GLP** | Yes | |
| **2.3** | **DEVIATIONS** | No | |
| | | **3 MATERIALS AND METHODS** | |
| **3.1** | **TEST MATERIAL** | $^{14}$C-BIT (1,2-benzisothiazolin-3-one) | |

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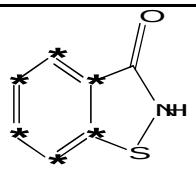
| | |  <br>* $^{14}$C label position |
| 3.1.1 | Lot/Batch number | Lot Number 1077.00 |
| 3.1.2 | Purity | Radiopurity = 97.7%; specific activity – 163.79 mCi/g |
| 3.1.3 | Further relevant properties | Water solubility is > 0.7 ppm<br>Vapor pressure = 2.3 x $10^{-4}$Pa at 25°C |
| 3.1.4 | TS inhibitory to microorganisms | Yes. Therefore, $^{14}$C-bit was employed as an attempt to obtain concentrations less than the minimal inhibitory concentration. |
| **3.2** | **REFERENCE SUBSTANCE** | |
| 3.2.1 | Sodium Benzoate | Sodium benzoate was employed as a reference compound for the test system. The dosing concentration was 15 mg of carbon/L (25.7 mg sodium benzoate/L) |
| 3.2.2 | $^{12}$C-BIT | Non-radiolabeled BIT ($^{12}$C-BIT) was from Rohm and Haas Company. The material lot number was 060309/1 (subsequently renamed MJB3738 by sponsor) and the purity was 100.1%. |
| **3.3** | **TESTING PROCEDURE** | |
| 3.3.1 | Inoculum | The details of the inoculum appear in Table A7.1.1.2.1/02-1. |
| 3.3.2 | Preparation of Solutions | <u>BIT</u><br>For the preliminary tests, an aqueous stock solution of $^{12}$C-BIT was prepared by adding 37.52 mg and making up to 250 mL |

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| | with water (final concentration, 150 mg/L). The required test concentration was achieved by addition of the appropriate volume of this stock solution to the test vessels<br><br>For the main test an aqueous stock solution of $^{14}$C-BITwas prepared by dissolving 4.580 mg in 50 mL water. The test vessels were dosed with 9.8 mL (0.971043 mg) of the stock solution resulting in a radioassayed vessel concentration of 0.3237 mg/L. For the toxicity controls, a $^{12}$C-BIT stock solution was prepared by dissolving 37.48 mg in 250 ml water and adding 6.25 mL to the appropriate vessel.<br><br><u>Sodium Benzoate</u><br><br>A stock solution of the reference compound was prepared by adding 3.859 g of sodium benzoate and making up to 1 liter using reverse-osmosis water. The reference and toxicity control vessels were dosed with 20 mL of this solution to give a nominal concentration of 15 mg carbon/L (25.7 mg sodium benzoate/L). | |
| 3.3.3 Preliminary Test | | |
| 3.3.3.1 Preliminary test 1 | The purpose of preliminary test 1 was to examine the effect of varying concentrations of $^{12}$C-BIT on viable cell counts and on the biodegradation of sodium benzoate. Two treatment vessels were prepared as controls containing only the mineral salt medium (Table A7.1.1.2.1/02-3) and two were references containing the mineral salt medium and sodium benzoate at 15 mgC/L. There were 5 toxicity controls identical to the references except that $^{12}$C-BIT was added at the following nominal concentrations; 0.313 mg/L, 0.625 mg/L, 1.25 mg/L, 2.5 mg/L, and 5 mg/L. All vessels were fitted with three 0.0125M $Ba(OH)_2$ traps and quantitation involved titration of the trap contents. Total viable cell counts were performed on Days 7 and | |

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**Annex Point IIA7.6.1.1**

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| | 14. The test duration was 14 days after which the cultures were acidified and purged for 1 day to remove dissolved $CO_2$ |
| 3.3.3.2 Preliminary test 2 | The purpose of preliminary test 2 was to examine the effect of varying concentrations of $^{12}$C-BIT on the respiration of standard cell cultures. Two treatment vessels were prepared as controls containing only the mineral salt medium (Table A7.1.1.2.1/02-3) and there were 5 test vessels containing the mineral salt medium and $^{12}$C-BIT at the following nominal concentrations; 0.313 mg/L, 0.625 mg/L, 1.25 mg/L, 2.5 mg/L and 5 mg/L. All vessels were fitted with three 0.125M $Ba(OH)_2$ traps which were quantitated by titration. The test duration was 9 days after which the cultures were acidified and purged for 1 day to remove dissolved $CO_2$. |
| 3.3.4 Main Test | |
| 3.3.4.1 Test system | The test system is described in Table A7.1.1.2.1/02-2. |
| 3.3.4.2 Test conditions | Table A7.1.1.2.1/02-3 describes the test conditions including the composition of the aqueous media, inoculum, temperature, pH and aeration. |
| 3.3.4.3 Initial Test Substance concentration | The initial nominal concentration of $^{14}$C-BIT was 0.313 ppm and the radioassayed (actual) concentration was 0.3237 mg/L |
| 3.3.4.4 Duration of test | The exposure period was 28 days. After sampling on Day 28 1 mL of concentrated HCl was added to every vessel except the two test vessels containing $^{14}$C-BIT. The vessels were aerated overnight to drive dissolved $CO_2$ into the alkali traps prior to final analysis. The two test vessels were not acidified to avoid metabolite artifacts as these solutions were being retained for additional chromatographic analysis. |

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| 3.3.4.5 Chemical and biochemical methods | Liquid scintillation spectrometry was employed to quantitate the $^{14}CO_2$ trapped in the NaOH traps.<br><br>$^{12}CO_2$ in the $Ba(OH)_2$ trapping solutions was quantitated by titration with standard HCl (0.05M) using phenolphthalein as an indicator. Titrations were performed on 20 mL aliquots until two matching (± 0.1 mL) titers were obtained<br><br>Inorganic carbon concentration of the inoculated salts medium was determined using a carbon analyzer. The sample is acidified with $H_3PO_4$, sparged with $CO_2$-free air, and quantitated by a non-dispersive infrared detector.<br><br>Air flow through the systems was measured weekly, adjusting if necessary, to maintain a flow rate of approximately 50 ml/min. This was accomplished with a bubble flow meter and a stopwatch.<br><br>Total viable cell counts in Preliminary Study 1 were determined on Day 7 and 14 by removing duplicate 0.1 mL aliquots from the test vessels and making $10^{-1}$ and $10^{-2}$ dilutions with phosphate buffer. The original solution and the dilutions were plated on a nutrient agar plate at 37°C for 2 days and subsequently scored manually.<br><br>Aliquots from the Test Flasks (dosed with $^{14}$C-BIT) were analyzed by HPLC using a modified C-18 column and a binary gradient composed of 0.5% aqueous formic acid and 0.5% methanolic formic acid. Detection employed a $^{14}$C-flow through monitor and/or UV detector (254 nm). |
| 3.3.4.6 Sampling | On Days 1, 3, 6, 8, 10, 13, 15, 16, 20, 22, 24, 28, and 29 the trap nearest the test vessel was removed for quantitation. The remaining two bottles in the series were moved up towards the test vessel and a fresh trap placed on the end of the series. Aliquots of the trapping solution were either radioassayed ($^{14}CO_2$) or titrated ($^{12}CO_2$). On Day 28, 1 mL of concentrated HCl was added to each culture vessel |

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| | (except the 2 containing $^{14}$C-BIT) and the flask aerated overnight to drive residual $CO_2$ into the traps thus accounting for dissolved $CO_2$. |
| 3.3.4.7 Intermediates/ degradation products | The test vessels containing $^{14}$C-BIT were chromatographed (HPLC ). |
| 3.3.4.8 Nitrate/nitrite measurement | No |
| 3.3.4.9 Controls | Toxicity Control: 0.313 mg $^{12}$C-BIT/L plus 25.7 mg sodium benzoate/L<br>Reference Control: 25.7 mg sodium benzoate/L<br>Inoculum Control: no BIT or sodium benzoate<br>Additional details are in Table A7.1.1.2.1/02-2. |
| 3.3.5 Calculations/ Statistics | The percent biodegradation was calculated as follows:<br>Percent Biodegradation $= \frac{\text{cumulative } CO_2 (\text{mg})}{\text{theoretical cumulative } CO_2 \text{ (mg)}} x100$<br>or<br>Percent Biodegradation $= \frac{\text{cumulative dpm}}{\text{total applied dpm}} \text{ x } 100$<br>where theoretical $CO_2$ =<br>mg of reference substance added x<br>percent of carbon content of the reference material x<br>3.667 (the weight (mg) of $CO_2$ produced from 1 mg of carbon) |
| | **4 RESULTS** |

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**Subsection A.7.1.1.1** **Abiotic**

**Section A7.1.1.2.1/02** **Ready Biodegradability**

**Annex Point IIA7.6.1.1**

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| **4.1** | **PRELIMINARY TEST** | | |
| 4.1.1 | Preliminary Test 1 | The purpose of Preliminary Test 1 was to examine the effect of varying concentrations of $^{12}$C-BIT on viable cell counts and on the biodegradation of sodium benzoate. Results from the total viable cell counts appear in Table A7.1.1.2.1/02-4. These results show that the microbial population was not reduced at any concentration of BIT applied and in fact increased with increasing concentration of BIT.<br><br>Biodegradation of sodium benzoate in the presence of BIT was only suppressed at the highest concentration, 5 mg BIT/L (Table A7.1.1.2.1/02-5). | |
| 4.1.2 | Preliminary Test 2 | The purpose of preliminary test 2 was to examine the effect of varying concentrations of $^{12}$C-BIT on the respiration of standard cell cultures. The results in Table A7.1.1.2.1/02-6 show that at BIT concentrations of 0.313 mg/L, 0.625 mg/L and 0.1.25 mg/L $CO_2$ evolution was similar to vessels with no added BIT. | |
| **4.2** | **Main Test** | | |
| 4.2.1 | Test Parameters | Based on the results of the preliminary tests, the main test was dosed at a nominal $^{14}$C-BIT concentration of 0.313 mg/L (actual $^{14}$C-BIT concentration was 0.3237 mg/L).<br><br>The inorganic carbon content of the inoculated mineral salts medium was 0.59 mg carbon/L culture solution, or 3.96% of the carbon loading from the addition of sodium benzoate.<br><br>The pH on Day 0 of the main test ranged from 7.40 – 7.56 and on Day 28, 7.22 -7.40. | |
| 4.2.2 | Biodegradation | A summary of the biodegradation results for the test compound | |

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| | $^{14}$C-BIT dosed at 0.313 mg/L, for the sodium benzoate reference control, and for the toxicity control (sodium benzoate plus 0.313 mg/L BIT) are presented in Table A7.1.1.2.1/02-7. Additionally the results are presented graphically in Figure A7.1.1.2.1/02-1.<br><br>After an initial lag phase of 8 days, biodegradation of $^{14}$C-BIT progressed steadily accounting for about 10% by Day 11. From Day 13 onward, the rate slowed reaching 20.1% on Day 16 and 23.8% at the end of the study. The maximum divergence between replicates was 0.5% on Day 20.<br><br>To be considered readily biodegradable the test substance must achieve 60% biodegradation by the end of the study and that 60% must be reached within 10 days of obtaining 10%. Figure A7.1.1.2.1/02-2 graphically shows the biodegradation of the test flasks with a 10-day window superimposed. This graphically demonstrates that BIT cannot be considered to be ready biodegradable.<br><br>The reference controls containing sodium benzoate rapidly evolved $CO_2$ reaching 64% by Day 8. Thereafter the rate slowed reaching 82% on Day 16 at which time the rate began to plateau. On Day 28, biodegradation level was 88%. The validity requirement is that biodegradation of sodium benzoate exceed 60% by Day 14, which was achieved.<br><br>The toxicity control measured the mineralization of sodium benzoate in the presence of BIT. BIT at 0.313 mg/L did not suppress the microbial degradation and thus the mineralization of sodium benzoate. The level of sodium benzoate biodegradation at study termination, 88%, was essentially the same as the reference control. | |
| 4.2.3 Abiotic Degradation | Abiotic vessels were not included because they had been examined in an earlier study (A7.1.1.2.1/01). Vessels dosed with | |

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| | BIT and $HgCl_2$ showed essentially no biodegradation. | |
| 4.2.4 Material Balance | The distribution of radioactivity and material balance are presented in Table A7.1.1.2.1/02-8. About 70% of the applied radioactivity was detected in the culture solution and about 24% in the NaOH traps. A wash of the culture vessels collected less than 0.5%. Recovery of applied radioactivity was about 95% which is an acceptable result. | |
| 4.2.5 Quantitation of Parent and Characterization of biodegradates- | Day 28 aliquots from the Test Flask (containing $^{14}$C-BIT) were examined by HPLC. No BIT was present and there were two major metabolites comprising about 22% and 49% of the applied activity. These results indicate that while BIT is not ready biodegradable, it is rapidly biodegraded. Assuming 100% BIT on Day 0 and 0.01% on Day 28, the half-life of BIT in this system is about 2.1 days. | |
| | **5 APPLICANT'S SUMMARY AND CONCLUSION** | |
| **5.1 MATERIALS AND METHODS** | This study employed OECD 301 B Ready Biodegradability, $CO_2$ Evolution (Modified Sturm Test).<br><br>Flasks containing mineral salts solution ($KH_2PO_4$, $K_2HPO_4$, $Na_2HPO_4$, $NH_4Cl$, $MgSO_4$, $CaCl_2$, and $FeCl_3$) plus activated sludge inoculum were prepared. Preliminary studies were performed to examine the effect of varying concentrations of BIT (0.313 mg/L to 5 mg/L) on microbial cell viability, biodegradation of sodium benzoate, and respiration in mineral salt solution.<br><br>In the main test, besides control flasks containing just the mineral salt solution there were flasks containing 0.313 mg $^{14}$C-BIT, flask containing sodium benzoate, and a flask containing sodium benzoate and BIT. All vessels were aerated and purged | |

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| | | with $CO_2$-free air. Evolved $^{14}CO_2$ from the test flasks and a set of controls was trapped in NaOH while $^{12}CO_2$ from the reference flasks, toxicity flask, and a set of control flasks were trapped in $Ba(OH)_2$. The flasks were incubated in the dark at 22 ± 2°C. On Days 1, 3, 6, 8, 10, 13, 15, 16, 20, 22, 24, 28 and 29 the traps were refreshed and aliquots of the solutions were removed for quantitation by either liquid scintillation spectroscopy or titration. On Day 28, aliquots from the Test Flask containing $^{14}$C-BIT were examined by HPLC. | |
| **5.2** | **RESULTS AND DISCUSSION** | BIT cannot be considered to be ready biodegradable, as it did not achieve 60% biodegradation to $CO_2$. Biodegradation plateaued at about 23-24% around Day 20. Sodium benzoate biodegradation was rapid and exceeded 60% by Day 8 demonstrating that the activated sludge culture was viable. BIT had no observable effect on the biodegradation of sodium benzoate since there was no observable difference in the biodegradation of sodium benzoate in the absence or presence of BIT. Chromatography of Day 28 solutions from the Test Flaks demonstrated that no BIT was still present in solution. The half-life of BIT in this system is about 2.1 days. Thus, while BIT is not ready biodegradable, it does rapidly biodegrade. | |
| **5.3** | **CONCLUSION** | This study fulfills the requirements and demonstrates that BIT cannot be considered to be readily biodegradable. However it can be considered to rapidly biodegrade since the half-life is about 2.1 days. | |
| 5.3.1 | Reliability | 1-valid without restrictions. | |
| 5.3.2 | Deficiencies | None. | |

| **Evaluation by Competent Authorities** | |
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| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
|---|---|
| **Date** | *March 2015.* |
| **Materials and Methods** | *Applicant's version is accepted with the following comments:*<br>*3.3.5. The test substance was tested a non-biocidal concentration (0,313 mg/L), and it is a non-biocidal concentration. According to the study report, this low concentration is employed because the substance is known to be inhibitory to the test systems routinely employed to assess biodegradation.* |
| **Results and discussion** | *Applicant's version is accepted with the following comments:*<br>*5.2. Preliminary test 1: Total viable cell count data at day 7 and day 14. The variability between replicates is too high to conclude than the cell density clearly increased with increased concentrations of BIT.* |
| **Conclusion** | *$^{14}$C-BIT cannot be considered to be readily biodegradable. Although $^{14}$C-BIT has failed to qualify for classification as readily biodegradable under the conditions employed in this study, based on the chromatography of the test solutions BIT does degrade rapidly.* |
| **Reliability** | *2* |
| **Acceptability** | *Acceptable* |
| **Remarks** | |

**Table A7.1.1.2.1/02-1: Inoculum**

| Criteria | Details |
|---|---|
| Nature | Activated sludge |
| Source | Return line of a sewage treatment works treating primarily domestic wastewater |
| Sampling site | Burley Menston Sewage Treatment Works, West Yorkshire, UK) |
| Preparation of inoculum | Sludge was blended and aerated. The suspended solids concentration was determined by filtration, oven drying the filtrate, and the weight of the dry sludge measured. |
| Pretreatment | The mineral salt medium was inoculated with activated sludge at 90 mg solid/L to provide a final solids concentration of 30 mg/L in each vessel. The solution was aerated with $CO_2$ free air overnight prior to addition of test compound |
| Concentration | 30 mg of sludge on a dry weight basis/L |

**Table 7.1.1.2.1/02-2: Test System for the Main Biodegradation Test**

| Criteria | Details |
|---|---|
| Composition of Culture Flask | Nine 3000 mL flask were dosed as below. |

| Identification | mg/L $^{14}$C-BIT | mg/L $^{12}$C Sodium Benzoate | mg/L $^{12}$C-BIT |
|---|---|---|---|
| Control ($^{12}$C) | | | |
| Control ($^{12}$C) | | | |
| Control ($^{14}$C) | | | |
| Control ($^{14}$C) | | | |
| Reference | | 25.7 | |
| Reference | | 25.7 | |
| Toxicity Control | | 25.7 | 0.313 |
| Test | 0.313[1] | | |
| Test | 0.313[1] | | |

| | |
|---|---|
| Aeration Device | $CO_2$-free air is passed through the flasks and into traps. |
| Measuring equipment | Evolved $^{14}CO_2$ measured by liquid scintillation spectrometry and $^{12}CO_2$ by titration with HCl using a phenolphthalein indicator |
| Trapping System | From the exit line of each flask dosed with $^{14}$C-BIT, three 0.0125M NaOH traps were placed in series to capture evolved $^{14}CO_2$. An identical procedure was employed for vessels dosed with $^{12}$C sodium benzoate except that 0.0125M $Ba(OH)_2$ was used instead of NaOH to capture evolved $^{12}CO_2$. |
| Test performed in closed vessels due to significant volatility of test substance | No |

[1] 0.313 mg $^{14}$C BIT/L was the nominal dose. Radioassayed concentration was 0.3237 mg $^{14}$C-BIT/L

**Table A7.1.1.2.1/02-3: Test Conditions**

| Criteria | Details |
|---|---|
| Composition of test medium | Stock solutions using analytical grade salts<br>a) $KH_2PO_4$: 8.50 g/L<br>$K_2HPO_4$: 21.75 g/L<br>$Na_2HPO_4 \cdot 2H_2O$: 33.40 g/L<br>$NH_4Cl$: 0.50 g/L<br>b) $CaCl_2 \cdot 2H_2O$: 36.40 g/L<br>c) $MgSO_4 \cdot 7H_2O$: 22.50 g/L<br>d) $FeCl_3 \cdot 6H_2O$: 0.25 g/L<br>The salts, a, b, c, and d were dissolved individually and made up to 1 L with water. The final testing solution was prepared containing 30 mL/L of solution a) and 3 mL/L of solutions b), c), and d). |
| Inoculum | The day before the addition of the test and reference substances, the mineral salt test medium was inoculated with activated sludge solids at 90 mg suspended solids/L. 1 liter of this mixture was added to each test vessel followed by 1.5L or 1.9 L of ultra pure water. Based on a volume of 3 L in each test volume at Day 0, the activated sludge solid concentration was 30 mg/L. |
| Additional substrates | No |
| Test temperature | nominal 21 ± 1°C |
| pH (main biodegradation study) | At Day 0 the pH ranged from 7.40 – 7.56. At termination (Day 28) the pH ranged from 7.22 – 7.40. |
| Aeration of dilution water | The test solutions were aerated through out the study using $CO_2$-free air |

**Table A7.1.1.2.1/02-4: Preliminary Test 1—Total Viable Cell Counts**

| Vessel | Mean Total Viable Cells (cells/mL) | |
|---|---|---|
| | Day 7 | Day 14 |
| Control 1<br>Control 2 | 1,042.5<br>840 | 647.5<br>2,150 |
| Reference 1<br>Reference 2 | 720<br>2,775 | 2,717.5<br>1,420 |
| 5 mg BIT/L | 670,250 | 100,625 |
| 2.5 mg BIT/L | 14,225 | 3,285 |
| 1.25 mg BIT/L | 3,525 | 2,482.5 |
| 0.625 mg BIT/L | 1,752.5 | 427.5 |
| 0.313 mg BIT/L | 1,595 | 505 |

**Table A7.1.1.2.1/02-5: Preliminary Test 1—Percent Biodegradation of Sodium Benzoate**

| BIT Concentration (mg/L) | Cumulative Percentage Biodegradation of Sodium Benzoate | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 6 | Day 8 | Day 10 | Day 14 | Day 15 |
| 0 (sodium benzoate only) | 8 | 37 | 47 | 62 | 67 | 72 | 79 | 86 |
| 5 | 0 | 20 | 37 | 61 | 65 | 68 | 72 | 76 |
| 2.5 | 0 | 28 | 43 | 68 | 75 | 80 | 85 | 88 |
| 1.25 | 0 | 32 | 45 | 70 | 77 | 81 | 87 | 91 |
| 0.625 | 3 | 36 | 48 | 68 | 73 | 78 | 84 | 88 |
| 0.313 | 6 | 36 | 46 | 63 | 68 | 73 | 81 | 86 |

**Table A7.1.1.2.1/02-6: Preliminary Test 2—Evolution of $^{12}CO_2$**

| BIT Concentration (mg/L) | Cumulative $CO_2$ Evolution in Vessels (mg) | | | | | |
|---|---|---|---|---|---|---|
| | Day 1 | Day 3 | Day 6 | Day 8 | Day 9 | Day 10 |
| 0 (control medium) | 4.2 | 13.5 | 26.2 | 35.9 | 42.4 | 51.6 |
| 5 | 3.8 | 9.5 | 17.3 | 22.7 | 26.6 | 32.8 |
| 2.5 | 4.0 | 11.2 | 20.5 | 27.7 | 33.1 | 41.8 |
| 1.25 | 4.2 | 12.4 | 24.5 | 34.3 | 41.1 | 51.2 |
| 0.625 | 4.3 | 13.0 | 25.4 | 34.8 | 41.1 | 50.7 |
| 0.313 | 4.6 | 14.0 | 27.1 | 38.0 | 44.9 | 55.9 |

**Table A7.1.1.2.1/02-7: Main Test—Cumulative Percent Biodegradation**

| Time (Days) | Cumulative Percent Biodegradation | | | | | | |
|---|---|---|---|---|---|---|---|
| | Test Vessels ($^{14}$C-BIT) | | | Reference Vessels (Sodium Benzoate) | | | Toxicity Control[1] |
| | 1 | 2 | Mean | 1 | 2 | Mean | |
| 1 | 0 | 0 | 0 | 7 | 7 | 7 | 3 |
| 3 | 0 | 0 | 0 | 44 | 44 | 44 | 42 |
| 6 | 0.2 | 0.2 | 0.2 | 58 | 57 | 58 | 58 |
| 8 | 0.6 | 0.6 | 0.6 | 65 | 64 | 64 | 67 |
| 10 | 6.6 | 7.6 | 7.1 | 70 | 68 | 69 | 72 |
| 13 | 16.0 | 16.3 | 16.2 | 76 | 74 | 75 | 78 |
| 15 | 18.8 | 19.2 | 19.0 | 79 | 77 | 78 | 81 |
| 16 | 19.9 | 20.3 | 20.1 | 83 | 81 | 82 | 84 |
| 20 | 21.8 | 22.3 | 22.1 | 84 | 82 | 83 | 84 |
| 22 | 22.6 | 22.8 | 22.7 | 86 | 84 | 85 | 85 |
| 24 | 23.0 | 23.2 | 23.1 | 87 | 85 | 86 | 86 |
| 28 | 23.7 | 23.8 | 23.8 | 87 | 86 | 87 | 87 |
| 29 | * | * | * | 89 | 88 | 88 | 88 |

[*] Samples saved for chromatographic analysis. Thus they were not acidified and purged overnight to prevent the potential for acid catalyzed metabolite artifacts.

**Table A7.1.1.2.1/02-8: Material Balance**

| Vessel | Percent of Applied Radioactivity | | | |
|---|---|---|---|---|
| | Culture Vessel | Vessel Wash | NaOH Traps | Recovery |
| Test Replicate 1 | 70.5 | 0.3 | 23.7 | 95 |
| Test Replicate 2 | 70.3 | 0.4 | 23.8 | 95 |

**Figure A7.1.1.2.1/02-1: Overview of Biodegradation of BIT, Sodium Benzoate (Reference Vessels), and Toxicity Control (Sodium Benzoate and BIT)**

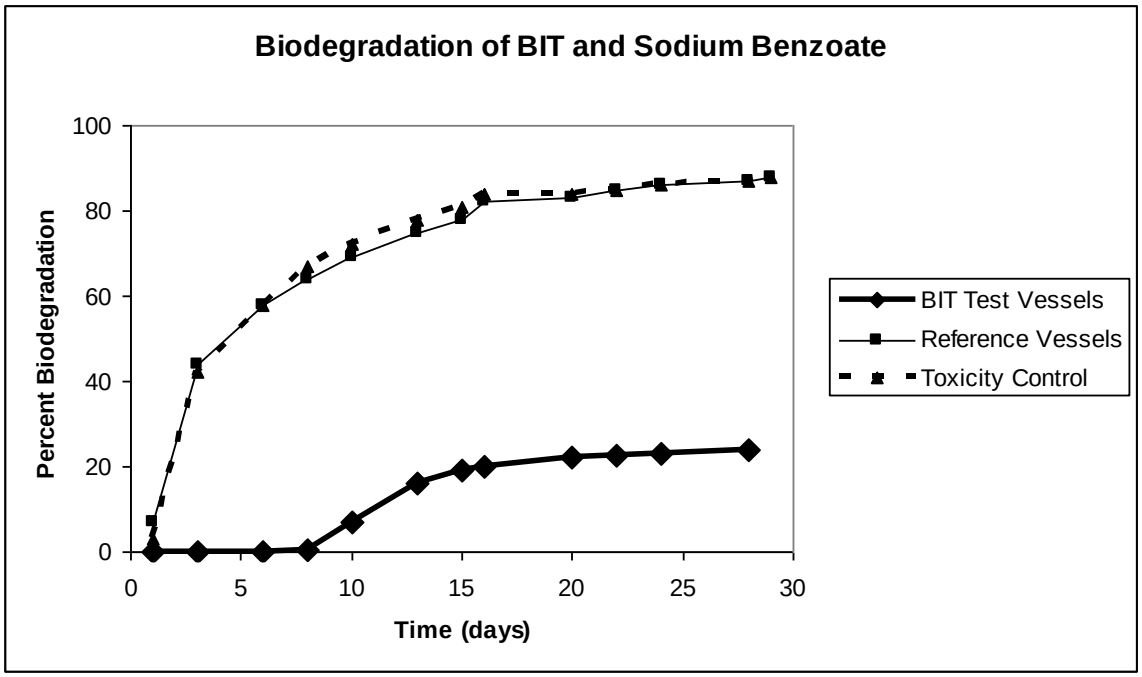
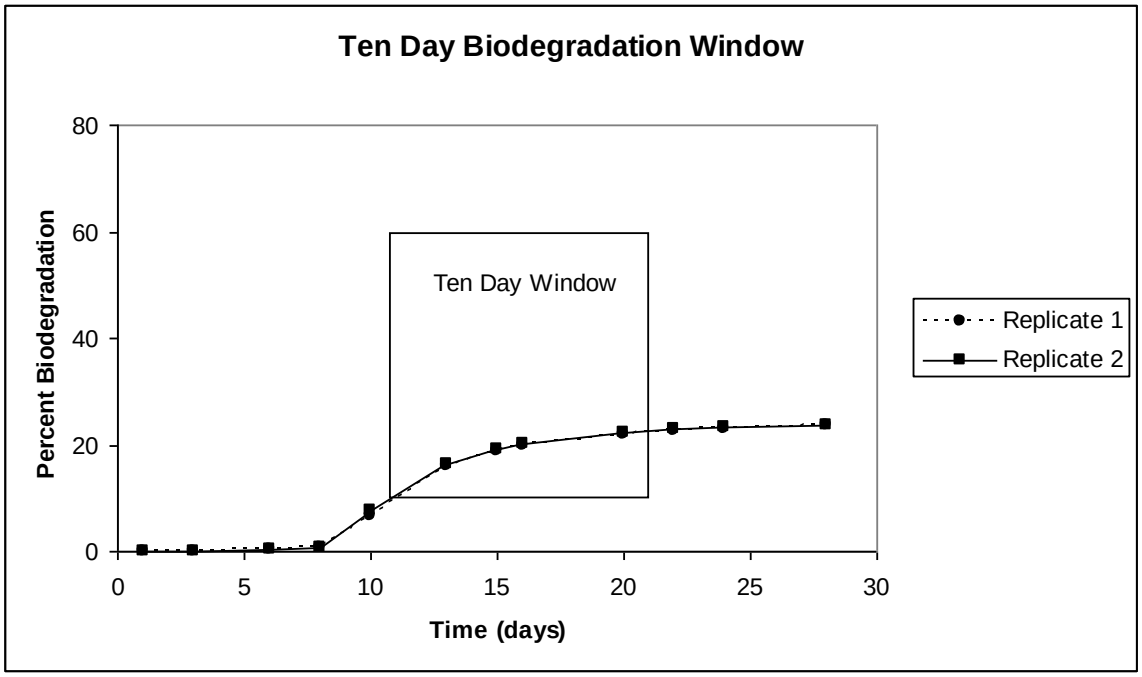


**Figure A7.1.1.2.1/02-2: Ten Day Window for the Biodegradation of $^{14}$C-BIT**



**Section A7** **Ecotoxicological Profile Including Environmental Fate and Behaviour**

**Subsection A7.1** **Fate and Behaviour in Water**

**Subsection A7.1.1** **Degradation, initial studies**

**Subsection A.7.1.1.1** **Abiotic**

**Subsection A7.1.1.2.1/03** **Ready Biodegradability**

**Annex Point IIA7.6.1.1** **1,2-Benzisothiazolin-3-one (BIT)**

| | | **1 REFERENCE** | **Official use only** |
|---|---|---|---|
| **1.1** | **REFERENCE** | **A7.1.1.2.1/03** [redacted] (2007) $^{14}$C-BIT: Assessment of primary biodegradation and biodegradation products at a non-biocidal concentration under the conditions of a "ready" biodegradation test, [redacted], Rohm and Haas Technical Report N° TR-07-037 (August 2007), Unpublished. | |
| **1.2** | **DATA PROTECTION** | Yes | |
| 1.2.1 | Data owner | Rohm and Haas Company | |
| 1.2.2 | Criteria for data protection | Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I/IA.<br>Data protection claimed in accordance with Article 12.1(c)(ii), as data generated after the entry into force of the Directive. | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **GUIDELINE STUDY** | Yes. OECD No. 301B, Ready Biodegradability, $CO_2$ Evolution (Modified Sturm Test) | |
| **2.2** | **GLP** | Yes | |
| **2.3** | **DEVIATIONS** | No | |
| | | **3 MATERIALS AND METHODS** | |
| **3.1** | **TEST MATERIAL** | $^{14}$C-BIT | |

**Section A7** **Ecotoxicological Profile Including Environmental Fate and Behaviour**

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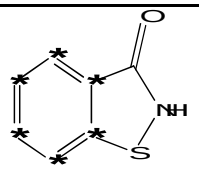
**Subsection A7.1.1** **Degradation, initial studies**

**Subsection A.7.1.1.1** **Abiotic**

**Subsection A7.1.1.2.1/03** **Ready Biodegradability**

**Annex Point IIA7.6.1.1** **1,2-Benzisothiazolin-3-one (BIT)**




* $^{14}$C label position

| | | |
|---|---|---|
| 3.1.1 | Lot/Batch number | Lot Number 1077.00 |
| 3.1.2 | Purity | Radiopurity = 97.7%; specific activity – 163.79 mCi/g |
| 3.1.3 | Further relevant properties | Water solubility is > 0.7 ppm<br>Vapor pressure = 2.3 x $10^{-4}$Pa at 25°C |
| 3.1.4 | TS inhibitory to microorganisms | Yes. Therefore, $^{14}$C-bit was employed as an attempt to obtain concentrations less than the minimal inhibitory concentration. |
| **3.2** | **REFERENCE SUBSTANCE** | |
| 3.2.1 | Sodium Benzoate | Sodium benzoate was employed as a reference compound for the test system. The dosing concentration was 15 mg of carbon/L (25.7 mg sodium benzoate/L) |
| 3.2.2 | $^{12}$C-BIT | Non-radiolabeled BIT ($^{12}$C-BIT) was from Rohm and Haas Company. The material lot number was 060309/1 (subsequently renamed MJB3738 by sponsor) and the purity was 99.8%. |
| **3.3** | **TESTING PROCEDURE** | |
| 3.3.1 | Note | This study was a continuation of the ready study, A7.1.1.2.1/03 and was designed primarily to investigate the metabolism of BIT in an activated sludge system. The control results (reference, toxicity, and background) also appear in study A7.1.1.2.1/03 and are provided here for |

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| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
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| **Subsection A.7.1.1.1** | **Abiotic** |
| **Subsection A7.1.1.2.1/03** | **Ready Biodegradability** |
| **Annex Point IIA7.6.1.1** | **1,2-Benzisothiazolin-3-one (BIT)** |

| | | |
|---|---|---|
| | completeness. | |
| 3.3.2 Inoculum | The details of the inoculum appear in Table A7.1.1.2.1/03-1. | |
| 3.3.3 Preparation of Solutions | <u>BIT</u><br>The $^{14}$C-BIT aqueous stock solution of $^{14}$C-BITwas prepared by dissolving 4.580 mg in 50 mL water. The test vessels were dosed with 9.8 mL (0.971043 mg) of the stock solution resulting in a radioassayed vessel concentration of 0.3237 mg/L.<br>The $^{12}$C-BIT aqueous solution used for the toxicity controls was prepared by dissolving 37.48 mg in 250 mL of water and adding 6.25 mL to the appropriate vessel.<br><u>Sodium Benzoate</u><br>A stock solution of the reference compound was prepared by adding 3.859 g of sodium benzoate and making up to 1 liter using reverse-osmosis water. The reference and toxicity control vessels were dosed with 20 mL of this solution to give a nominal concentration of 15 mg carbon/L (25.7 mg sodium benzoate). | |
| 3.3.4 Test system | The test system is described in Table A7.1.1.2.1/03-2. | |
| 3.3.5 Test conditions | Table A7.1.1.2.1/03-3 describes the test conditions including the composition of the aqueous media, inoculum, temperature, pH and aeration. | |
| 3.3.6 Initial Test Substance concentration | The initial nominal concentration of $^{14}$C-BIT was 0.313 ppm and the radioassayed (actual) concentration was 0.3237 mg/L | |
| 3.3.7 Duration of test | The background control, reference control, and toxicity control exposure period was 28 days. After sampling on Day | |

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| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
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| **Subsection A.7.1.1.1** | **Abiotic** |
| **Subsection A7.1.1.2.1/03** | **Ready Biodegradability** |
| **Annex Point IIA7.6.1.1** | **1,2-Benzisothiazolin-3-one (BIT)** |

| | | |
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| | 28, 1 mL of concentrated HCl was added to these vessels. The vessels were aerated overnight to drive dissolved $CO_2$ into the alkali traps prior to final analysis. The two test vessels containing $^{14}$C-BIT were exposed for 16 days. In order not to introduce artifacts, these two vessels were not acidified. | |
| 3.3.8 Chemical and biochemical methods | Liquid scintillation spectrometry was employed to quantitate the $^{14}CO_2$ trapped in the NaOH traps.<br><br>$^{12}CO_2$ in the $Ba(OH)_2$ trapping solutions was quantitated by titration with standard HCl (0.05M) using phenolphthalein as an indicator. Titrations were performed on 20 mL aliquots until two matching (± 0.1 mL) titers were obtained<br><br>Inorganic carbon concentration of the inoculated salts medium was determined using a carbon analyzer. The sample is acidified with $H_3PO_4$, sparged with $CO_2$-free air, and quantitated by a non-dispersive infrared detector.<br><br>Air flow through the systems was measured weekly, adjusting if necessary, to maintain a flow rate of approximately 50 mL/min. This was accomplished with a bubble flow meter and a stopwatch.<br><br>Aliquots from the Test Flasks (dosed with $^{14}$C-BIT) were analyzed by HPLC using a modified C-18 column and a binary gradient composed of 0.5% aqueous formic acid and 0.5% methanolic formic acid. Detection employed a $^{14}$C-flow through monitor and/or UV detector (254 nm).<br><br>Metabolites were analyzed by LC-MS using a C-18 column and a binary gradient composed of either 0.5% aqueous formic acid and 0.5% methanolic formic acid or 0.5% aqueous formic acid and 0.5% acetonitrile-formic acid. The flow was split between the mass spectrometer and a | |

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| **Subsection A.7.1.1.1** | **Abiotic** |
| **Subsection A7.1.1.2.1/03** | **Ready Biodegradability** |
| **Annex Point IIA7.6.1.1** | **1,2-Benzisothiazolin-3-one (BIT)** |

| | | |
|---|---|---|
| | radioactive flow monitor. | |
| 3.3.9 Sampling | Sample analysis for the background controls, reference controls (containing sodium benzoate) and the toxicity controls (containing sodium benzoate and $^{12}$C-BIT) took place on Days 1, 3, 6, 8, 10, 13, 15, 16, 20, 22, 24, 28, and 29. For the test vessels (containing $^{14}$C-BIT) sampling occurred on Days 1, 3, 6, 8, 10, 13, 15, and 16. At these intervals the trap nearest the test vessel was removed for quantitation. The remaining two bottles in the series were moved up towards the test vessel and a fresh trap placed on the end of the series. Aliquots of the trapping solution were either radio assayed ($^{14}CO_2$) or titrated ($^{12}CO_2$). On Day 28, 1 mL of concentrated HCl was added to each of the six remaining control vessels and the flasks aerated overnight to drive residual $CO_2$ into the traps thus accounting for dissolved $CO_2$. | |
| 3.3.10 Nitrate/nitrite measurement | No | |
| 3.3.11 Controls | Toxicity Control: 0.313 mg $^{12}$C-BIT/L plus 25.7 mg sodium benzoate/L<br>Reference Control: 25.7 mg sodium benzoate/L<br>Inoculum Control: no BIT or sodium benzoate<br>Additional details are in Table A7.1.1.2.1/03-2. | |
| 3.3.12 Calculations/ Statistics | The percent biodegradation was calculated as follows:<br>$$\text{Percent Biodegradation} = \frac{\text{cumulative } CO_2\text{(mg)}}{\text{theoretical cumulative } CO_2\text{ (mg)}} x100$$<br>or<br>$$\text{Percent Biodegradation} = \frac{\text{cumulative dpm}}{\text{total applied dpm}} \text{ x } 100$$<br>where theoretical $CO_2$ = | |

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| **Subsection A7.1.1.2.1/03** | **Ready Biodegradability** |
| **Annex Point IIA7.6.1.1** | **1,2-Benzisothiazolin-3-one (BIT)** |

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| | mg of reference substance added x<br>percent of carbon content of the reference material x<br>3.667 (the weight (mg) of $CO_2$ produced from 1 mg of carbon) |
| | **4 RESULTS** |
| **4.1 Test Parameters** | The inorganic carbon content of the inoculated mineral salts medium was 0.59 mg carbon/L culture solution, or 3.96% of the carbon loading from the addition of sodium benzoate.<br>The pH on Day 0 of the main test ranged from 7.40 – 7.47 and on Day 28, 7.22 -7.40. |
| **4.2 Biodegradation** | A summary of the biodegradation results for the test compound $^{14}$C-BIT dosed at 0.3237 mg/L, for the sodium benzoate reference control, and for the toxicity control (sodium benzoate plus 0.313 mg/L BIT) are presented in Table A7.1.1.2.1/03-4. Additionally the results are presented graphically in Figure A7.1.1.2.1/03-1.<br>After an initial lag phase of 8 days, biodegradation of $^{14}$C-BIT progressed steadily until Day 13 when the rate of degradation slowed. By Day 16 approximately 20% of the applied $^{14}$C-activity was present as $^{14}CO_2$. The maximum divergence between replicates was 0.3% observed on Days 8 and10.<br>The reference controls containing sodium benzoate rapidly evolved $CO_2$ reaching 64% by Day 8. Thereafter the rate slowed reaching 82% on Day 16 at which time the rate began to plateau. On Day 28, biodegradation level was 88%. The validity requirement is that biodegradation of sodium |

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| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
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| **Subsection A.7.1.1.1** | **Abiotic** |
| **Subsection A7.1.1.2.1/03** | **Ready Biodegradability** |
| **Annex Point IIA7.6.1.1** | **1,2-Benzisothiazolin-3-one (BIT)** |

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| | benzoate exceed 60% by Day 14, which was achieved.<br><br>The toxicity control measured the mineralization of sodium benzoate in the presence of BIT. $^{12}$C-BIT at 0.313 mg/L did not suppress the microbial degradation and thus the mineralization of sodium benzoate. The level of sodium benzoate biodegradation at study termination, 88%, was essentially the same as the reference control. |
| **4.3 Abiotic Degradation** | Abiotic vessels were not included because they had been examined in an earlier study (A7.1.1.2.1/01). Vessels dosed with BIT and $HgCl_2$ showed essentially no biodegradation. |
| **4.4 Material Balance** | The distribution of radioactivity and material balance are presented in Table A7.1.1.2.1/03-5. About 68% of the applied radioactivity was detected in the culture solution and about 20% in the NaOH traps. A wash of the culture vessels collected less than 0.5%. Recovery of applied radioactivity was 95.2 ± 2.6% which is an acceptable result. |
| **4.5 Quantitation of Parent and Characterization of biodegradates-** | On Day 16 no BIT was detected in the test flask culture solutions (containing $^{14}$C-BIT) but two major metabolites were observed. The metabolites were identified by LC-MS (Table A7.1.1.2.1/03-6). 2-methylthiobenzamide was present at 61.47% of the applied activity on Day 16 and 2-methylsulfinyl-benazmide as 16.34%. In addition, about 20% of the applied activity was present as $^{14}CO_2$ which indicates that cleavage of the benzene ring did occur. Assuming 100% BIT on Day 0 and 0.01% on Day 16, the half-life of BIT in this system is about 1.2 days. |
| | **5 APPLICANT'S SUMMARY AND CONCLUSION** |
| **5.1 MATERIALS AND** | This study employed OECD 301B Ready Biodegradability, |

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| **Subsection A.7.1.1.1** | **Abiotic** |
| **Subsection A7.1.1.2.1/03** | **Ready Biodegradability** |
| **Annex Point IIA7.6.1.1** | **1,2-Benzisothiazolin-3-one (BIT)** |

| | | |
|---|---|---|
| | **METHODS** | $CO_2$ Evolution (Modified Sturm Test).<br><br>Flasks containing mineral salts solution ($KH_2PO_4$, $K_2HPO_4$, $Na_2HPO_4$, $NH_4Cl$, $MgSO_4$, $CaCl_2$, and $FeCl_3$) plus activated sludge inoculum were prepared. The following systems were prepared: control vessels containing just the mineral salt solution, duplicate test vessels containing 0.313 mg $^{14}$C-BIT, duplicate reference control vessels containing sodium benzoate, and a single toxicity control vessel containing sodium benzoate and BIT. All vessels were aerated and purged with $CO_2$-free air. Evolved $^{14}CO_2$ from the test vessels and a set of controls was trapped in NaOH while $^{12}CO_2$ from the reference control, toxicity control, and set of control vessels were trapped in $Ba(OH)_2$. All vessels were incubated in the dark at 22 ± 2°C. On Days 1, 3, 6, 8, 10, 13, 15, 16, 20, 22, 24, 28 and 29 the traps from the reference control, toxicity control, and a set of control vessels were refreshed and aliquots of the solutions were removed for quantitation by either liquid scintillation spectroscopy or titration. Test vessel traps were refreshed and on Days 1, 3, 6, 8, 10, 13, 15, and 16. On Day16, aliquots from the Test Flasks containing $^{14}$C-BIT were examined by LC-MS. |
| **5.2** | **RESULTS AND DISCUSSION** | As part of a traditional OECD 301B ready test, two additional vessels containing $^{14}$C-BIT were prepared. After 16 days approximately 20% of the applied $^{14}$C-BIT was present as $^{14}CO_2$. Per the protocol, these two test vessels were then analyzed by LC-MS in order to evaluate the metabolic pathway. Two major metabolites were present:<br><br>2-methylthiobenzamide: ~61% of the activity<br>2-methylsulfinyl-benzamide: ~16% of the activity<br><br>No BIT was detected. The half-life in this system is about 1.2 days. |

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| **Subsection A.7.1.1.1** | **Abiotic** |
| **Subsection A7.1.1.2.1/03** | **Ready Biodegradability** |
| **Annex Point IIA7.6.1.1** | **1,2-Benzisothiazolin-3-one (BIT)** |

| | | | |
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| | | Sodium benzoate biodegradation was rapid and exceeded 60% by Day 8 demonstrating that the activated sludge culture was viable. BIT had no observable effect on the biodegradation of sodium benzoate since there was no observable difference in the biodegradation of sodium benzoate in the absence or presence of BIT. | |
| **5.3** | **CONCLUSION** | This study identifies the metabolites of BIT in a biological system. By Day 16 BIT had completely degraded to 2-methylthiobenzamide, 2-methylsulfinyl-benzamide, or $CO_2$. The half-life of BIT in this system is about 1.2 days. | |
| 5.3.1 | Reliability | 1-valid without restrictions. | |
| 5.3.2 | Deficiencies | None. | |

| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *November 2010* |
| **Materials and Methods** | *3.1.3. Further relevant properties*<br>*Water solubility should be > 0.7 g/L* |
| **Results and discussion** | *Applicant's version is accepted* |
| **Conclusion** | *This study identifies the metabolites of BIT in a biological system. By Day 16 BIT had completely degraded to 2-methylthiobenzamide, 2-methylsulfinyl-benzamide, or $CO_2$. The half-life of BIT in this system is about 1.2 days* |
| **Reliability** | *2* |

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| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
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| **Subsection A.7.1.1.1** | **Abiotic** |
| **Subsection A7.1.1.2.1/03** | **Ready Biodegradability** |
| **Annex Point IIA7.6.1.1** | **1,2-Benzisothiazolin-3-one (BIT)** |

| | |
|---|---|
| **Acceptability** | *Acceptable* |
| **Remarks** | *General remark: Numbering of the sections should be checked.*<br>*This study was a continuation of the ready study, A7.1.1.2.1/02 and not of 7.1.1.2.1/03 as cited along the test.* |

**Table A7.1.1.2.1/03-1: Inoculum**

| Criteria | Details |
|---|---|
| Nature | Activated sludge |
| Source | Return line of a sewage treatment works treating primarily domestic wastewater |
| Sampling site | Burley Menston Sewage Treatment Works, West Yorkshire, UK) |
| Preparation of inoculum | Sludge was blended and aerated. The suspended solids concentration was determined by filtration, oven drying the filtrate, and the weight of the dry sludge measured. |
| Pretreatment | The mineral salt medium was inoculated with activated sludge at 90 mg solid/L to provide a final solids concentration of 30 mg/L in each vessel. The solution was aerated with $CO_2$ free air overnight prior to addition of test compound |
| Concentration | 30 mg of sludge on a dry weight basis/L |

**Table 7.1.1.2.1/03-2: Test System for the Main Biodegradation Test**

| Criteria | Details | | | |
|---|---|---|---|---|
| Composition of Culture Flask | Nine 3000 mL flask were dosed as below. | | | |
| | **Identification** | **mg/L $^{14}$C-BIT** | **mg/L $^{12}$C Sodium Benzoate** | **mg/L $^{12}$C-BIT** |
| | Control ($^{12}$C) | | | |
| | Control ($^{12}$C) | | | |
| | Control ($^{14}$C) | | | |
| | Control ($^{14}$C) | | | |
| | Reference | | 25.7 | |
| | Reference | | 25.7 | |
| | Toxicity Control | | 25.7 | 0.313 |
| | Test | 0.313[1] | | |
| | Test | 0.313[1] | | |
| Aeration Device | $CO_2$-free air is passed through the flasks and into traps. | | | |
| Measuring equipment | Evolved $^{14}CO_2$ measured by liquid scintillation spectrometry and $^{12}CO_2$ by titration with HCl using a phenolphthalein indicator | | | |
| Trapping System | From the exit line of each flask dosed with $^{14}$C-BIT, three 0.0125M NaOH traps were placed in series to capture evolved $^{14}CO_2$. An identical procedure was employed for vessels dosed with $^{12}$C sodium benzoate except that 0.0125M $Ba(OH)_2$ was used instead of NaOH to capture evolved $^{12}CO_2$. | | | |
| Test performed in closed vessels due to significant volatility of test substance | No | | | |

[1] 0.313 mg $^{14}$C BIT/L was the nominal dose. Radioassayed concentration was 0.3237 mg $^{14}$C-BIT/L

**Table A7.1.1.2.1/03-3: Test Conditions**

| Criteria | Details |
|---|---|
| Composition of test medium | Stock solutions using analytical grade salts<br>a) $KH_2PO_4$: 8.50 g/L<br>$K_2HPO_4$: 21.75 g/L<br>$Na_2HPO_4 \cdot 2H_2O$: 33.40 g/L<br>$NH_4Cl$: 0.50 g/L<br>b) $CaCl_2 \cdot 2H_2O$: 36.40 g/L<br>c) $MgSO_4 \cdot 7H_2O$: 22.50 g/L<br>d) $FeCl_3 \cdot 6H_2O$: 0.25 g/L<br>The salts, a, b, c, and d were dissolved individually and made up to 1 L with water. The final testing solution was prepared containing 30 mL/L of solution a) and 3 mL/L of solutions b), c), and d). |
| Inoculum | The day before the addition of the test and reference substances, mineral salt test medium was inoculated with activated sludge solids at 90 mg suspended solids/L. 1 liter of this mixture was added to each test vessel followed by 1.5L or 1.9 L of ultra pure water. Based on a volume of 3 L in each test volume at Day 0, the activated sludge solid concentration was 30 mg/L. |
| Additional substrates | No |
| Test temperature | nominal 21 ± 1°C |
| pH | At Day 0 the pH ranged from 7.40 – 7.47. At termination (Day 28) the pH ranged from 7.22 – 7.40. |
| Aeration of dilution water | The test solutions were aerated through out the study using $CO_2$-free air |

**Table A7.1.1.2.1/03-4: Main Test—Cumulative Percent Biodegradation**

| Time (Days) | Cumulative Percent Biodegradation | | | | | | |
|---|---|---|---|---|---|---|---|
| | Test Vessels ($^{14}$C-BIT) | | | Reference Vessels (Sodium Benzoate) | | | Toxicity Control[1] |
| | 1 | 2 | Mean | 1 | 2 | Mean | |
| 1 | 0 | 0 | 0 | 7 | 7 | 7 | 3 |
| 3 | 0 | 0.1 | 0.1 | 44 | 44 | 44 | 42 |
| 6 | 0.2 | 0.3 | 0.3 | 58 | 57 | 58 | 58 |
| 8 | 0.9 | 1.2 | 1.1 | 65 | 64 | 64 | 67 |
| 10 | 9.0 | 8.7 | 8.9 | 70 | 68 | 69 | 72 |
| 13 | 16.3 | 16.2 | 16.3 | 76 | 74 | 75 | 78 |
| 15 | 18.9 | 18.9 | 18.9 | 79 | 77 | 78 | 81 |
| 16 | 19.9 | 19.9 | 19.9 | 83 | 81 | 82 | 84 |
| 20 | | | | 84 | 82 | 83 | 84 |
| 22 | | | | 86 | 84 | 85 | 85 |
| 24 | | | | 87 | 85 | 86 | 86 |
| 28 | | | | 87 | 86 | 87 | 87 |
| 29 | | | | 89 | 88 | 88 | 88 |

**Table A7.1.1.2.1/03-5: Material Balance from Vessels Dosed With $^{14}$C-BIT Only**

| Vessel | Percent of Applied Radioactivity | | | | |
|---|---|---|---|---|---|
| | Culture Vessel | Vessel Wash | NaOH Traps | Residue | Recovery |
| Test Replicate 1 | 67.7 | 0.2 | 19.9 | 5.5 | 93.3 |
| Test Replicate 2 | 68.1 | 0.2 | 19.9 | 8.8 | 97.0 |
| | | | | Mean | 95.2 ± 2.6 |

**Table A7.1.1.2.1/03-6: Identification and Quantitation of Metabolites**

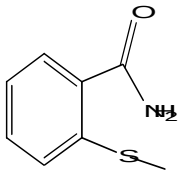
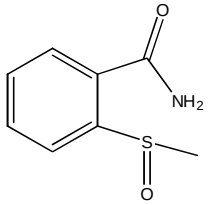
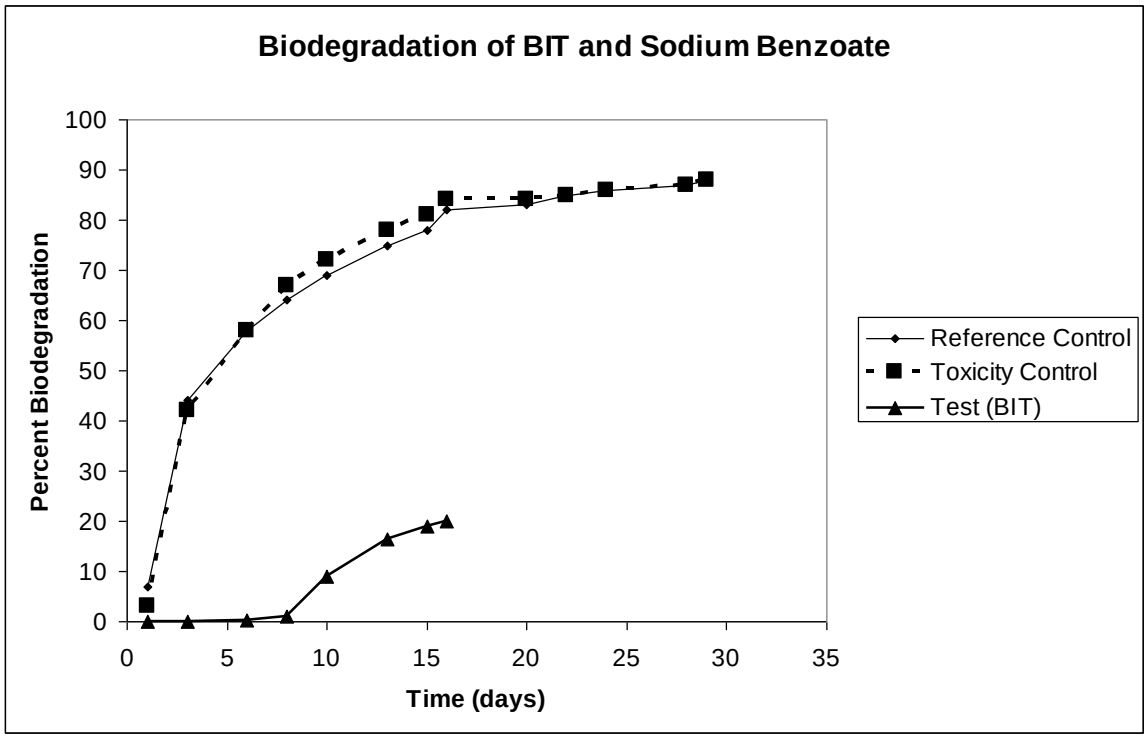
| Structure/Name | Rt (HPLC) | Average Percentage on Day 16 |
|---|---|---|
|  2-methylthiobenzamide | 21 min | 61.47 |
|  2-methylsulfinyl-benzamide | 19 min | 16.34 |

**Figure A7.1.1.2.1/03-1: Overview of Biodegradation of BIT, Sodium Benzoate (Reference Vessels), and Toxicity Control (Sodium Benzoate and BIT)**



**Section A7** **Ecotoxicological Profile Including Environmental Fate and Behaviour**

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**Subsection A7.1.1** **Degradation, initial studies**

**Subsection A.7.1.1.1** **Abiotic**

**Inherent Biodegradability**

**Subsection A.7.1.1.2.2.**

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| | | | Official use only |
|---|---|---|---|
| | | **1 REFERENCE** | |
| **1.1** | **Reference** | **A7.1.1.2.2** [redacted]. (2006) 1,2-Benzisothiazolin-3-one: Inherent Biodegradability in a Manometric Respirometry Test; [redacted] Rohm and Haas Report N° GLP-2006-090 (October 02, 2006), unpublished. | |
| **1.2** | **Data protection** | Yes | |
| 1.2.1. | Data owner | Rohm and Haas Company | |
| 1.2.2. | Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into Annex I/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **Guideline study** | Yes.<br>OECD No. 302C, Inherent Biodegradability: Modified MITI Test (II) with the following modifications<br>• Activated sludge was from only one source.<br>• Activated sludge was not fed during holding period.<br>• Holding period was maximum seven days.<br>• Test water prepared according to OECD 301F.<br>• Test run at 22°C.<br>• Only BOD monitored. No test specific analysis performed. | |
| **2.2** | **GLP** | Yes. | |

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**Subsection A.7.1.1.2.2.** **Inherent Biodegradability**

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| | | |
|---|---|---|
| **2.3 Deviations** | No. | **X** |
| | **3 MATERIALS AND METHODS** | |
| **3.1 Test material** | 1,2-Benzisothiazolin-3-one (BIT) | |
| 3.1.1 Lot/Batch number | 220904 | |
| 3.1.2 Specification | As given in section 2. | |
| 3.1.3 Purity | Purity : 100 % | |
| 3.1.4 Further relevant properties | Solubility in water : > 0.7 g/L<br>Vapor pressure : 2.3 x $10^{-4}$ Pa at 25°C | |
| 3.1.5 Composition of Product | Not applicable. | |
| 3.1.6 TS inhibitory to microorganisms | In an activated sludge respiration inhibition test (OECD 209), BIT had an NOEC of 1-3 mg/L (see section A7.4.1.4). BIT is a biocidal active substance and as such, inhibitory to microorganisms (see section A5). | |
| 3.1.7 Specific chemical analysis | The biodegradation process consumes dissolved oxygen and subsequently generates $CO_2$. By adsorbing the $CO_2$ with soda lime, a pressure drop can be measured using a manometric electrode and this calibrated to oxygen consumption (mg/L) | |
| **3.2 Reference substance** | Yes. Sodium Benzoate. | |
| 3.2.1 Initial concentration of reference substance | 100 mg/L | |

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**Subsection A.7.1.1.1** **Abiotic**

**Subsection A.7.1.1.2.2.** **Inherent Biodegradability**

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| **3.3 Testing procedure** | | |
|---|---|---|
| 3.3.1 Inoculum/ test species | Aerobic activated sludge was obtained from a wastewater treatment facility (ARA Ergolz II, Füllinsdorf, Switzerland) treating primarily domestic wastewater (Table A7.1.1.2.2-1). The sludge was washed twice via centrifugation with tap water and the liquid supernatant phase was decanted. A homogenized aliquot of the final sludge suspension was weighed, thereafter dried and the ratio of wet to dry weight was calculated. Sludge was used at a final concentration of 100 mg dry material per liter. | |
| 3.3.2 Test system | The test system is described in Table A7.1.1.2.2-2 | |
| 3.3.3 Test conditions | Table A7.1.1.2.1-3 describes the test conditions including the composition of the aqueous mineral salts medium, temperature, pH, and aeration.<br>Eight 500 ml airtight flasks where filled with 250 mL of mineral salt water (Table A7.1.1.2.2-3) which contained 25 mg of activated sludge inoculum. The reference compound (sodium benzoate) and test compound (BIT) were dissolved in the mineral salt medium and added as described in Table A7.1.1.2.2-2. | |
| 3.3.4 Initial TS concentration | 17.9 -18.2 mg/L (10- 10.1 mg total organic carbon/L). See Table A7.1.1.2.2-2 | |
| 3.3.5 Duration of test | 28 days (exposure period). | |
| 3.3.6 Analytical parameter | Biochemical oxygen demand. Pressure drop due to the consumption of oxygen (Table A7.1.1.2.2-2). | |
| 3.3.7 Sampling | Oxygen consumption was measured daily. | |
| 3.3.8 Intermediates/ degradation products | Not identified | |
| 3.3.9 Nitrate/nitrite | Theoretical oxygen demand for BIT was calculated with and without | |

| | |
|---|---|
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| **Subsection A.7.1.1.1** | **Abiotic** |
| **Subsection A.7.1.1.2.2.** | **Inherent Biodegradability** |
| **IIA VII 7.6.1.2** | |

| | |
|---|---|
| measurement | nitrification.. |
| 3.3.10 Controls | Toxicity control: 31 mg/L BIT (Test item) and 100 mg/L Sodium Benzoate (Reference item).<br>Procedure control: 100 mg/L Sodium Benzoate (Reference item)<br>Abiotic control: 30 mg/L BIT(test item) poisoned with 10 mg/L $HgCl_2$<br>Inoculum control: neither test item nor reference item |
| 3.3.11 Calculations/ Statistics | Percent biodegradation<br>$\text{Biodegradation (\%)} = \frac{\text{BOD (mg } O_2\text{/mg chemical)}}{\text{ThOD}_{NH4 \text{ or } NH3}\text{(mg } O_2\text{/mg chemical)}} \times 100$<br>where:<br>BOD = Biochemical oxygen demand of the test or reference compound<br>$\frac{\text{(mg } O_2 \text{ uptake/L test or reference cmpd) - (mg } O_2\text{/L inoculum con}}{\text{mg test and/or reference compound/L}}$<br>$ThOD_{NH4 \text{ or } NO3}$ = Theoretical oxygen demand of the test or reference compound without or with nitrification.<br>The theoretical oxygen demand is the total amount of oxygen required to oxidize a chemical completely. It is calculated from the molecular formula, assuming the turnover of H into $H_2O$, C into $CO_2$, S into $SO_3$, Na into $Na_2O$, and N into $NH_3$ and/or $NO_3$.<br>The calculated theoretical oxygen demain is tabulated below.<br>**Theoretical Oxygen Demand in mg $O_2$/L** |

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**Subsection A7.1.1** **Degradation, initial studies**

**Subsection A.7.1.1.1** **Abiotic**

**Subsection A.7.1.1.2.2.** **Inherent Biodegradability**

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| BIT | | Sodium Benzoate |
|---|---|---|
| $ThOD_{NH4}$ | $ThOD_{NO3}$ | ThOD |
| 1.80 | 2.22 | 1.67 |

**4 RESULTS**

**4.1 Degradation of test substance**

4.1.1 Biodegradation of the test compound, BIT

The biodegradation of the test compound, BIT is presented in Tables A7.1.1.2.2-4 and A7.1.1.2.2-5.

During the study period of 28 days the biochemical oxygen demand (BOD) of BIT in the test media was less than the normal range found for the inoculum controls. Therefore, BIT was not biodegraded under the test conditions.

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**Subsection A7.1.1** **Degradation, initial studies**

**Subsection A.7.1.1.1** **Abiotic**

**Subsection A.7.1.1.2.2.** **Inherent Biodegradability**

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| | | |
|---|---|---|
| 4.1.2 | Biodegradation of the reference compound, sodium benzoate | Biodegradation in the procedure controls which contained only the reference compound, sodium benzoate is presented in Tables A7.1.1.2.2-4 and A7.1.1.2.2-5 as well as Figures A7.1.1.2.2-1 and A7.1.1.2.2-2.<br><br>In the procedure controls, sodium benzoate was biodegraded by an average of 72% and 81% on Days 7 and 14, respectively. These results confirm the suitability of the activated sludge used in this study. By the end of the study (Day 28), the reference compound was biodegraded by an average of 86%. |
| 4.1.3 | Biodegradation in the toxicity control | The percent biodegradation in the toxicity control which contained both the test compound (BIT) and the reference compound (sodium benzoate) was calculated based on the sum of the theoretical oxygen demand of the test item (with and without nitrification, $ThOD_{NO3}$ and $ThOD_{NH4}$) and the reference compound. The results appear in Tables A7.1.1.2.2-4 and A7.1.1.2.2-5 as well as Figures A7.1.1.2.2-1 and A7.1.1.2.2-2.<br><br>In the toxicity control, the biochemical oxygen demand (BOD) over the 28 day study period showed a similar course as the BOD of the procedure controls which contained only the reference compound. However, after Day 5 the BOD in the toxicity control was consistently lower than the procedure controls. According to the test guidelines, BIT is assumed to have no relevant inhibitory effect on activated sludge microorganisms at the tested concentration of 32 mg/L because biodegradation in the toxicity control was greater than 25% on Day 14. On Day 14 the biodegradation was 41% and 39% based on the $ThOD_{NH4}$ and $ThOD_{NO3}$, respectively. The percent biodegradation was nearly the same at the end of the exposure period, Day 28. |
| 4.1.4 | Percent biodegradation summary | |

| Percent Biodegradation on Day 28 | | | | |
|---|---|---|---|---|
| BIT | | Sodium Benzoate | Toxicity Control (BIT + Sodium Benzoate) | |
| $ThOD_{NH4}$ | $ThOD_{NO3}$ | | $ThOD_{NH4}$ | $ThOD_{NO3}$ |
| 0 | 0 | 86 | 39 | 37 |

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**Subsection A7.1.1** **Degradation, initial studies**

**Subsection A.7.1.1.1** **Abiotic**

**Subsection A.7.1.1.2.2.** **Inherent Biodegradability**

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| | | |
|---|---|---|
| | | **5 APPLICANT'S SUMMARY AND CONCLUSION** |
| **5.1** | **Materials and methods** | BIT was investigated for its inherent biodegradability in a 28-day Biochemical Oxygen Demand (BOD) test according to a modified version of OECD Guideline for testing of Chemicals N° 302C, Inherent Biodegradability: Modified MITI Test (II).<br><br>Eight 500 mL airtight flasks were prepared containing 250 mL of test water containing mineral salts ($KH_2PO_4$, $K_2HPO_4$, $Na_2HPO_4$, $NH_4Cl$, $MgSO_4$, $CaCl_2$, and $FeCl_3$) and 25 mg of activated sludge inoculum were added. The flask were dosed as follows:<br>• 2 flasks contained 31 mg/L BIT.<br>• 2 flasks contained 100 mg/L sodium benzoate.<br>• 2 flasks were controls (no BIT or sodium benzoate).<br>• 1 flask contained 30 mg/L BIT + 10 mg/L $HgCl_2$.<br>• 1 flask contained 32 mg/L BIT + 100 mg/L sodium benzoate.<br><br>Biochemcial oxygen demand was measured on Days 0 – 28 using a manometric electrode. |
| **5.2** | **Results and discussion** | The test item, BIT, was found to be not inherently biodegradable under the test conditions within 28 days.<br><br>In the procedure controls, sodium benzoate was degraded to an average extent of 72% and 81% by Days 7 and 14, respectively, confirming the suitability of the activated sludge. By the end of the test (Day 28) sodium benzoate had biodegraded by 86%<br><br>In the toxicity control containing both BIT and the reference item sodium benzoate, biodegradation had a similar course as the BOD of sodium benzoate alone. However, the BOD of the toxicity control was consistently lower from Day 5 onward. |
| **5.3** | **Conclusion** | BIT was not inherently biodegradable under the tests conditions within 28 days. However testing biocides for inherent biodegredability may not be relevant since biocides which are toxic to the inoculum may give false |

| | |
|---|---|
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| **Subsection A.7.1.1.2.2.** | **Inherent Biodegradability** |
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| | | |
|---|---|---|
| | negative test results which may lead to requirements for further tests. | |
| 5.3.1 Reliability | 1-valid without restrictions. | |
| 5.3.2 Deficiencies | No. | |

| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *March 2013* |

| | |
|---|---|
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| **Subsection A.7.1.1.2.2.** | **Inherent Biodegradability** |

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**Materials and Methods**

*Applicant's version is accepted with the following comments:*

*2.3 Deviations*

1. *The basal culture medium contained different quantities of $Na_2HPO_42H_2O$, $NH_4Cl$ and $CaCl_22H_2O$ and the final solution consisted of different volumes of each stock solution to that of the guideline.. Culture medium is prepared following OECD guidelines 301F for ready biodegradability (manometric respirometry test).*
2. *The sludge sampling did not take place in at least 10 places throughout the country. According to the study report, only one sample of activated sludge was taken, from a domestic wastewater treatment plant.*
3. *No reference is made to the mixing of old and new activated sludge samples. Only one sample was taken for the test, and the holding period was maximum seven days.*
4. *The number and type of test flasks prepared differed to that of the guideline. The study was performed using 500-ml Erlenmeyer flasks, with a final volume of 250 ml per flask.*

*3.1.6. BIT cannot be assumed to be inhibitory on the activity of the sludge following the OECD criteria, because degradation of reference substance in toxicity control is higher than 25% (based on total ThOD) within 14 days. However, the decrease in biodegradation in toxicity control compared to procedure control could indicate a certain inhibitory effect of BIT. This inhibitory effect could also explain the fact that BOD for BIT in the test media was lower than the normal range found for inoculums controls.*

*3.3.5. Eight 500 mL Airtight flask were dosed as dosed as below. The dosed material was mixed into the Mineral Salt Solution*

| ***Identification*** | ***Replicate No.*** | ***Amount of Test Item (BIT)*** | | ***Amount of Reference Item (Sodium Benzoate)*** | | ***$HgCl_2$ (mg/L)*** |
|---|---|---|---|---|---|---|
| | | ***mg/L*** | ***$ThOD_{NH4/NO3}$[a]*** | ***mg/L*** | ***ThOD[b]*** | |
| *Test Item* | *1* | *31* | *56/69* | | | |
| *Test Item* | *2* | *31* | *56/69* | | | |
| *Inoculum Control* | *1* | | | | | |

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**Subsection A.7.1.1.1** **Abiotic**

**Inherent Biodegradability**

**Subsection A.7.1.1.2.2.**

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| | |
|---|---|
| **Results and discussion** | *Accepted* |
| **Conclusion** | *BIT was not inherently biodegradable under the tests conditions within 28 days.*<br>*Nevertheless, BIT at the concentration used seems to be toxic to the inoculum:*<br>*TS inhibitory to microorganisms: In an activated sludge respiration inhibition test (OECD 209), BIT has a NOEC of 1-3 mg/L.* |
| **Reliability** | *2* |
| **Acceptability** | *Acceptable* |
| **Remarks** | |

**Table A7.1.1.2.2-1: Inoculum**

| Criteria | Details |
|---|---|
| Nature | Activated sludge |
| Source | Wastewater treatment plant treating predominantly domestic wastewater |
| Sampling site | ARA Ergolz II, Füllinsdorf, Switzerland |
| Preparation of inoculum | Sludge was washed twice with tap water by centrifugation and the supernatant liquid phase decanted. |
| Pretreatment | Sludge was added to mineral salt solution and aerated with $CO_2$ free air overnight prior to addition of test compound |
| Concentration | 100 mg of washed sludge on a dry weight basis/L |

**Table A7.1.1.2.2-2: Test System Including Flask Composition and Dosing Concentrations**

| **Eight 500 mL Airtight flask were dosed as dosed as below. The dosed material was mixed into the Mineral Salt Solution** | | | | | | |
|---|---|---|---|---|---|---|
| **Identification** | **Replicate No.** | **Amount of Test Item (BIT)** | | **Amount of Reference Item (Sodium Benzoate)** | | **$HgCl_2$ (mg/L)** |
| | | **mg/L** | **$ThOD_{NH4/NO3}$[a]** | **mg/L** | **ThOD[b]** | |
| Test Item | 1 | 31 | 56/69 | | | |
| Test Item | 2 | 31 | 56/69 | | | |
| Inoculum Control | 1 | | | | | |
| Inoculum Control | 2 | | | | | |
| Procedure Control | 1 | | | 100 | 167 | |
| Procedure Control | 2 | | | 100 | 167 | |
| Abiotic Control | 1 | 30 | 55/68 | | | 10 |
| Toxicity Control | 1 | 32 | 57/70 | 100 | 167 | |

| | |
|---|---|
| Aeration Device | Consumed oxygen was replaced by electrolysis of cupper sulfate |
| Measuring equipment | manometric electrode |
| Measurement Principle | The biodegradation process consumes the dissolved oxygen in the test liquid and generates $CO_2$. The $CO_2$ is adsorbed by soda lime and the total pressure decreases in the airtight test flask. The pressure drop is detected and converted into an electrical signal by means of an electrode type manometer. The consumed oxygen is replaced by electrolytically generated oxygen from a copper sulfate solution |

[a] Theoretical oxygen demand in mg $O_2$/L ($NH_4$/$NH_3$; without/with nitrification)

**Table A7.1.1.2.2-3: Test Conditions**

| Criteria | Details |
|---|---|
| Composition of test medium | Stock solutions using analytical grade salts<br>a) $KH_2PO_4$: 8.50 g/L<br>$K_2HPO_4$: 21.75 g/L<br>$Na_2HPO_4 \cdot 2H_2O$ 33.40 g/L<br>$NH_4Cl$: 0.50 g/L<br>b) $MgSO_4 \cdot 7H_2O$: 22.50 g/L<br>c) $CaCl_2 \cdot 2H_2O$: 36.40 g/L<br>d) $FeCl_3 \cdot 6H_2O$: 0.25 g/L<br>**One drop of concentrated HCl was added to solution d) as a preservative.**<br>The final testing solution was prepared by adding 10 mL of solution a) and 1 mL of solutions b), c), and d) to 800 ml of purified water. The solution was then made up to 1000 mL with purified water and the pH adjusted to 7.4 with dilute HCl. |
| Additional substrates | HgCl to the abiotic control |
| Test temperature | 22°C (temperature controlled room) |
| pH | At the start the pH in the test samples was 7.4. At termination, the pH ranged from 7.3-8.0 |
| Aeration of dilution water | Not Applicable |

**Table A7.1.1.2.2-4: Oxygen Consumption**

| Time (days) | Cumulative Oxygen Consumption (mg/L) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Test Compound (BIT) | | Inoculum Control | | Reference Compound (Sodium Benzoate) | | Abiotic Control | Toxicity Control |
| | Rep 1 | Rep 2 | Rep 1 | Rep 2 | Rep 1 | Rep 2 | | |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 | 0 | 0 | 3 | 2 | 29 | 27 | 0 | 0 |
| 2 | --[1] | -- | -- | -- | -- | -- | -- | -- |
| 3 | 0 | 0 | 13 | 12 | 113 | 107 | 0 | 110 |
| 4 | 0 | 0 | 16 | 15 | 122 | 115 | 0 | 115 |
| 5 | 0 | 2 | 20 | 18 | 134 | 126 | 0 | 119 |
| 6 | 0 | 3 | 22 | 20 | 141 | 133 | 0 | 120 |

| Time (days) | Cumulative Oxygen Consumption (mg/L) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Test Compound (BIT) | | Inoculum Control | | Reference Compound (Sodium Benzoate) | | Abiotic Control | Toxicity Control |
| | Rep 1 | Rep 2 | Rep 1 | Rep 2 | Rep 1 | Rep 2 | | |
| 7 | 0 | 3 | 25 | 23 | 147 | 140 | 0 | 120 |
| 8 | 1 | 4 | 27 | 25 | 152 | 146 | 0 | 120 |
| 9 | 3 | 4 | 29 | 27 | 156 | 151 | 0 | 120 |
| 10 | 6 | 5 | 32 | 29 | 160 | 156 | 0 | 120 |
| 11 | 6 | 5 | 33 | 30 | 163 | 159 | 0 | 120 |
| 12 | 7 | 6 | 35 | 32 | 166 | 163 | 0 | 126 |
| 13 | 8 | 8 | 37 | 34 | 168 | 165 | 0 | 127 |
| 14 | 8 | 8 | 37 | 34 | 171 | 169 | 0 | 127 |
| 15 | 8 | 8 | 39 | 35 | 174 | 172 | 0 | 127 |
| 16 | 8 | 8 | 40 | 36 | 175 | 174 | 0 | 127 |
| 17 | 8 | 8 | 41 | 37 | 177 | 176 | 0 | 127 |
| 18 | 8 | 8 | 42 | 37 | 178 | 177 | 0 | 127 |
| 19 | 8 | 8 | 43 | 38 | 180 | 179 | 0 | 128 |
| 20 | 8 | 8 | 44 | 39 | 182 | 181 | 0 | 129 |
| 21 | 8 | 9 | 45 | 40 | 183 | 183 | 0 | 130 |
| 22 | 8 | 9 | 45 | 40 | 184 | 183 | 0 | 130 |
| 23 | 8 | 9 | 46 | 40 | 186 | 185 | 0 | 131 |
| 24 | 8 | 9 | 46 | 41 | 187 | 185 | 0 | 131 |
| 25 | 8 | 9 | 47 | 41 | 188 | 186 | 0 | 132 |
| 26 | 8 | 9 | 47 | 41 | 188 | 186 | 0 | 132 |
| 27 | 8 | 10 | 48 | 42 | 189 | 187 | 0 | 132 |
| 28 | 8 | 10 | 48 | 42 | 190 | 188 | 0 | 132 |

[1] No reading taken

**Table A7.1.1.2.2.-5: Percent Biodegradation**

| Time (days) | Percent Biodegradation[1] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Test Compound (BIT) | | | | Reference (Sodium Benzoate) | | Toxicity Control | |
| | $ThOD_{NH4}$ | | $ThOD_{NO3}$ | | ThOD | | $ThOD_{NH4}$ | $ThOD_{NO3}$ |
| | Rep 1 | Rep 2 | Rep 1 | Rep 2 | Rep 1 | Rep 2 | | |

| Time (days) | Percent Biodegradation[1] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Test Compound (BIT) | | | | Reference (Sodium Benzoate) | | Toxicity Control | |
| | $ThOD_{NH4}$ | | $ThOD_{NO3}$ | | ThOD | | $ThOD_{NH4}$ | $ThOD_{NO3}$ |
| | Rep 1 | Rep 2 | Rep 1 | Rep 2 | Rep 1 | Rep 2 | | |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 | *[2] | * | * | * | 16 | 15 | -1 | -1 |
| 2 | --[3] | -- | -- | -- | -- | -- | -- | -- |
| 3 | * | * | * | * | 60 | 57 | 44 | 41 |
| 4 | * | * | * | * | 64 | 60 | 44 | 42 |
| 5 | * | * | * | * | 69 | 64 | 45 | 42 |
| 6 | * | * | * | * | 72 | 67 | 44 | 42 |
| 7 | * | * | * | * | 74 | 69 | 43 | 40 |
| 8 | * | * | * | * | 75 | 72 | 42 | 40 |
| 9 | * | * | * | * | 77 | 74 | 41 | 39 |
| 10 | * | * | * | * | 78 | 75 | 40 | 38 |
| 11 | * | * | * | * | 79 | 76 | 40 | 37 |
| 12 | * | * | * | * | 79 | 78 | 41 | 39 |
| 13 | * | * | * | * | 79 | 78 | 41 | 39 |
| 14 | * | * | * | * | 81 | 80 | 41 | 39 |
| 15 | * | * | * | * | 82 | 81 | 40 | 38 |
| 16 | * | * | * | * | 82 | 81 | 40 | 38 |
| 17 | * | * | * | * | 83 | 82 | 39 | 37 |
| 18 | * | * | * | * | 83 | 82 | 39 | 37 |
| 19 | * | * | * | * | 84 | 83 | 39 | 37 |
| 20 | * | * | * | * | 84 | 84 | 39 | 37 |
| 21 | * | * | * | * | 84 | 84 | 39 | 37 |
| 22 | * | * | * | * | 85 | 84 | 39 | 37 |
| 23 | * | * | * | * | 86 | 85 | 39 | 37 |
| 24 | * | * | * | * | 86 | 85 | 39 | 37 |
| 25 | * | * | * | * | 86 | 85 | 39 | 37 |
| 26 | * | * | * | * | 86 | 85 | 39 | 37 |
| 27 | * | * | * | * | 86 | 85 | 39 | 37 |
| 28 | * | * | * | * | 87 | 86 | 39 | 37 |

[1] Percent Biodegradation corrected for the mean oxygen update in the inoculum controls

[2] * Negative value due to higher oxygen consumption in inoculum controls than in the test compound
[3] -- No readings taken

**Figure A7.1.1.2.2-1: Biodegradation in Test Flasks Without Nitrification**

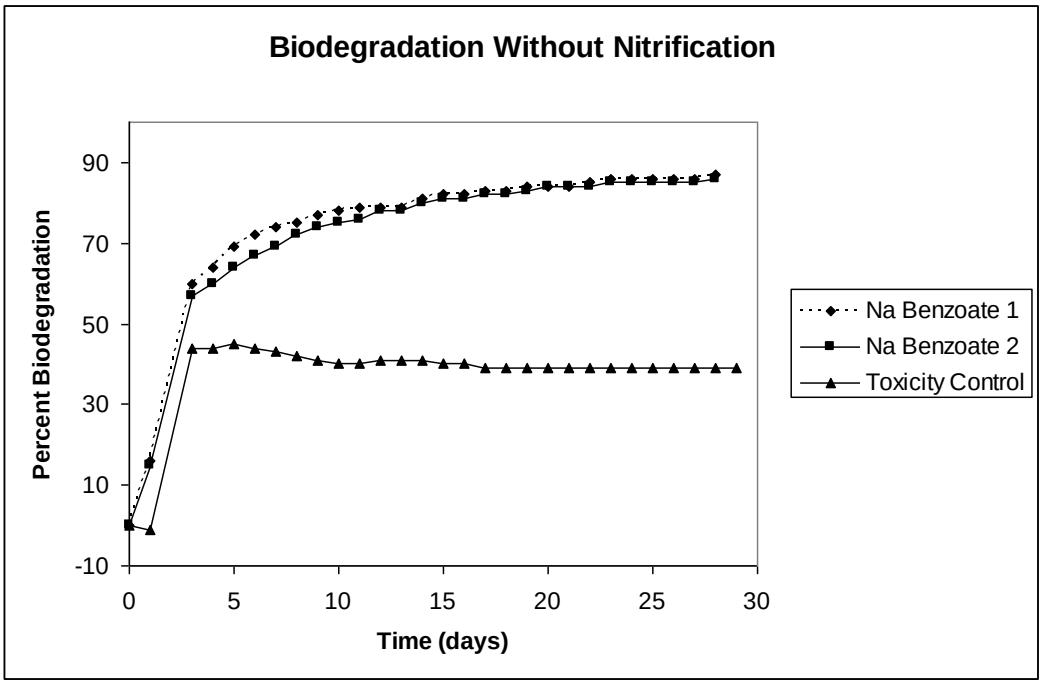
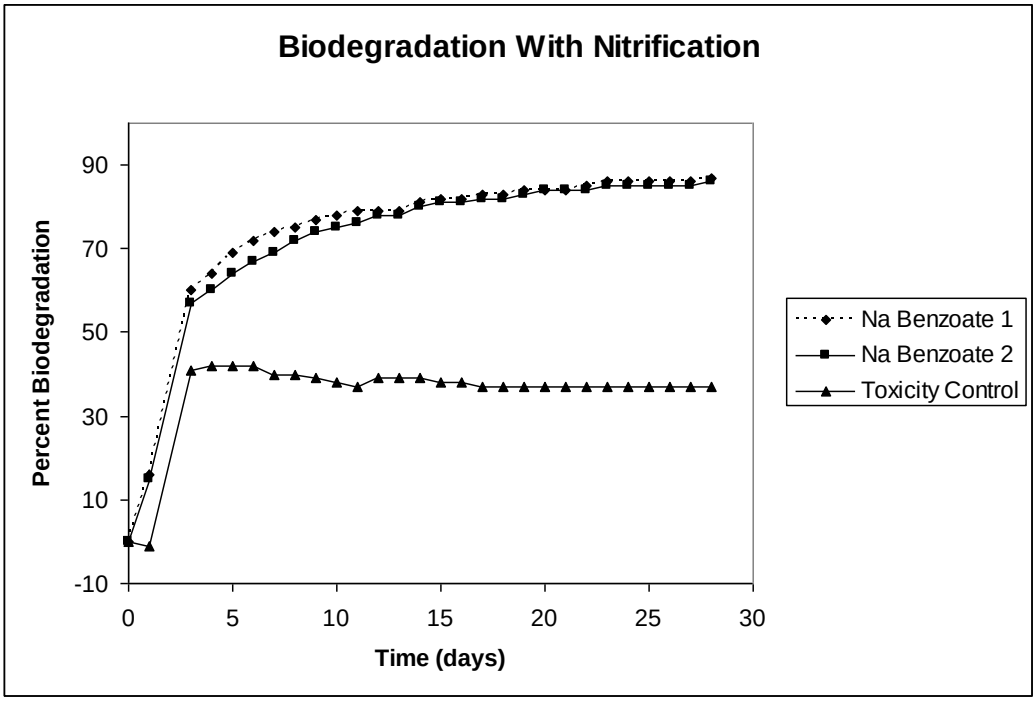



**Figure A7.1.1.2.2-2: Biodegradation in Test Flasks With Nitrification**



**Section A7** **Ecotoxicological Profile Including Environmental Fate and Behaviour**

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**Subsection A7.1.1** **Degradation, initial studies**

**Subsection A7.1.1.2** **Biotic**

**Subsection A7.1.1.2.3** **Biodegradation in Sea Water**

**Annex Point**

| | | 1. REFERENCE | Official use only |
|---|---|---|---|
| **1.1** | **REFERENCE** | **A7.1.1.2.3** [redacted] (2009). Aerobic Transformation of 1,2-Benzisothiazolin-3-one (BIT) in Sea Water; Rohm and Haas Technical Report N° GLP-2009-063 (November 19, 2009), Unpublished. | |
| **1.2** | **DATA PROTECTION** | Yes | |
| 1.2.1 | Data owner | Rohm and Haas Company | |
| 1.2.2 | Companies with letter of access | | |
| 1.2.3 | Criteria for data protection | Data submitted for the first time in support of the first inclusion into Annex I/IA.<br>Data protection claimed in accordance with Article 12.1(c) (ii). | |
| | | **2. GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **GUIDELINE STUDY** | Yes. OECD Guideline for Testing of Chemicals 309: Aerobic Mineralization in Surface Water-Simulation Biodegradation Test (April 2004) | |
| **2.2** | **GLP** | Yes. | |
| **2.3** | **DEVIATIONS** | Four minor GLP deviations:<br>1) plate counts determining microbial activity were not conducted under GLP;<br>2) total organic carbon and nutrients of the surface water were analyzed at Midwest Laboratories, Omaha, NB, USA;<br>3) due to a power failure the temperature in the incubator slightly exceeded that listed in the protocol on two days; and | |

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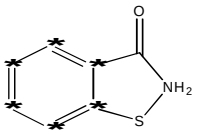
| | | 4) the range finding study was conducted non-GLP.<br>These deviations are not expected to impact the quality or integrity of the study. |
| | | **3. METHOD** |
| **3.1** | **TEST MATERIAL** | |
| 3.1.1 | Test material name | BIT, 1,2-benzisothiazolin-3-one<br>  <br>* site of $^{14}C$ label |
| 3.1.2 | Lot/Batch number | Lot 1069.00 and sublot 1069.0008; $^{14}C$ labeled uniformly in the benzene ring; Specific activity : 53.57 mCi/g. |
| 3.1.3 | Purity | Radiopurity = 98.61% |
| 3.1.4 | Further relevant properties | • Water solubility >0.7g/L<br>• Half-life in soil is 0.23 days (20°C and 5.0 ppm)<br>• Half-life in fresh water at 20°C is 30.8 (25.6 µg/L) and 41.8 (105 µg/L). |
| **3.2** | **REFERENCE SUBSTANCE** | *N*-methyl malonamic acid was used to tune the mass spectrometer in the negative ion mode. |
| **3.3** | **TEST ING PROCEDURE** | |
| 3.3.1 | Water | The water used for the definitive study was sea water obtained from |

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| | characterization | the top 15 cm at Dollar Point Pier, Laporte, Texas, USA. Water parameters including pH, temperature, oxygen content, bacteria cell count, conductivity, total organic carbon, and nutrients, were measured during the experiment and at the end of the study. The results are presented in Table A7.1.1.2.3-1. |
| 3.3.2 | Test system | Experimental System<br><br>Both the range finding and definitive studies employed a flow through system placed in the dark. Sea water was added to 250 mL glass jars which were sealed with a 2-hole stopper containing glass tubes. Following the test water containers were a series of traps the first containing ethylene glycol to capture volatile organics and then two more containing 1.0N NaOH to capture evolved $CO_2$. The test water jars and traps were connected together and to the house vacuum with plastic tubing. The house vacuum which help maintain aerobic conditions in the test jars and remove headspace gases, was adjusted to maintain a slight vacuum (~30 mm Hg).<br><br>The test system was maintained in the dark in an incubator at 20 ± 2°C and the temperature monitored.<br><br>Range Finding Study<br><br>A range finding study was performed to identify the appropriate dosing concentration and sampling intervals. Water was dosed at nominal 20 ppb and 100 ppb (actual concentrations were 19.9 ppb and 99.5 ppb). Ten glass jars containing 100 ml of sea water were dosed at 19.9 ppb and 10 jars containing 50 ml at 99.5 ppb. Duplicate jars were removed at Hours 4, 24, 48, 96, and 216. At harvest, aliquots were radioassayed and aliquots were processed using a preconditioned Oasis Max SPE cartridge. SPE eluants were analyzed by TLC. |

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| | | Definitive Study<br><br>The nominal dosing rate for the definitive study was 20 ppb and 100 ppb and the actual concentration was 22 ppb and 105.2 ppb. At 22 ppb, 20 jars were prepared with 50 mL of sea water and at 105.2 ppb, 26 jars with 50 ml of sea water. Test solution was added slowly to the water surface and then the jar gently swirled before being placed in the flow through apparatus and positioned on an orbital shaker.<br><br>Sterile samples were prepared in an identical manner except that prior to dosing with $^{14}$C-BIT 100 ppm $HgCl_2$ was added and mixed thoroughly. Duplicate sterile samples dosed at both concentrations were taken after 48 hours and duplicate 105.2 ppb samples were taken on Day 10.<br><br>To assist with metabolite identification, 500 mL of sea water was placed into a 1 L glass bottle and dosed at about 1000 ppb $^{14}$C-BIT. This was stored in the dark at 20°C. | |
| 3.3.3 | Method of preparation of test solution | A stock solution was prepared by dissolving 10.34 mg of $^{14}$C-BIT in 2 mL of methanol. Based on radioassay, the concentration of the dosing solution was 259 µg/mL.<br><br>Nominal 20 ppb<br><br>A dosing solution was prepared by diluting 25 µL of stock solution with 2.975 mL of methanol. Radioassay yielded a concentration of 78.5 ppm. To each jar, 14 µL of dosing solution was added.<br><br>Nominal 100 ppb<br><br>A dosing solution was prepared by diluting 25 µL of stock solution with 2.975 mL of methanol. Radioassay yielded a concentration of 82.2 ppm. To each jar, 64 µL of dosing solution was added.<br><br>Nominal 1000 ppb (metabolite identification) | |

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| | | To 500 mL of sea water, 60 µL of the stock solution was added.<br><br>LOD/LOQ Solution<br><br>This was prepared by diluting 0.5 mL of the nominal 20 ppb dosing solution with 2 mL of methanol. The resulting concentration was 14.2 ppm. To 200 mL of sea water, 30 µL was added with an average concentration in four samples of 2.2 ppb. The 1 ppb samples ere prepared by adding 15 µL to 200 mL of seawater. |
| 3.3.4 | Initial TS concentration | The initial test concentration for the definitive study was 22 ppb and 105.2 ppb. In addition, an exaggerated dose of ~1000 ppb was employed to assist with metabolite identification. |
| 3.3.5 | Duration of test | The duration of the definitive test was as listed below.<br><br>**Concentration** **Duration**<br>22 ppb: 30 days<br>105.2 ppb: 31 days<br>1000 ppb: 10 days |
| 3.3.6 | Sampling | |

| Dosing Level | Sampling Intervals-Hours |
|---|---|
| 22 ppb | 0, 2, 6, 24, 48, 72, 96, 144, and 720 |
| 105.2 ppb | 0, 10, 24, 48, 72, 96, 120, 168, 336, and 744 |
| 1000 ppb metabolite identification | 120 and 240 |

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| | | Sterile controls | |
| | | 22 ppb | 48 |
| | | 105.2 ppb | 48 and 240 |
| 3.3.7 | Replicates | Duplicate samples were taken at each interval for the 22 ppb and 105.2 ppb dosing level. Duplicate samples were also taken at each interval for the sterile system. For the LOD/LOQ determination, four replicates at each concentration were measured. | |
| 3.3.8 | Extraction and chromatography | At each sampling interval, duplicate samples were removed and radioassayed. The samples were applied to a preconditioned solid phase extraction cartridge (SPE; Max Oasis). The initial eluant and a 2 ml water wash were radioassayed and if it contained greater than 10% of the applied activity it was chromatographed (TLC). The radioactivity remained at the origin on the plate which is indicative in this system of small polar acids. After the water was the cartridge was subsequently eluted with 10 mL of methanol followed by 10 mL of methanol/ethyl acetate/acetic acid (50/50/1). The combined eluant was concentrated and analyzed by TLC.<br><br>At the 24 h and later sampling intervals, the SPE cartridge underwent an additional elution with 10 mL of methanol/1N HCl (100/1) and methanol/1N KOH (100/1). The eluants were concentrated and analyzed by TLC.<br><br>Quantitation of parent and metabolites was performed using TLC. | |
| 3.3.9 | Analysis of trapped volatiles | Ethylene glycol and NaOH traps were analyzed at every sample interval. | |
| 3.3.10 | Analytical Methods | Thin layer chromatography (TLC) was used to quantitate parent and metabolites as well as isolate metabolites. Extract aliquots were applied to silica gel TLC plates and eluted with ethyl acetate:acetonitrile: methanol:acetic acid (90:5:5:1, v/v/v/v). The location of radioactivity on the TLC plates was determined using a phosphorimager. Zones on the plate were demarcated and the silica gel scrapped and transferred to a liquid scintillation vial for radioassay. For metabolite isolation, plates | |

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| | | were developed as above, and the silica from appropriate zone of radioactivity, identified by the phosphorimager, was scraped and transferred to a megabore Pasteur pipette containing a glass wool plug in the neck. The $^{14}C$-activity was eluted with methanol and concentrated with a stream of nitrogen prior to LC-MS analysis.<br><br>Parent confirmation and metabolite identification was performed on an LC-ion trap-MS. A Metasil AQ-C18 column was employed using a gradient of 0.5% aqueous acetic acid and 0.5% acetic acid in methanol. An electrospray interface was used to introduce the LC flow into the MS and both positive and negative ionization were employed. A radioactivity detector was employed to locate the $^{14}C$-peaks. Additional spectrometry was performed using LC-TOF/MS which can supply exact mass data. Conditions were similar to the LC-ion trap-MS conditions except acetonitrile was used as a solvent instead of methanol.<br><br>Radioactivity from liquid fractions was measured by liquid scintillation counting/spectrometry. Samples were counted 3 times for 5 minutes each (total of 15 minutes) and counting efficiency was determined by an external $Ba^{133}$ standard. Data analysis was performed by validated Rohm and Haas developed software. | |
| 3.3.11 | Degradation products | As described above, surface water was dosed at an exaggerated rate of ~1000 ppb to assist with metabolite isolation and identification. Samples were prepared as describe above using a SPE cartridge. SPE organic eluants were either applied to a TLC plate to isolate the metabolite or injected directly into the LC-MS. | |
| | | **4. RESULTS** | |
| **4.1** | **RANGE FINDING STUDY** | A preliminary experiment at 19.9 ppb and 99.5 ppb at 20°C was performed to estimate the half-life and determine the appropriate sampling intervals. The results are summarized in Table A7.1.1.2.3-2. | |

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| | Within 48 hour BIT had decreased to about 45% of the applied activity. After 96 hours 11.1% and 5.3% of the applied dose was BIT at 19.9 ppb and 99.5 ppb, respectively. The half-life of BIT was 53.3 hours and 21.1 hours at 19.9 ppb and 99.5 ppb, respectively. | |
| **4.2 DEFINITIVE EXPERIMENT** | | |
| 4.2.1 System feasibility | The results from sterile surface water describe the abiotic degradation of BIT. The results presented in Table A7.1.1.2.3-3 show that even after 48 hours of exposure BIT was very stable in a sterilized system as over 93% of the applied activity remained as BIT. At the higher dose over 92% remained as BIT after 10 days. As demonstrated below, biodegradation is the route of BIT dissipation in surface water.<br><br>The average temperature for the 22 ppb experiment was 18.29 ± 0.69°C and for the 105.5 ppb, 19.32 ± 2.30°C. There were a small number of readings which deviated from the desired range of 20 ± 2°C<br><br>The results from the limit of quantitation (LOQ) determination are presented in Table A7.1.1.2.3-4. The TLC LOQ for $^{14}$C-BIT in sea water was determined as 1.2 ppb. The limit of detection (LOD) was 0.4 ppb.<br><br>The region on the TLC plate that co-chromatographed with BIT was confirmed as BIT by LC-MS. | |
| 4.2.2 Distribution and recovery of $^{14}$C-activity | Table A7.1.1.2.3-5 summarizes the distribution between the SPE eluants and volatiles as well as the recovery of applied radioactivity. The amount of radioactivity in the initial SPE eluant from bottles dosed at 22 ppb decreased with time going from 91.4% at of the applied radioactivity at Time 0 to 49.8% at Time 144 hours and 43.8% at 720 hours. At 24 h of treatment and later it was deemed necessary to perform an additional elution of the SPE cartridges using acidic and basic methanol. The radioactivity in this fraction increased with time from 5.7% at Time 24 hours to 18.1% at 96 hours. Less than 0.1% of | |

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| | the applied activity was present as $^{14}CO_2$ after 720 hours and there was no detectable volatile organics in the ethylene glycol trap. Recovery of applied $^{14}$C-activity averaged 89.0 ± 8.2%.<br><br>For bottles dosed at 105.2 ppb $^{14}$C-BIT the percent of applied activity in the initial SPE eluant at 0 hours was 89.5% and that increased to 92.1 after 48 hours. The percentage then decreased to 47.3% of applied at 744 hours. The activity in the additional SPE eluant increased from 3.2% of applied at 24 hours to 16.6% at 744 hours. After 744 hours, 1.2% of the applied activity was detected as $^{14}CO_2$ while there was no detectable volatile organics. Recovery of applied $^{14}$C-activity averaged 96.2 ± 5.3%. | |
| 4.2.3 Quantitation of parent and metabolites | Quantitation of BIT and its metabolites is presented in Table A7.1.1.2.3-6. At 22 ppb, BIT decreased from 86.9% of the applied activity at time 0 to 18.1% at 720 hours. At 105.2 ppb, parent decreased from 85.9% at time 0 to 2.8% after 744 hours.<br><br>At 22 ppb there were 6 metabolites in surface water detected; M1, M2, M3, M4, M5 and M6. Only M1 and M4 exceeded 10% of the applied dose. Metabolite M1 increased with time from 17% of the applied activity at Time 0 to 14.9% at 96 hours and then down to 8.8% at 720 hours. Metabolite M4 was not detected until 96 hours where it comprised 14.5% of the applied dose. M4 increased to 29.1% at 144 hours and then decreased to 19.8% at 720 hours<br><br>At 105.2 ppb there were 7 metabolites detected: M1, M2, M3, M4, M5, M6 and Mx. Mx is nonspecific radioactivity representing all the areas on the TLC plate not corresponding to parent, M1, M2, M3, M4, M5, and M6. Thus it is not a single compound but comprises multiple components. Mx was less than 5% of the applied activity at all sampling intervals. Similar to the lower dose results, Metabolites M1 and M4 exceeded 10% of the applied activity as did M5 for the higher dose level. Metabolite M1 increased from 3.5% of the applied at 0 hours to 13.2% after 744 hours. Metabolite M4 increased from 3.1% of applied at 48 hours to 16.6% at 168 hours and then decreased to 11.9% at 744 hours while M5 increased from 10.3% at 120 hours to 16.3% at 744 hours.<br><br>The amount of $^{14}CO_2$ evolved (Table A7.1.1.2.3-5) was less than 0.1% for the lower dose and for the higher dose, 1.2% at 744 hours. This indicates that the benzene ring remained intact. | |

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4.2.4 Half-life

Quantitation of BIT at each sampling interval is presented in Table A7.1.1.2.3-6 and graphical presentations of the decline of BIT with time appear in Figures A7.1.1.2.3-1 and A7.1.1.2.3-2. The kinetic analysis of $^{14}$C-BIT in sea water is tabulated below:

| Parameter | 22ppb | 105.2 ppb |
|---|---|---|
| K | -0.0103 | -0.0045 |
| $r^2$ | 0.9578 | 0.942 |
| $DT_{50}$ | 67.3 hours | 154.0 hours |
| $DT_{90}$ | 223.3 hours | 511.1 hours |

These results demonstrate that BIT biodegrades very quickly in sea water. The longer half-life observed at the higher concentration is at least partly due to the lower microbial activity present in this water sample (560 cfu/mL versus 12000 cfu/mL for the 22 ppb dose). The OECD Guidelines suggest that the 22 ppb dose level is the more environmentally relevant concentration

4.2.5 Identification of metabolites

Table A7.1.1.2.3-7 summarizes the metabolite identification. The metabolites, which exceeded 10% of the applied were identified by mass spectroscopy as the following:

- M1, 2-sulfobenzamide,
- M4, 2-methylthio-benzamide,
- M5, 2-(4-hydroxyphenylsulfanyl)-benzamide.

In addition, Metabolite M3 which was less than 5% of applied was identified as:

- M3, 2-methylsufinyl-benzamide,

Structural identification was based on fragmentation and exact mass analysis.

Metabolite M5 is most likely the result of a halogenated phenol

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| | | being present in the sampled Houston Bay sea water. Similar to other nucleophiles such as $SH^-$, $CN^-$, and cysteine which have been shown to initiate a nucleophilic attack on the N-S bond of isothiazolones, phenolic compounds should also be able to initiate a nucleophilc attack on the electrophilic BIT molecule. Thus Metabolite M5 is a product of this particular environmental condition which would explain its absence at the lower dose. | |
| 4.2.6 | Metabolic pathway | A metabolic pathway is presented in Figure A7.1.1.2.3-3. | |
| | | **5. APPLICANT'S SUMMARY AND CONCLUSION** | |
| **5.1** | **MATERIALS AND METHODS** | The test guideline followed was the OECD Guideline for the Testing of Chemicals 309: Aerobic Mineralization in Surface Water - Simulation Biodegradation Test, April 13, 2004<br><br>Bottles containing 50 mL of surface sea water collected from Dollar Point Pier, Laporte, Texas, USA were dosed at either 22 ppb or 105.2 ppb. The samples were placed on an orbital shaker in a dark incubator at 20°C. A vacuum was applied to maintain aerobic conditions and remove volatiles which were trapped in ethylene glycol and KOH. The measured mean temperature of the dark incubator was 18.29 ± 0.69°C for water dosed at 22 ppb and 19.32 ± 2.30°C for 105.2 ppb. Sterile systems were prepared in a similar manner except $HgCl_2$ was added.<br><br>Duplicate nonsterile samples were removed on Hours 0, 2, 6, 24, 48, 72, 96, 144, and 720 for the 22 ppb dosing level and Hours 0, 10, 24, 48, 72, 96, 120, 168, 336, and 744 for the 105.2 ppb level. Sterile flasks were removed at Hours 48 and 240. After radioassaying, the aqueous sample was applied to an SPE cartridge and eluted with methanol followed by methanol/ethyl acetate/acetic acid From 24 hours and later, an additional elution of the cartridge was performed using methanol:HCl | |

followed by methanol:KOH. The organic phases were concentrated and chromatographed for quantitation of parent and metabolites and for identification of metabolites. The ethylene glycol and KOH traps were also radioassayed. To aid in metabolite identification, sea water was dosed at approximately 1000 ppb $^{14}$C-BIT and processed with SPE cartridges as described above. Metabolites were identified by LC-MS.

**5.2 RESULTS AND DISCUSSION**

The amount of $^{14}$C activity initially eluted from the SPE cartridge generally decreased with time for both dosing levels. For samples dosed at 22 ppb the $^{14}$C-activity eluted with the additional solvents increased between 24 and 96 hours and then decreased slightly while for the 105.2 ppb the applied activity increased with time. This indicates that BIT is being quickly degraded. Under sterile conditions, there was essentially no degradation of BIT demonstrating that biodegradation is the primary route of dissipation for BIT in sea water.

BIT biodegrades very fast in the sea water studied. The half-lives at 20°C were 67.3 hours at 22 ppb and 154 hours at 105.2 ppb.

The table below provides the metabolites detected at greater than 10% of applied and their maximum percentage. They were identified by mass spectroscopy.

| Metabolite | Maximum Percent of Applied Dose | |
|---|---|---|
| | 22 ppb | 105.2 ppb |
| 2-sulfobenzamide | 14.9 | 13.2 |
| 2-methylthio-benzamide | 29.1 | 16.6 |
| 2-(4-hydroxyphenylsulfanyl)-benzamide | Not present | 16.3 |
| $^{14}CO_2$ | <0.1 | 1.2 |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.1** | **Fate and Behaviour in water** |
| **Subsection A7.1.1** | **Degradation, initial studies** |
| **Subsection A7.1.1.2** | **Biotic** |
| **Subsection A7.1.1.2.3** | **Biodegradation in Sea Water** |
| **Annex Point** | |

| | | | |
|---|---|---|---|
| | | . | |
| **5.3** | **CONCLUSION** | Similar to the results in other media (*e.g.* fresh water, soil and STP), BIT quickly biodegrades in sea water. The half-life at 20°C was 67.3 hours at 22 ppb and 154 hours at 105.2 ppb. Sterile samples were essential stable. Metabolism involved cleavage of the isothiazolone ring, leading to the formation of benzamide metabolites. . | |
| 5.3.1 | Reliability | 1-valid without restrictions | |
| 5.3.2 | Deficiencies | No | |

| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *November 2010* |
| **Materials and Methods** | *Applicant's version is accepted with the following remarks:*<br>*The applicant uses the OECD guide 309, while for seawater, the OECD 306 is recommended. However, the RMS understands that the OECD guideline used was also appropriated for the cited study.* |
| **Results and discussion** | *Applicant's version is adopted with the following remarks:*<br>*Figure A7.1.1.2.3-3: "Metabolic Pathway of CMIT in Sea Water" should read "**Proposed** Metabolic Pathway for **BIT** in Sea Water"* |

## Section A7 Ecotoxicological Profile Including Environmental Fate and Behaviour

## Subsection A7.1 Fate and Behaviour in water

## Subsection A7.1.1 Degradation, initial studies

## Subsection A7.1.1.2 Biotic

## Subsection A7.1.1.2.3 Biodegradation in Sea Water

**Annex Point**

| | |
|---|---|
| **Conclusion** | *BIT quickly biodegrades in sea water. Three major transformation products (> 10% of applied radioactivity) were identified in the present study: 2-sulfobenzamide, 2-methylthiobenzamide and 2-(4-hydroxyphenylsulfany1)-benzamide.*<br>*The half-life at 20°C was 67.3 hours at 22 ppb and 154 hours at 105.2 ppb. Sterile samples were essential stable. Metabolism involved cleavage of the isothiazolone ring, leading to the formation of benzamide metabolites. Ultimate biodegradation of BIT did not occur at the conditions and concentration tested.* |
| **Reliability** | *2* |
| **Acceptability** | *Acceptable.* |
| **Remarks** | |

**Table A7.1.1.2.3-1: Parameters of Test Water**

| Parameter | Method Development /Range Finding | 22 ppb Dosing | 105.2 ppb Dosing |
|---|---|---|---|
| pH | 8.01 | 7.80 | 8.48 |
| Temperature (°C)[2] | 21.1 | 19.6 | 21.0 |
| Dissolved Oxygen (ppm) | NA[1] | 6.50 | 6.34 |
| Calcium (ppm) | NA | 178 | 268 |
| Magnesium (ppm) | NA | 507 | 869 |
| Sodium (ppth) | 36.8 | 48.9 | 81.4 |
| Conductivity (mmhos/cm) | NA | 23.1 | 367 |
| Sodium Adsorption Ratio | NA | 42 | 54 |
| Total Dissolved Solids (ppm) | NA | 15015 | 23885 |
| Nitrogen/Phosphorus/ Potassium (mg/L) | NA | 1.43/0.3/155 | 1.2/ND[2]/270 |
| Total Organic Carbon (mg/L) | NA | 3.6 | 4.2 |
| Microbial Activity (cfu/ml) | 1808 | 12000 | 560 |

[1] NA= not analyzed
[2] ND = not determined

**Table A7.1.1.2.3-2: Range Finding Study—Quantitation of Parent**

| Sample Interval (hours) | Percent BIT[1] | |
|---|---|---|
| | 19.9 ppb | 99.5 ppb |
| 4 | 85.5 | 86.9 |
| 24 | 84.1 | 87.4 |
| 48 | 44.5 | 46.4[a] |
| 96 | 11.1 | 5.3 |
| 216 | 6.5 | 5.5[a] |

[1] Average of duplicate samples
[2] Not used in half-life calculation due to insufficient data recorded to verify recovery values

**Table A7.1.1.2.3-3: Stability of BIT in Sterile Water**

| Study Type | BIT in Sterile Water as Percent of Applied Activity[1] | |
|---|---|---|
| | 48 hours | 10 day |
| 22 ppb Definitive | 100 | --[2] |
| 105.2 ppb Definitive | 93.1 | 92.2 |

[1] Average of duplicate samples.
[2] Not done at Day 10.

**Table A7.1.1.2.3-4: LOD Determination of BIT in Sea Water**

| Dose Rate | Percent Recovered | Percent BIT |
|---|---|---|
| 2.2 ppb | 87.3 | 80.5 |
| | 97.1 | 83.9 |
| | 83.9 | 78.1 |
| | 95.1 | 89.8 |
| *2.2 ppb Average* | 90.8 ± 6.3 | 83.1 ± 5.1 |
| 1.2 ppb | 94.1 | 81.9 |
| | 96.7 | 85.9 |
| | 92.3 | 81.0 |
| | 93.5 | 79.4 |
| *1.2 ppb Average* | 94.2 ± 1.9 | 82.1 ± 2.8 |

**Table A7.1.1.2.3-5: Distribution of $^{14}C$-Activity in Sea Water**

| Sample Time (hrs) | Percent of Applied $^{14}C$-Activity (average of duplicate samples) | | | | |
|---|---|---|---|---|---|
| | Initial SPE Eluant | Additional SPE Eluant | $CO_2$ | Organic Volatiles | Recovery |
| **22 ppb** | | | | | |
| 0 | 91.4 | NA[1] | 0.0 | ND[2] | 93.2 |
| 2 | 90.6 | NA | 0.0 | ND | 92.9 |
| 6 | 91.4 | NA | 0.0 | ND | 94.3 |
| 24 | 87.2 | 5.7 | 0.0 | ND | 97.5 |
| 48 | 73.8 | 8.8 | 0.0 | ND | 89.7 |
| 48 STERILE | 88.7 | NA | 0.0 | ND | 92.1 |
| 72 | 62.8 | 13.5 | 0.0 | ND | 88.6 |
| 96 | 55.9 | 18.1 | 0.0 | ND | 89.3 |
| 144 | 49.8 | 13.6 | 0.0 | ND | 82.4 |
| 720 | 43.8 | 12.1 | 0.0 | ND | 69.8 |
| | | | Average Recovery: | | 89.0 ± 8.2% |
| **105.5 ppb** | | | | | |
| 0 | 89.5 | NA | 0.0 | ND | 90.9 |
| 10 | 91.5 | NA | 0.0 | ND | 92.8 |

| Sample Time (hrs) | Percent of Applied $^{14}$C-Activity (average of duplicate samples) | | | | |
|---|---|---|---|---|---|
| | Initial SPE Eluant | Additional SPE Eluant | $CO_2$ | Organic Volatiles | Recovery |
| 24 | 94.6 | 3.2 | 0.0 | ND | 101.9 |
| 48 | 92.1 | 4.0 | 0.0 | ND | 101.9 |
| 48 STERILE | 95.0 | NA | 0.0 | ND | 99.3 |
| 72 | 86.5 | 5.1 | 0.0 | ND | 100 |
| 96 | 85.9 | 5.4 | 0.0 | ND | 102.4 |
| 120 | 77.5 | 6.1 | 0.0 | ND | 96.6 |
| 168 | 69.4 | 7.1 | 0.0 | ND | 95.0 |
| 240 STERILE | 93.1 | NA | 0.0 | ND | 95.6 |
| 336 | 57.7 | 9.9 | 0.17 | ND | 93.7 |
| 774 | 47.3 | 16.6 | 1.2 | ND | 84.6 |
| | | | Average Recovery: | | 96.2 ± 5.3% |

[1] NA = not applicable
[2] ND = not detected

**Table A7.1.1.2.3-6: Quantitation of Parent and Metabolites**

| Sample Time | Percent of Applied $^{14}$C-Activity (average of duplicate samples) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | BIT | M1 | M2 | M3 | M4 | M5 | M6 | Mx[1] |
| **22 ppb** | | | | | | | | |
| 0 | 86.9 | 1.7 | NS[2] | NS | NS | NS | 2.8 | |
| 2 | 83.3 | 5.0 | NS | NS | NS | NS | 2.4 | |
| 6 | 82.2 | 3.6 | NS | NS | NS | NS | 3.6 | |
| 24 | 72.2 | 6.6 | NS | NS | NS | NS | 8.4 | |
| 48 | 62.0 | 11.5 | 7.3 | NS | NS | NS | 2.3 | |
| 72 | 49.2 | 13.0 | 5.4 | NS | NS | NS | 1.9 | |
| 96 | 37.9 | 14.9 | 5.8 | NS | 14.5 | NS | 0.9 | |
| 144 | 17.7 | 11.7 | 3.5 | 0.6 | 29.1 | NS | 0.8 | |
| 720 | 18.1 | 8.8 | 9.2 | NS | 19.8 | NS | NS | |
| **105.5 ppb** | | | | | | | | |
| 0 | 85.9 | 3.5 | NS | NS | NS | NS | NS | NS |
| 10 | 88.3 | 3.2 | NS | NS | NS | NS | NS | NS |
| 24 | 89.8 | 4.8 | NS | NS | NS | NS | NS | NS |
| 48 | 84.7 | 4.2 | NS | NS | 3.1 | NS | NS | NS |
| 72 | 70.9 | 4.7 | 1.3 | 0.8 | 1.8 | NS | 9.0 | 2.7 |
| 96 | 77.2 | 5.3 | 1.4 | 1.4 | 1.8 | NS | 0.9 | 3.4 |
| 120 | 50.6 | 6.7 | 1.6 | 1.0 | 6.8 | 10.3 | 5.0 | 3.0 |
| 168 | 37.1 | 6.0 | 0.4 | 3.4 | 16.6 | 9.0 | 0.3 | 3.6 |
| 336 | 40.9 | 7.3 | 3.2 | 0.8 | 10.5 | NS | 0.4 | 4.5 |
| 774 | 2.8 | 13.2 | 5.3 | 4.8 | 11.9 | 16.3 | 4.3 | 4.6 |

[1] Mx = nonspecitic radioactivity recovered from the TLC palte areas other than the spots designated for M1, M2, M3, M4, M5, M6, and BIT. Significant Mx was not detected in the 22 ppb dosed samples.

[2] NS = less than LOD

**Table A7.1.1.2.3-7: Structure of Metabolites Produced in Sea Water Dosed with BIT**

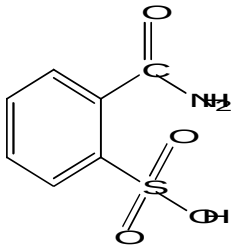
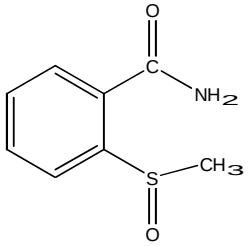
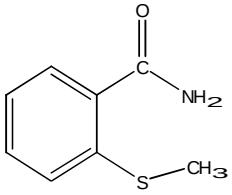
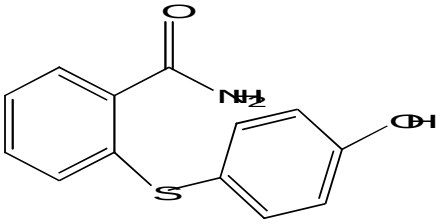
| Component | Structure | Name | Maximum Percent of Applied $^{14}$C-Activity |
|---|---|---|---|
| M1 |  | 2-Sulfobenzamide | 14.9% at 96 hours (22 ppb)<br>13.2% at 744 hours (105.2 ppb) |
| M3 |  | 2-methylsulfinyl-benzamide | 0.6% at 144 hours (22 ppb)<br>4.8% at 744 hours (105.2 ppb) |
| M4 |  | 2-methylthio-benzamide | 29.1% at 144 hours (22 ppb)<br>16.6% at 168 hours (105.2 ppb) |
| M5 |  | 2-(4-hydroxyphenylsulfanyl)-benzamide | Not present (22 ppb)<br>16.3% at 744 hours (105.2 ppb) |

**Figure A7.1.1.2.3-1: Kinetic Analysis of BIT in Sea Water Dosed at 22 ppb**

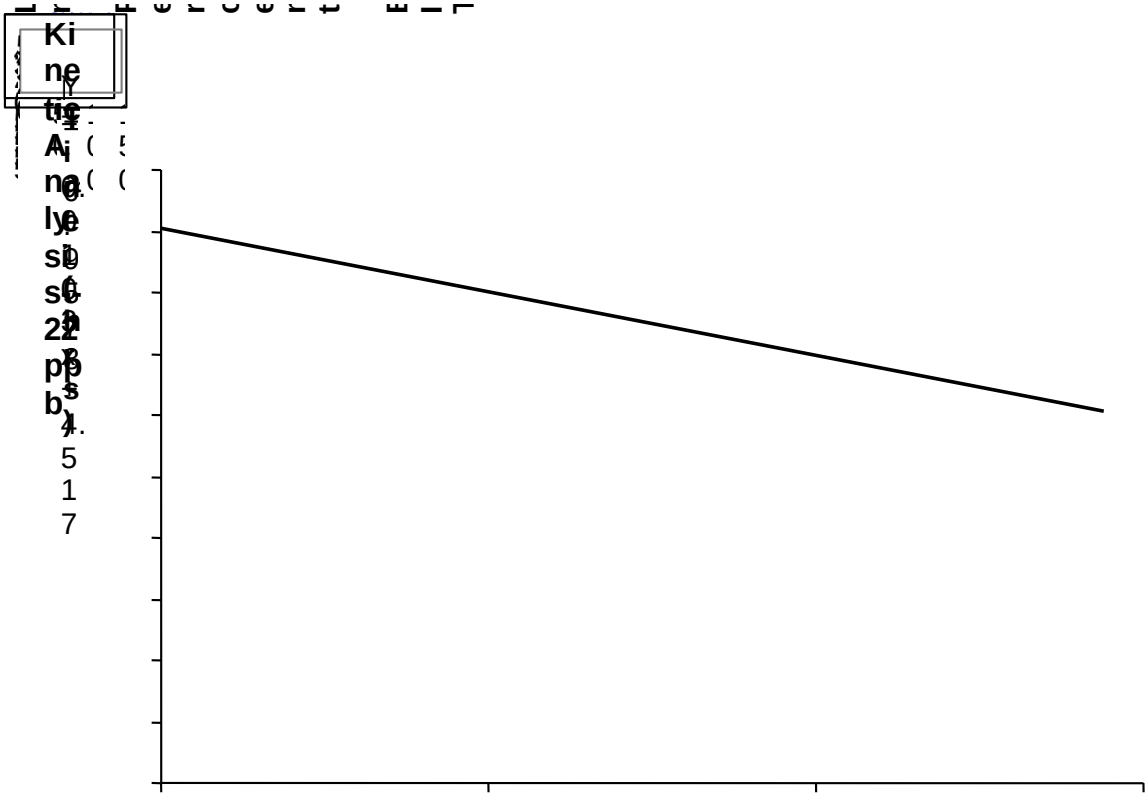



**Figure A7.1.1.2.3-2: Kinetic Analysis of BIT in Sea Water Dosed at 105.2 ppb.**

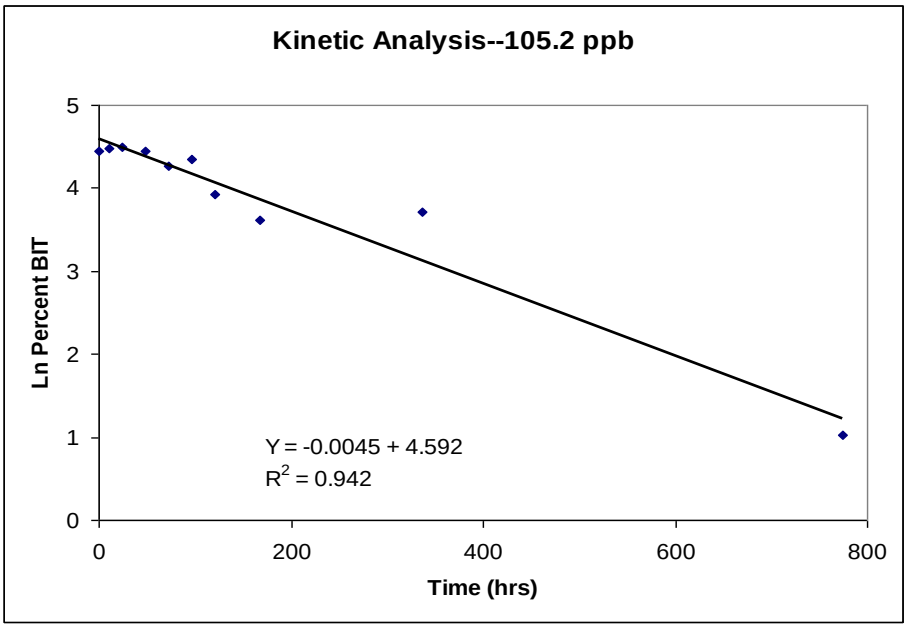
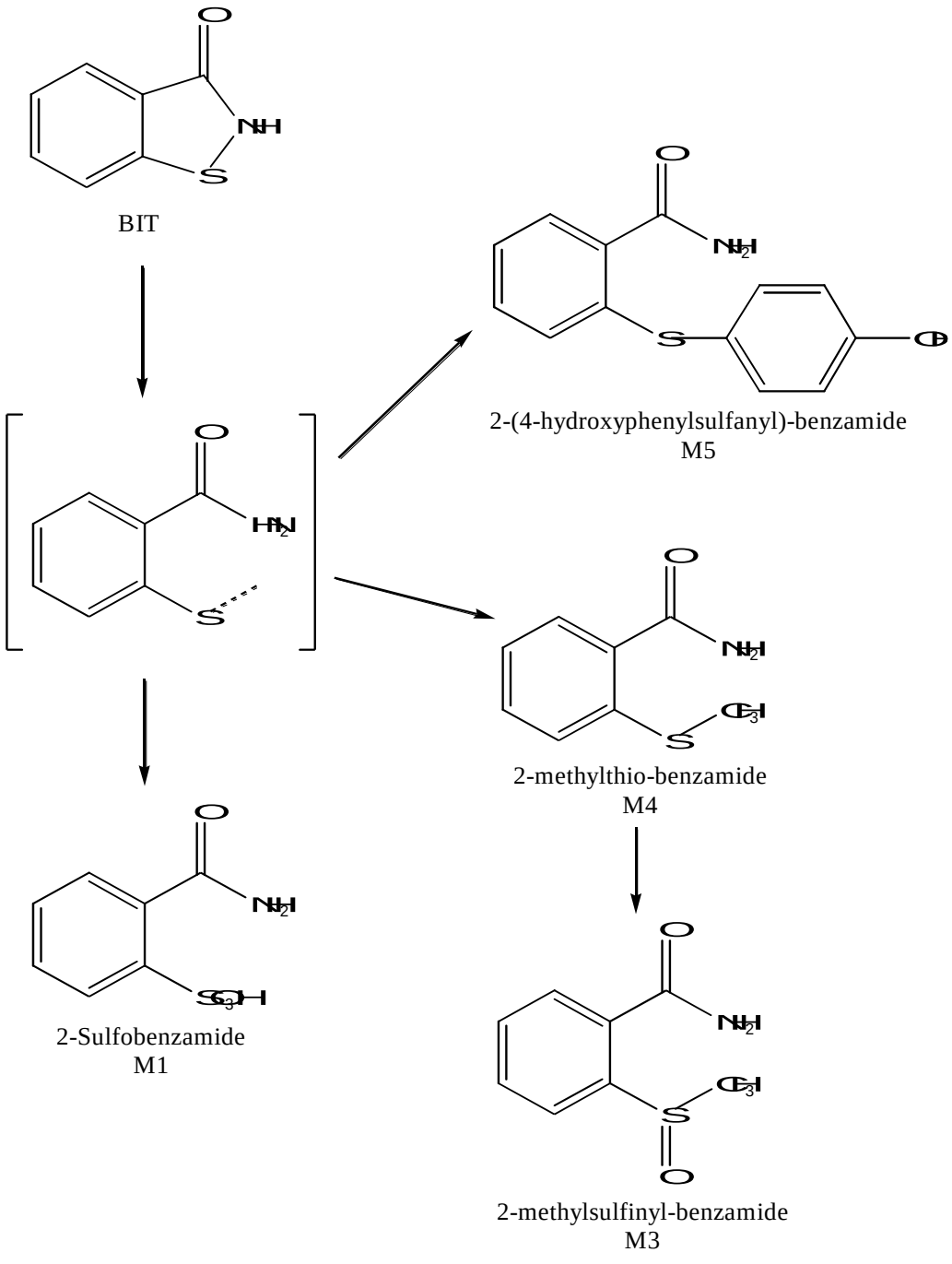


**Figure A7.1.1.2.3-3: Metabolic Pathway of CMIT in Sea Water**



**Section A7** **Ecotoxicological Profile Including Environmental Fate and Behaviour**

**Subsection A7.1** **Fate and Behaviour in Water**

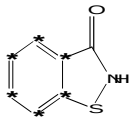
**Subsection A7.1.2.1** **Biological Sewage Treatment**

**Subsection A7.1.2.1.1** **Aerobic**

**Annex Point**

| | | 1 REFERENCE | Official use only |
|---|---|---|---|
| **1.1** | **Reference** | **A7.1.2.1.1/01** [redacted] (2008) $^{14}$C-1,2,Benzisothiazolin-3-one: Porous Pot Test Method for Assessing the Biodegradability of the Test Substance During Wastewater Treatment Simulation. [redacted] Rohm and Haas Technical Report No. TR-008-002, (September 9, 2008) Unpublished.<br><br>**A7.1.2.1.1/02** [redacted] (2008) Kinetic Analysis to Determine the Half-Life of BIT in an STP Simulation Study: Supplemental to Rohm and Haas Technical Report No. TR-08-002, Rohm and Haas Technical Report No. TR-008-053 (29 September 2008) Unpublished.<br><br>**A7.1.2.1.1/03** [redacted] (2009) Metabolite Identification for Samples Generated from BIT Wastewater Treatment Simulation Study (TR-08-002), Rohm and Haas GLP Report No. GLP-2009-046 (04 August 2009), Unpublished. | |
| **1.2** | **Data protection** | Yes | |
| 1.2.1 | Data owner | Rohm and Haas Company | |
| 1.2.2 | Companies with letter of access | | |
| 1.2.3 | Criteria for data protection | Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I/IA.<br><br>Data protection claimed in accordance with Article 12.1(c) (ii), as data generated after the entry into force of the Directive. | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **Guideline Study** | A7.1.2.1.1/01<br><br>Yes. OECD Guideline 303A, Simulation Test—Aerobic Sewage Treatment: Activated Sludge and U.S. Environmental Protection Agency Office of Prevention, Pesticides, and Toxic Substances 835.3220. | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.1** | **Fate and Behaviour in Water** |
| **Subsection A7.1.2.1** | **Biological Sewage Treatment** |
| **Subsection A7.1.2.1.1** | **Aerobic** |

| **Annex Point** | | | |
|---|---|---|---|
| | | A7.1.2.1.1/02 and A7.1.2.1.1/03<br>No applicable guidelines | |
| **2.2** | **GLP** | A7.1.2.1.1/01: Yes<br>A7.1.2.1.1/02: Not applicable (calculations only)<br>A7.1.2.1.1/03: Yes | |
| **2.3** | **Deviations** | A7.12.1.1/01<br>Two minor GLP deviations. 1) Characterization and stability of test material under site specific storage conditions were not performed in accordance with GLP guidelines (however chemical characterization was performed under GLP by the sponsor) and 2) analysis of water (purified and municipal) for contaminants were not performed by a GLP certified laboratory (however were performed by a certified laboratory using U.S. EPA analytical methods).<br>A7.1.2.1.1/02: Not Applicable<br>A7.1.2.1.1/03: None | |
| | | **3 MATERIAL AND METHODS** | |
| **3.1** | **Test Material (A7.1.2.1.1/01)** | $^{14}$C-BIT<br>  <br>* position of the $^{14}$C-label | |
| 3.1.1 | Lot/Batch number | 1069.00 | |
| 3.1.2 | Purity | Radiopurity > 98% | |
| 3.1.3 | Further relevant properties | • Soil adsorption $K_f$ = 55.6<br>• Water solubility (deionized water) > 0.7 g/L<br>• Half-life in aerobic soil simulation study is 5.6 hours (20°C)<br>• Half-life in aerobic surface water simulation study is 31 hours | |

**Section A7** **Ecotoxicological Profile Including Environmental Fate and Behaviour**

**Subsection A7.1** **Fate and Behaviour in Water**

**Subsection A7.1.2.1** **Biological Sewage Treatment**

**Subsection A7.1.2.1.1** **Aerobic**

**Annex Point**

| | | | |
|---|---|---|---|
| | | (20°C) | |
| **3.2** | **Reference substances** | No reference substances were employed to validate the STP system. | |
| **3.3** | **Sludge** | | |
| 3.3.1 | Test inoculum | Fresh settled sewage was collected from the Cambridge Wastewater Facility, Cambridge, Maryland, USA and sieved through a 2 mm sieve. This facility treats sewage of predominantly domestic origin. The total suspended solids concentration was measured and adjusted to approximately 2500 mg/L | **X** |
| 3.3.2 | Domestic sewage | Domestic sewage was collected weekly from Cambridge Wastewater Facility, Cambridge, Maryland, USA and sieved through a 1 mm sieve. This sewage provides nutrients for the bacterial metabolism. The sewage was maintained refrigerator and continuously stirred. | |
| **3.4** | **Test procedures** | | |
| 3.4.1 | Test system | A bioreactor was comprised of a "porous pot"; a glass vessel containing a porous polyethylene membrane that retains the solids but allows the liquid to flow through the system. The test contained two bioreactors that were continuously dosed with $^{14}$C-BIT and a single control reactor that was not exposed to the test substance but allowed measurements of the operational parameters.<br><br>Approximately 1.13 L of test inoculum (adjusted to 2500 mg/L of total suspended solids) was added to each bioreactor. During the Stabilization Period, 2.4 mL/min of domestic sewage was added plus 0.3 mL/min water while during the Acclimation Period and Steady State Period 2.4 mL/min of domestic sewage and 0.3 mL/min of 0.25 ppm $^{14}$C-BIT solution (or 0.3 mL/min water to the control reactor) were added to each bioreactor. The resulting hydrolytic retention time (HRT) was approximately 7 hours.<br><br>Approximately 113 mL/day of the activated sludge/domestic sewage was removed from each bioreactor per day yielding a sludge retention time (SRT) of approximately 10 days.<br><br>Test temperature, measured daily was maintained at 20°C – 22°C. The pH was measured at least twice a week and if necessary adjusted to 7.5 ± 0.5. Dissolved oxygen was also measured at least twice a week and aeration rates were adjusted so that the dissolved oxygen concentration | |

**Section A7** **Ecotoxicological Profile Including Environmental Fate and Behaviour**

**Subsection A7.1** **Fate and Behaviour in Water**

**Subsection A7.1.2.1** **Biological Sewage Treatment**

**Subsection A7.1.2.1.1** **Aerobic**

**Annex Point**

| | | |
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| | was greater than 2 mg/L.<br><br>A stabilization period during which the sludge becomes adjusted to the test system lasted 8 days. During this period all three bioreactors received 0.3 mL/min of water (instead of $^{14}$C-BIT in the two test reactors). The stabilization period ended once the DOC and/or COD removal was greater than or equal to 80% (actually achieved in 4 days).<br><br>After the stabilization period the two test bioreactors were dosed continuously at nominal 0.25 mg/L $^{14}$C-BIT (the BIT was substituted with water in the control bioreactor). The acclimation period lasted 12 days. DOC and COD concentration were measured twice weekly and the influent, effluent and mixed liquor samples were radioassayed periodically.<br><br>At the termination of the acclimation period a steady state period was initiated lasting 22 days. During this period the effluent from each bioreactor was collected in a sealed container. The effluent gases from the containers were passed through a 1.5N KOH trap. The dosing solution, the combined influent, effluent, mixed liquor, and KOH traps were collected three times each week and radioassayed. | |
| 3.4.2 Preparation of test solution | A7.1.2.1.1/01<br><br>A stock solution was prepared containing 103.37 mg of $^{14}$C-BIT dissolved in 10 mL of ethanol. The stock solution was stored frozen.<br><br>A dosing solution was prepared using 1.58 mL of the stock solution and diluting with 7 L of nitrogen purged water to obtain a final concentration of approximately 2.3 mg $^{14}$C-BIT/L. Concentration was verified by radioassay and the percentage of BIT in the solution analyzed by HPLC. The results are in Table A7.1.2.1.1-1. Average concentration was 2.35 mg/L (102% of nominal value) and the solutions averaged 97% BIT. Dosing solutions were prepared at least weekly and were continuously refrigerated and mixed. Additionally they were maintained in the dark and in a nitrogen atmosphere to prevent oxidation.<br><br>Water was administered to the control bioreactor under the same conditions as the BIT dosed bioreactors.<br><br>A7.1.2.1.1/03<br><br>A stock solution was prepared by dissolving 10.34 mg $^{14}$C-BIT into 2 ml of methanol. A dosing solution was prepared by combining 40 µL of this stock solution with 3.960 mL of methanol. The final concentration based on radioassay was 94.1 ppm. Both the stock | |

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| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.1** | **Fate and Behaviour in Water** |
| **Subsection A7.1.2.1** | **Biological Sewage Treatment** |
| **Subsection A7.1.2.1.1** | **Aerobic** |
| **Annex Point** | |

| | | | |
|---|---|---|---|
| | | solution and dosing solution were stored in the freezer until needed. | |
| 3.4.3 | Dosing of test unit | The 2.35 mg/L $^{14}$C-BIT dosing solution was delivered by volumetric addition at a rate of 0.3 mL/min and this was combined with domestic sludge at a rate of 2.4 mL/min. The resulting nominal dosing concentration was 0.25 mg $^{14}$C-BIT/L. The flow rates for both the $^{14}$C-BIT and the domestic sewage was measured each working day and adjusted if necessary.<br>In the control units, 0.3 mL/min of water was substituted for the $^{14}$C-BIT. | |
| 3.4.4 | Duration of test | The unit was operated for 8 days (stabilization period) before dosing.<br>Dosing with $^{14}$C-BIT continued for a period of 33 days; 12 days acclimation and 22 days steady state. | |
| 3.4.5 | DOC/COD analysis | DOC was measured using a carbon analyzer. COD was measured using Hach Method 8000 and a Hach DR/890 colorimeter with preprogrammed calibrations. | |
| 3.4.6 | Sampling analysis: dosing solution and influent | The dosing solution was analyzed periodically by removing triplicate aliquots and radioassaying. Additionally, aliquots were diluted for HPLC quantitation of percent parent.<br>Periodically replicate aliquots of the influent were obtained and radioasssayed. | |
| 3.4.7 | Sample analysis: effluent | A7.1.2.1.1/01<br>The effluent was analyzed on Days 10, 13, 14, and 16 during the stabilization period and all on non-weekend days throughout the steady test period. Aliquots were radioassayed to determine total $^{14}$C-activity. An additional 10 mL aliquot was removed and 1 mL of acetonitrile added. The sample was filtered and chromatographically analyzed by HPLC to quantitate the amount of BIT remaining in the effluent.<br>The KOH traps were radioassayed three times a week.<br>A7.1.2.1.1/03 (Metabolite Identification)<br>Six ml effluent samples from Days 1 through 41of the original simulation study (A7.1.2.1.1/01) were sent frozen to Rohm and Haas Technical Center where they were temporarily stored in a freezer. Samples as listed below were selected for metabolite identification: | |

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| | Porous Pot Reactor #2: Days 13, 31, 36, 38, and 41<br><br>Porous Pot Reactor #3: Days 13, 20 , 28, 36, 37, and 38<br><br>Samples to be analyzed were removed from the freezer, radioassayed and preserved with $HgCl_2$. The sample was concentrated to about 1 mL, filtered, and analyzed by either HPLC (for metabolite profiling/quantitation) or LC-MS (metabolite identification).<br><br>As part of the metabolite identification, the storage stability of BIT in effluent was examined. Control effluent (150 mL) was mixed with 15 mL of acetonitrile mimicking the procedure done in the initial study. $^{14}$C-BIT was added to give a concentration of 0.25 µg/L, the sample mixed, and 4 mL aliquots transferred into vials and stored either in a refrigerator (~4°C) or a freezer (~18°C). Periodically over 89 days, duplicate vials were removed from the refrigerator and freezer, 1 mL aliquots transferred to autosampler vials, and the analyzed by HPLC. |
| 3.4.8 Sample analysis: Mixed liquor | A7.1.2.1.1/01<br><br>A mixed liquor sample was taken every workday during the steady test period. A 40 mL aliquot of mixed liquor was centrifuged and the supernatant radioassayed. To a 10 mL aliquot of the supernatant, 1 mL of acetonitrile was added, the sample filtered, and chromatographed (HPLC).<br><br>The solids resulting from centrifugation were extracted 3 times with acetonitrile and the combined volume determined and aliquots radioassayed. Aliquots of the remaining solids were combusted prior to radioassay. A 25 mL portion of the acetonitrile extract was concentrated to dryness, redissolved in 0.2 – 0.5 mL of acetonitrile followed by 1.8 to 4.5 mL of 0.1% aqueous $H_3PO_4$. The resulting samples were chromatographed (HPLC).<br><br>A7.1.2.1.1/03<br><br>Six ml aliquots of the supernatant that was produced by centrifugation of the mixed liquor sludge from Days 1 through 41 of the original simulation study (A7.1.2.1.1/01) were sent frozen to Rohm and Haas Technical Center where they were temporarily stored in a freezer. A number samples as listed below were selected for metabolite identification:<br><br>Porous Pot Reactor #2: Days 21 and 37<br><br>Porous Pot Reactor #3: Day 24<br><br>Samples to be analyzed were removed from the freezer, radioassayed |

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| | | and preserved with $HgCl_2$. The sample was concentrated to about 1 mL, filtered, and analyzed by either HPLC (for metabolite profiling/quantitation) or LC-MS (metabolite identification).<br><br>Even though ACN extracts were provided, it was decided to analyze only the supernatant from the mixed liquor sludge. The activities in the ACN extracts were too low for metabolite identification.<br><br>To examine the stability of 14C-BIT, control sludge was centrifuged and the 150 mL of the supernatant was mixed with 15 mL of acetonitrile, again mimicking the procedure employed in the original study. $^{14}$C-BIT was added to give a final concentration of 0.26 µg/L, the sample mixed, placed into a plastic bottle, and stored in a refrigerator. Periodically over 89 days, 1 mL aliquots were removed, transferred to autosampler vials, and duplicate analysis by HPLC was performed.<br><br>The sludge remaining after centrifugation was transferred to a centrifuge tube, 10 mL of acetonitrile added, mixed, centrifuged, and the acetonitrile supernatant removed. The sludge was extracted two more times with acetonitrile and placed in the refrigerator. The next day the extract was dosed with $^{14}$C-BIT for a concentration of 0.24 µg/L and returned to the refrigerator. Duplicate samples were analyzed periodically over 89 days | |
| 3.4.9 | Analytical methods | A7.1.2.1.1/01<br><br>Radioassay of solutions was performed using liquid scintillation counters. Solid samples were first combusted in a sample oxidizer to yield $^{14}CO_2$ which was trapped in a liquid adsorbent. The resulting sample was then quantitated by liquid scintillation spectrometry.<br><br>HPLC employed a modified C-18 column and a binary gradient consisting of 0.1% aqueous $H_3PO_4$ and acetonitrile. Detection employed a UV detector at 313 nm and a radioactive flow through monitor using a 1000 µL cell.<br><br>A7.1.2.1.1/03<br><br>Radioassay of solutions was performed using liquid scintillation counters.<br><br>Metabolite profiling/quantitation was performed by HPLC using a radioactivity flow through detector with a 100 µL cell. HPLC employed a modified C-18 column and a binary gradient consisting of acetic acid in water and acetic acid in methanol. | |

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| **Annex Point** | |
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| | Liquid Chromatography-Mass Spectroscopy (LC-MS) was performed with a modified C-18 column and a binary gradient consisting of acetic acid in water and acetic acid in methanol. The mass spectrometer was an ion trap employing an electrospray interface. |
| 3.4.10 Half-Life Calculations (A7.1.2.1.1/02) | Rohm and Haas calculated the half-life of BIT in the simulated STP study using the data in Reference 1 (A7.1.2.1.1/01). Kinetics were calculated using the data in the steady test period only and assuming first order degradation. The calculations were based on the previous published work: Nyholm et al., Water Research 26(3): 339-353 (1992). |
| | **4 RESULTS** |
| | Note: Section 4.1 to 4.5 refers to Reference A7.1.2.1.1/01<br>Section 4.6 refers to Reference A7.1.2.1.1/02 |
| **4.1 Temperature, pH, dissolved oxygen, and operational parameters** | The temperature range recorded during the test was 20°C to 22°C which is within the specified limits of 20 ± 2°C for the duration of the study.<br>The average pH, dissolved oxygen, mixed liquor total suspended solids, DOC, and COD for the control and two $^{14}$C-BIT dosed bioreactors is presented in Table A7.1.2.1.1-2. The mean pH in the two dosed bioreactors was 7.4- 7.5 and the mean dissolved oxygen, 3.4 mg $O_2$/L. .<br>For both reactors dosed with $^{14}$C-BIT, the average sludge retention time was 10 days and the hydraulic retention time, 6.8 hours. These observed parameters were acceptable for good operational performance of the test system. |
| **4.2 Organic carbon removal** | As shown in Table A7.1.2.1.1-2 mean COD as a percent removal averaged greater than 90% for the two dosed bioreactors. This demonstrates that the microbial activity in the test system was operating satisfactorily. |
| **4.3 Distribution and recovery of radioactivity** | The sampled daily distribution of radioactivity between the effluent, mixed liquor, and evolved $^{14}CO_2$ for the two test reactors are presented in Tables A7.1.2.1.1-3 and A7.1.2.1.1-4. The mean distribution during the steady test period is tabulated below. |

| **Reactor** | **Percent Distribution of Applied Radioactivity Mean ± Standard Deviation** |
|---|---|

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| | | Effluent | Mixed Liquor | $^{14}CO_2$ | Mass Balance |
|---|---|---|---|---|---|
| | 2 | 74.7 ± 5.5 | 17.7 ± 1.6 | 0.3 ± 0.2 | 92.7 ± 4.9 |
| | 3 | 82.9 ± 7.0 | 15.0 ± 1.2 | 0.2 ± 0.1 | 98.1 ± 6.3 |

Thus most of the applied activity was present in the effluent and very little as evolved $CO_2$. The cumulative $^{14}CO_2$ during the steady test period (Days 20-41) in reactor 2 was 3.4% of the applied activity and in reactor 3, 2.4%.

The mixed liquor fraction was centrifuged to remove the supernatant and the resulting solids extracted with acetonitrile. The sampled daily distribution of $^{14}C$-activity in the mixed liquor fractions is presented in Table A7.1.2.1.1-5. Most of the $^{14}C$-activity remained associated with the sludge solids after centrifugation and ACN extraction. Approxiamtely 2% of the applied activity was in the sludge solution after centrifugation and about 0.7% was extractable with ACN.

**4.3.**1 Recovery of $^{14}C$-activity

The mean recovery of applied $^{14}C$-activity during the steady test period for Reactor 2 was 92.7 ± 4.9% and for reactor 3, 98.1 ± 6.3%. The average recovery from the two reactors was 95.4 ± 6.2%.

**4.4 Chromatographic analysis**

The effluent, supernatant resulting form centrifugation of the mixed liquor, and the acetonitrile extract of the mixed liquor solids were chromatographed (HPLC). A summary of the chromatographic results are presented in Table A7.1.2.1.1-6. There were 5 chromatographic regions detected. BIT had a retention time of about 7.5 minutes (Region 4).

4.4.1 Effluent

There were two major peak regions in the effluent. Parent (Region 4) was present a about 22-25% of the applied activity (NOTE: subsequent analysis described below demonstrated that parent percentage was actually about 3.3% of applied activity). There was a major polar metabolite with a retention time of 4.4-5.2 minutes (Region 2) that represented about 32-33% of applied activity. The metabolite (or metabolites) was not identified. About 10% of the applied activity was present in the system void volume (ca. 2 minutes; Region 1) and this polar and poorly retentive fraction generally contains multiple compounds. The other two Regions, 3 and 5, accounted for less than 10% each.

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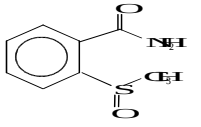
| **Annex Point** | | |
|---|---|---|
| 4.4.2 Mixed liquor supernatant | The total activity in the mixed liquor was significantly less than in the effluent. Thus the supernatant from the mixed liquor had less than 3% of the total applied activity. About 0.7% of the applied activity was parent and the polar Region 2 contained about 1%. | |
| 4.4.3 ACN Extract of sludge solids | Similar to the mixed liquor supernatant, the acetonitrile extract of the sludge solids accounted for much less than the effluent; less than 1% of the total applied activity (Table A7.1.2.1.1-5). BIT in this extracted accounted for 0.3% of the applied activity (Table A7.1.2.1.1-6) (NOTE: subsequent analysis described below demonstrated that parent percentage was actually less than 0.1% of the applied activity). The remaining regions contained less than 0.3% of the applied activity. | |
| **4.5 Degradation kinetics**<br>**(A7.1.2.1.1/02)** | A summary of the degradation kinetics calculations for $^{14}$C-BIT are presented in Tables A7.1.2.1.1-7. The kinetics were calculated assuming the steady state kinetics accounting for the direct dissipation in the aqueous, solids, and volatile phases. The kinetic results are summarized below.<br><br>**Half-life (hours)**<br>Test Reactor #2: 1.9<br>Test Reactor #3: 2.4<br><br>These results show that there is a very fast turnover of parent and total $^{14}$C-activity in the system having a half-life of less than 3 hours. | |
| **4.7 Metabolite Identification** | As described above, In the initial study two major chromatography peaks were observed; one assigned as a metabolite and the other as BIT. Using the HPLC conditions described in the initial report (A7.1.2.1.1/01) the chromatography was essentially replicated for metabolite identification. However, instead of using a 1000 µL radioactivity detector flow cell a 100 µL cell was employed. This resulted in the two major peaks, a metabolite and BIT, being split into multiple peaks due to the increased resolution caused by a narrower peak width. Thus the samples were reanalyzed using the smaller flow cell and an enhanced gradient to assist with separation. The BIT results of this analysis, as well as the | |

previous results, are presented in Table A7.1.2.1.1-08. In the initial study, BIT accounted for about 24% (Table A7.1.2.1.1-06) of the applied activity in the effluent, however, with reanalysis using improved HPLC conditions, the average BIT concentration was 3.3% (also see Table A7.1.2.1.1-09). Originally in the ACN extract of the sludge solids BIT comprised 0.3% (Table A7.1.2.1.1-06) of the applied activity but with reanalysis this was less than 0.1% (Table A7.1.2.1.1-09).

In the effluent, reanalysis of the original BIT peak with improved HPLC conditions showed that besides BIT this peak also contained several metabolites of which one was greater than 10% (M3 = 11.6%, Table A7.1.2.1.1-09). Reanalysis of the metabolite that originally had a retention time of 4.1 – 5.2 minutes (Table A7.12.1.1-06) had primarily on major metabolite, M2, at an average of 45.5% of applied activity. Similar results were seen in the ACN extract of the sludge solids but the percent of applied was significantly small due to less activity residing in the solids (Table A7.1.2.1.1-09).

Major metabolites M2 and M3 were identified by LC-MS as noted below.

| Structure/Name | Average Percent of Applied Activity | |
|---|---|---|
| | Effluent | Supernatant |
|  2-methylsulfinyl-benzamide | 45.53 | 1.39 |

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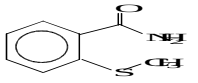
|  2-methylthio-benzamide | 11.57 | 0.61 |

Since metabolite identification did not commence immediately storage stability was examined. $^{14}$C-BIT was spiked into effluent, mixed liquor sludge supernatant, and an acetonitrile extract of the mixed liquor sludge solids. The results from the HPLC analysis are presented in Table A7.1.2.1.1-10. In all situations examined BIT was stable for up to 89 days.

The results from the storage stability study show that under the storage conditions examined, BIT was stable in the effluent, mixed liquor sludge supernatant, and an acetonitrile extract of the mixed liquor sludge solids. Thus the reduction of BIT observed in the metabolite identification is due to improved chromatography and not due to degradation of BIT in the samples.

**5 APPLICANT'S SUMMARY AND CONCLUSION**

**5.1 Materials and methods**

The test guideline was OECD 303, Simulation Test-Aerobic Sewage Treatment: Activated Sludge Units.

The test unit was a porous pot bioreactor which consists of a glass vessel housing a polyethylene membrane that retains the sludge solids but allows the liquid to flow through. Three reactors were prepared; a control dosed with water and two test reactors dosed with $^{14}$C-BIT. About 1.13L of activated sludge was added to the reactors and domestic sewage was pumped into the system at 2.4 mL/min. A 2.35 mg/L solution of $^{14}$C-BIT was added to the porous pot system at a flow rate of 0.3 mL/min for a resulting concentration in the porous pot of 0.25 mg/L. About 113 mL of activated sludge was removed per day. The hydraulic retention time in the aeration vessel was 6.8 hours and the sludge retention time, 10 days. The effluent was collected in a refrigerated container.

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| | The unit was allowed to equilibrate (stabilization period) for 8 days prior to dosing with $^{14}$C-BIT during which the DOC/COD became greater than 80%. A 12 day acclimation period followed the stabilization period and during this time the systems were dosed with BIT (the control with a similar volume of water). The effluent, mixed liquor and dosing solution were radioassayed. After 12 days the system had reached equilibrium and a 22 day steady test period was commenced. During the steady test period, the effluent, mixed liquor, mixed liquor supernatant, acetonitrile extract of the mixed liquor solids, and dosing solution were radioassayed. The system temperature was maintained between 20°C and 22°C.<br><br>Dissolved organic carbon, pH, temperature, and oxygen content were monitored throughout the study.<br><br>During the steady test period volatile traps consisting of NaOH were connected to the effluent to collect evolved $^{14}CO_2$. Aliquots of the NaOH were taken periodically for radioassay.<br><br>The effluent, the supernatant result from centrifugation of the mixed liquor, and an acetonitrile extract of the sludge solids were chromatographed using HPLC. | |
| **5.2 Results and Discussion** | | |
| 5.2.1 Distribution and recovery of applied $^{14}$C-acitivity | Average recovery of applied radioactivity from the two reactors dosed with BIT was 95.4 ± 6.2%. Over 74% of the applied activity was in the effluent and 15% to 18% was in the mixed liquor continuously removed from the porous pot system. Volatiles averaged about 0.2-0.3% of the applied activity per steady test period study day and the total accumulated during this period was less than 3.5%. | |
| 5.2.2 Quantitation of parent | Parent present in the effluent accounted for about 22%-25% of the total applied activity while in the mixed liquor and acetonitrile extract of the mixed liquor sludge solids, 0.7% and 0.3%, respectively. | |
| 5.2.3 Metabolites | There was one major metabolite detected by HPLC and it was more polar than parent. It was present in the effluent at 32%-33% of the total applied activity while in the mixed liquor supernatant and acetonitrile extract of the mixed liquor sludge | |

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| | solids, 0.9% and 0.2%, respectively. | |
| 5.2.4 Half-life | The half-life of total applied radioactivity (parent and metabolites) in the sewage treatment system studied was calculated in the two test reactors to be less than 3 hours. | |
| 5.2.3 Organic carbon turnover | The average COD was 90.1% which satisfies the OECD guideline requirement | |
| **5.3 Conclusion** | In a sewage treatment plant simulation system dosed with $^{14}$C-BIT over 74% of the applied activity was in the effluent and 15%-18% in the mixed liquor. Evolved $CO_2$ totaled less than 3.5% of the total applied radioactivity.<br>The half-life of BIT in the simulated STP systems was less than 3 hours.<br>The half-life of BIT in the simulated STP systems was less than 3 hours.<br>Less than 25% of the total applied activity in the effluent was parent. In the supernatant resulting from centrifugation of the mixed liquor and in the acetonitrile extraction of the sludge solids, BIT accounted for approximately 0.7% and 0.3% of the applied activity, respectively. | |
| 5.3.1 Reliability | 1-valid without restrictions | |
| 5.3.2 Deficiencies | None | |

| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *December 2012* |
| **Materials and Methods** | *Applicant's version is accepted with the following remarks:*<br>*Deviations from GLP:*<br>*1) Characterization and stability of test material under site specific storage* |

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**Annex Point**

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| | *conditions were not performed in accordance with GLP guideline, however chemical characterization was performed under GLP by the sponsor.*<br><br>*2) Analysis of water (purified and municipal) for contaminants were not performed by a GLP certified laboratory, however RMS accepts that this was performed by a certified laboratory using U.S. EPA analytical method).*<br><br>*3.2. No reference substances were employed to validate the STP system.*<br><br>*3.3.1. Test inoculum is collected from one single source. However, to get as many different species of bacteria as possible, it is adviseable to add inocula from various other sources, for example surface water.*<br><br>*In addition, the solid concentration in the test (3.6 g/L) was higher than required in the guideline (1-3 g/L). However, because of the relatively low adsorption property of the substance, this deviation should not have any consequences on the result of the study.*<br><br>*3.4.3. Concentration of BIT is lower than recommended in OECD guidelines. However, the choice of this low concentration may be justified to avoid possible toxicity effects in the inoculum. In addition, the test substance is radiolabelled.* |
| **Results and discussion** | *Applicant's version is adopted with the following remarks:*<br><br>*4.7.(it should be 4.6) Metabolite Identification. Initial analysis of effluent samples, performed using high-performance liquid chromatography (HPLC) coupled with a RAM radioactivity detector (HPLC/RAM), suggested the presence of parent [$^{14}$C]-BIT at approximately 24% (Table A7.1.2.1.1-06) of the applied concentration. Samples were later re-analyzed at the Rohm and Haas Technical Center using improved HPLC/RAM methodologies. Re-analysis resulted in more pronounced chromatographic resolution and resolved the peak initially identified as [$^{14}$C]-BIT into multiple peaks. Quantification of the newly identified [$^{14}$C]-BIT peak indicated a concentration of approximately 3.3%. % (also see Table A7.1.2.1.1-09). The applicant provided a technical rationale supporting use of the 3.3% rather than 24% from this study for risk assessment.*<br><br>*5.2.3 Metabolites. The improved HPLC/RAM methodologies resulted in the detection of two major metabolites: M2, at an average of 45.5% of applied activity and M3 at 11.57%.*<br><br>*Section 5.2.3 Organic carbon turnover should be numbered as section 5.2.5* |
| **Conclusion** | *Applicant's version with the following remarks:*<br><br>*A typing error was detected in 5.1. "During the steady test period volatile traps consisting of NaOH were connected to the effluent to collect evolved $^{14}CO_2$.* |

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| | *Aliquots of the NaOH were taken periodically for radioassay." The traps are actually consisting in KOH, not in NaOH.* |
| **Reliability** | *2* |
| **Acceptability** | *Acceptable* |
| **Remarks** | |

**Table A7.1.2.1.1-1: Dosing Concentration of $^{14}$C-BIT**

| Study Day | mg $^{14}$C-Activity/mL[1] | Percent Recovery | $^{14}$C-BIT Peak Area Percent |
|---|---|---|---|
| 8[2] | 2.36 | 103 | 97.3 |
| 15A[3] | 2.34 | 102 | 98.2 |
| 15B | 2.33 | 101 | 98.6 |
| 22A | 2.34 | 102 | 99.3 |
| 22B | 2.37 | 103 | 92.8 |
| 29A | 2.34 | 102 | 98.9 |
| 29B | 2.38 | 103 | 95.9 |
| 36A | 2.36 | 103 | 96.7 |
| 36B | 2.34 | 102 | 98.4 |
| 41 | 2.33 | 101 | 97.2 |
| | | | |
| Average | 2.35 ± 0.02 | 102 ± 0.74 | 97.3 ± 1.91 |

[1] Average of three replicate LSC analysis

[2] Day 8 was the start of dosing with $^{14}$C-BIT

[3] A = analysis before changing dosing solution. B = analysis on freshly prepared dosing solution.

**Table A7.1.2.1.1-2: Summary of Test Reactor Operational Paramters**

| Test Unit | Mean ± Standard Deviation | | | | |
|---|---|---|---|---|---|
| | pH | Dissolved Oxygen (mg $O_2$/L | Mixed Liquor Total Suspended Solids (mg/L) | DOC (% Removal) | COD (% Removal) |
| Control (Bioreactor #1) | 7.3 ± 0.1 | 3.6 ± 1.2 | 3586 ± 791 | 68.1 ±14.8 | 90.1 ± 5.2 |
| Treatment Replicate #1 | 7.4 ± 0.1 | 3.3 ± 1.0 | 3655 ± 589 | 68.3 ± 11.6 | 90.4 ± 5.9 |
| Treatment Replicate #2 | 7.5 ± 0.2 | 3.5 ± 1.0 | 3681 ± 435 | 67.7 ± 11.0 | 90.1 ±5.1 |

**Table A7.1.2.1.1-3: Distribution of Radioactivity—Test Reactor #2**

| Day | Percent of Applied Activity | | | | |
|---|---|---|---|---|---|
| | Effluent | Percent Removal | Mixed Liquor | NaOH Trap[2] | Mass Balance |
| **Acclimation Period** | | | | | |
| 10 | 68.3 | 31.7 | 6.5 | | 74.8 |
| 13 | 69.5 | 30.5 | 12.2 | | 81.7 |
| 14 | 72.8 | 27.2 | 12.6 | | 85.3 |
| 16 | 76.9 | 23.1 | 13.2 | | 90.2 |
| **Steady Test Period** | | | | | |
| 20 | 69.9 | 30.1 | 17.6 | 0.0 | 87.5 |
| 22 | 70.9 | 29.1 | 19.5 | 0.1 | 90.5 |
| 24 | 67.1 | 32.9 | 19.3 | 0.2 | 86.5 |
| 27 | 69.4 | 30.6 | 17.8 | 0.4 | 87.7 |
| 29 | 80.4 | 19.6 | 18.0 | 0.6 | 98.9 |
| 31 | 82.6 | 17.4 | 14.2 | 0.3 | 97.1 |
| 34 | 72.4 | 27.6 | 16.7 | 0.5 | 89.6 |
| 36 | 76.9 | 23.1 | 16.4 | 0.4 | 93.7 |
| 38 | 80.6 | 19.4 | 17.8 | 0.3 | 98.7 |
| 41 | 76.6 | 23.4 | 19.3 | 0.6 | 96.5 |
| Mean | 74.7 ± 5.5[1] | 25.3 ± 5.5[1] | 17.7 ± 1.6[1] | 0.3 ± 0.2[1,2] | 92.7 ± 4.9[1] |

[1] Mean and Standard Deviation during Study Test Period.

[2] Values presented are the daily $^{14}CO_2$ determinations. Cumulative $^{14}CO_2$ was 3.4 at study termination.

**Table A7.1.2.1.1-4: Distribution of Radioactivity—Test Reactor #3**

| Day | Percent of Applied Activity | | | | |
|---|---|---|---|---|---|
| | Effluent | Percent Removal | Mixed Liquor | NaOH Trap[2] | Mass Balance |
| **Acclimation Period** | | | | | |
| 10 | 75.8 | 24.2 | 6.1 | | 81.9 |
| 13 | 82.1 | 17.9 | 10.6 | | 92.7 |
| 14 | 89.3 | 10.7 | 11.3 | | 100.6 |
| 16 | 83.7 | 16.3 | 11.6 | | 95.2 |
| **Steady Test Period** | | | | | |
| 20 | 76.8 | 23.2 | 15.5 | 0.0 | 92.3 |
| 22 | 76.9 | 23.1 | 17.1 | 0.2 | 94.1 |
| 24 | 75.1 | 24.9 | 14.9 | 0.3 | 90.4 |
| 27 | 75.9 | 24.1 | 16.5 | 0.4 | 92.8 |
| 29 | 83.5 | 16.5 | 14.4 | 0.3 | 98.2 |
| 31 | 85.3 | 14.7 | 13.1 | 0.1 | 98.6 |
| 34 | 82.8 | 17.2 | 15.8 | 0.2 | 98.8 |
| 36 | 85.2 | 14.8 | 14.1 | 0.3 | 99.6 |
| 38 | 91.1 | 8.9 | 13.8 | 0.3 | 105.2 |
| 41 | 96.2 | 3.8 | 14.7 | 0.2 | 111.1 |
| Mean | 82.9 ± 7.0[1] | 17.1 ± 7.0[1] | 15.0 ± 1.2[1] | 0.2 ± 0.1[1,2] | 98.1 ± 6.3[1] |

[1] Mean and Standard Deviation during Study Test Period.

[2] Values presented are the daily $^{14}CO_2$ determinations. Cumulative $^{14}CO_2$ was 2.4% of applied activity at study termination

**Table A7.1.2.1.1-5: Distribution of Applied Radioactivity in Mixed Liquor Fractions During Steady Test Period**

| Day | Percent of Applied Radioactivity | | | | | |
|---|---|---|---|---|---|---|
| | Supernatant | | Acetonitrile Extract | | Sludge Solids | |
| | Reactor #2 | Reactor #3 | Reactor #2 | Reactor #3 | Reactor #2 | Reactor #3 |
| 20 | 2.6 | 2.2 | 0.7 | 0.5 | 25.1 | 17.7 |
| 21 | 2.8 | 2.6 | 0.7 | 0.6 | 26.8 | 22.5 |
| 22 | 2.7 | 2.2 | 0.9 | 0.4 | 24.9 | 18.6 |
| 23 | 2.8 | 2.3 | 1.0 | 0.6 | 26.3 | 20.1 |
| 24 | 2.6 | 2.3 | 1.0 | 0.7 | 27.3 | 21.4 |
| 27 | 2.6 | 2.0 | 0.8 | 0.6 | 26.5 | 19.2 |
| 28 | 2.1 | 1.9 | 0.8 | 1.5 | 21.0 | 17.0 |
| 29 | 1.7 | 1.3 | 0.5 | 0.5 | 15.7 | 12.2 |
| 30 | 1.6 | 1.4 | 0.6 | 1.0 | 11.9 | 10.0 |
| 31 | 1.5 | 1.3 | 0.4 | 0.3 | 12.3 | 9.4 |
| 34 | 2.4 | 1.7 | 0.7 | 0.4 | 22.1 | 13.5 |
| 35 | 2.4 | 1.6 | 0.9 | 1.0 | 23.3 | 12.9 |
| 36 | 2.1 | 1.5 | 0.5 | 0.3 | 19.0 | 11.3 |
| 37 | 1.7 | 0.8 | 0.4 | 0.1 | 13.5 | 5.9 |
| 38 | 1.8 | 1.0 | 0.4 | 0.2 | 15.2 | 6.2 |
| 41 | 2.2 | 0.8 | 0.6 | 0.2 | 20.4 | 5.5 |
| Mean | 2.2 ± 0.5 | 1.7 ± 0.6 | 0.7 ± 0.2 | 0.6 ± 0.4 | 20.7 ± 5.5 | 14.0 ± 5.7 |

**Table A7.1.2.1.1-06: BIT as a Percent of Applied in the Effluent and the Supernatant and Acetonitirile Mixed Liquor Fractions**

| Reactor | TLC Regions—Mean Percent of Applied Radioactivity During Steady Test Period | | | | |
|---|---|---|---|---|---|
| | Region 1 (Rt 2.0 – 4.4) | Region 2 (Rt 4.4 – 5.2) | Region 3 (Rt 5.2 – 7.3) | Region 4 BIT (Rt 7.3 – 8.0) | Region 5 (Rt 8.0 – 10.0) |
| **Effluent** | | | | | |
| 2 | 7.7 ± 2.1 | 31.9 ± 5.6 | 6.6 ± 2.8 | 22.4 ± 2.8 | 7.7 ± 0.9 |
| 3 | 11.6 ± 2.9 | 32.9 ± 2.7 | 8.5 ± 3.3 | 24.5 ± 3.1 | 6.3 ± 1.3 |
| **Mixed Liquor Supernatant** | | | | | |
| 2 | 0.3 ± 0.1 | 1.0 ± 0.2 | 0.2 ± 0.1 | 0.7 ± 0.2 | 0.2 ±0.1 |
| 3 | 0.3 ± 0.1 | 0.8 ± 0.3 | 0.2 ± 0.1 | 0.7 ± 0.3 | 0.1 ± 0.1 |
| **Acetonitrile Extract of Mixed Liquor Solids** | | | | | |
| 2 | 0.1 ± 0.0 | 0.2 ± 0.1 | 0.1 ± 0.0 | 0.3 ± 0.1 | 0.1 ± 0.0 |
| 3 | 0.0 ± 0.0 | 0.2 ± 0.1 | 0.0 ± 0.0 | 0.3 ± 0.3 | 0.1 ± 0.1 |

**Table A7.1.2.1.1-7: Steady State Degradation Kinetics**

| | Unit 2 | | Unit 3 | |
|---|---|---|---|---|
| | **Value** | **Unit** | **Value** | **Units** |
| Reactor Volume (V) | 1.13 | L | 1.13 | L |
| Influent flow rate (Qi) | 3.888 | L/day | 3.888 | L/day |
| Effluent flow rate (Qo) | 3.775 | L/day | 3.775 | L/day |
| Volume of wasted sludge (Qw) | 113.0 | ml/day | 113.0 | ml/day |
| Concentration of Suspended Solids in Wasted Sludge (Xw) | 4320.6 | mg dry wt/L | 4178.4 | mg dry wt/L |
| Concentration of Suspended Solids in Effluent (Xo) | 112.0 | mg dry wt/L | 112.0 | mg dry wt/L |
| Test Substance Concentration in Influent (Ci) | 261.1 | µg/L | 261.1 | µg/L |
| Total BIT Concentration in Effluent (Co) | 45.0 | µg/L | 54.6 | µg/L |
| Test Substance Concentration in Sludge Solids (Wss) | 422.3 | µg/g | 418.5 | µg/g |
| Mineralization Rate (Mo) | 34.5 | µg/day | 24.4 | µg/day |
| | | | | |
| Kd (=Wss/Co) | 9384.9 | | 7662.0 | |
| Fi (=Qi x Ci) | 1015.2 | µg/day | 1015.2 | µg/day |
| Fo,diss (=Qi x Co) | 175.0 | µg/day | 212.4 | µg/day |
| Fo, part (=Kd x Co x (Qo x Xo + Qw x Xw) | 384.7 | µg/day | 374.5 | µg/day |
| Rbio (=Fi-Fo,diss-Fo,part/V) | 403.1 | µg/L/day | 379.0 | µg/L/day |
| k (=Rbio/Co) | 9.0 | $day^{-1}$ | 6.9 | $day^{-1}$ |
| Half-life | 0.1 | Days | 0.1 | days |
| Half-life | 1.9 | Hours | 2.4 | hours |

Note: Kinetic calculations incorporate steady test period only

| | | |
|---|---|---|
| **Section A7**<br>**Subsection A7.1**<br>**Subsection A7.1.2.1**<br>**Subsection A.7.1.2.1.2**<br>**Annex Point IIIA XII.2.1** | **Ecotoxicological Profile Including Environmental Fate and Behaviour**<br>**Fate and Behaviour in Water**<br>**Biological Sewage Treatment**<br>**Anaerobic biodegradation** | |
| | **JUSTIFICATION FOR NON-SUBMISSION OF DATA** | **Official use only** |
| **Other existing data [ ]** | **Technically not feasible [ ] Scientifically unjustified [ ]** | |
| **Limited exposure [ X]** | **Other justification [X]** | |
| **Detailed justification:** | 7.1.2.1.2 Anaerobic Biological Sewage Treatment<br>A waiver from performing an anaerobic biological sewage treatment simulation study for BIT in Product Type 12 is requested. As noted in the Chapter 3, Section 7.1.2.1.2 for the Guidance on Data Requirements in the Technical Guidance Document, an Anaerobic study is only required if exposure to anaerobic conditions is likely. For the Product Types PT 6 and 13 in question, this exposure is unlikely. | |
| **Undertaking of intended data submission [ ]** | No further studies planned | |
| | | |
| | | |
| | **Evaluation by Competent Authorities** | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | *December 2010* | |
| **Evaluation of applicant's justification** | *Applicant's justification is accepted due to the unlikely anaerobic exposure of BIT* | |
| **Conclusion** | *Accepted* | |
| **Remarks** | | |

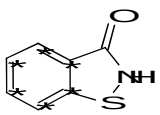
**Section A7** **Ecotoxicological Profile Including Environmental Fate and Behaviour**

**Subsection A7.1** **Fate and Behaviour in Water**

**Subsection A7.1.2.2** **Aerobic aquatic degradation study**

**Subsection A7.1.2.2.1** **Biodegradation in Estuarine Surface Water**

**Annex Point IIIA XII.2.1**

| | | | Official use only |
|---|---|---|---|
| | | **1 REFERENCE** | |
| **1.1 Reference** | | **A7.1.2.2.1/01** [redacted] Aerobic Transformation of 1,2-Benzisothiazolin-3-one (BIT) in Surface Water; Rohm and Haas Technical Report N° GLP-2008-078 (August 20, 2008), Unpublished. | |
| **1.2 Data protection** | | Yes | |
| 1.2.1 | Data owner | Rohm and Haas Company | |
| 1.2.2 | Companies with letter of access | | |
| 1.2.3 | Criteria for data protection | Data submitted for the first time in support of the first inclusion into Annex I/IA.<br>Data protection claimed in accordance with Article 12.1(c) (ii). | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1 Guideline study** | | Yes. OECD Guideline for Testing of Chemicals 309: Aerobic Mineralization in Surface Water-Simulation Biodegradation Test (April 2004) | |
| **2.2 GLP** | | Yes. | |
| **2.3 Deviations** | | Three minor GLP deviations: 1) plate counts determining microbial activity were not conducted under GLP; 2) total organic carbon and nutrients of the surface water were analyzed after the kinetic test was completed; and 3) the temperature in the constant temperature room was out of the desired range, 20±2°C at four intervals (17.02, 17.86, 22.18, and 22.94°C). | |
| | | **3 METHOD** | |
| **3.1 Test material** | | | |
| 3.1.1 | Test material name | BIT, 1,2-benzisothiazolin-3-one<br>  <br>* site of $^{14}C$ label | |

| | | |
|---|---|---|
| 3.1.2 | Lot/Batch number | Lot 1069.00 and sublot 1069.0008; $^{14}C$ labeled uniformly in the benzene ring; Specific activity: 53.57 mCi/g. |
| 3.1.3 | Purity | Radiopurity = 98.61% |
| 3.1.4 | Further relevant properties | • Water solubility >0.7g/L<br>• Half-life in soil is 0.23 days (20°C and 5.0 ppm) |
| **3.2 Reference substance** | | Aniline (Fisher Scientific, 99.8%) was employed as a system reference standard to insure the bioactivity of the water substrate. *N*-methyl malonamic acid was used to tune the mass spectrometer in the negative ion mode. |
| **3.3 Testing procedure** | | |
| 3.3.1 | Water characterization | The water used for the definitive study was estuarine water obtained from Wissahickon Creek, Ambler, Pennsylvania, USA (GPS Coordinates: N40°08.674' and W075° 13.220') Water parameters including pH, temperature, oxygen content, bacteria cell count, conductivity, total organic carbon, and nutrients, were measured during the experiment and at the end of the study. The results are presented in Table A7.1.2.2.1-1. |
| 3.3.2 | Test system | <u>Range Finding Study</u><br><br>A range finding study was performed to identify the appropriate dosing concentration and sampling intervals. Water was dosed at nominal 20 ppb and 100 ppb (actual concentrations were 20.7 ppb and 104 ppb). For dosing at 20 ppb, 100 mL of water was added to twelve glass 250 ml bottles while for 100 ppb dosing, 50 mL was added to 12 bottles. The bottles were sealed with 2-hole stoppers containing glass tubes connected together and to the house vacuum with plastic tubing. The house vacuum was adjusted to maintain a slight vacuum (~30 mm Hg). Bottles were maintained in the dark at 20 ± 2°C. Duplicate samples for both concentrations were removed at Hours 0, 2, 6, 24, 48, and 120. At harvest, aliquots were radioassayed and aliquots were processed using a preconditioned Oasis Max SPE cartridge. SPE cartridges were eluted with water, methanol, and methanol:ethyl acetate:acetic acid. Aliquots of the organic eluants were concentrated, chromatographed (TLC), and parent quantitated.<br><br><u>Definitive Study</u><br><br>The nominal dosing rate for the definitive study was 20 ppb and 100 ppb and the actual concentration was 25.6 ppb and 105 ppb. In addition, samples for metabolite identification only were dosed at 500 ppb and 1000 ppb. Fourteen 250 mL glass bottles contain 50 mL surface water for samples dosed at 105 ppb and 100 mL water for samples dosed at 25.6 ppb were prepared.<br><br>The bottles were placed in a dark incubator maintained at 20 ± 2°C |

| | | | |
|---|---|---|---|
| | | (mean temperature over the entire study was 20.69 ± 0.11°C). At the front end of each series of bottles was a glass bottle containing water to provide moistened air to the system. Following each series of bottles containing the BIT dosed surface water were three traps; the first containing ethylene glycol and the next two containing 1N KOH. After dosing the bottles were given a gentle swirl and then sealed with 2-hole stoppers containing glass tubes connected together and to the house vacuum with plastic tubing. The house vacuum was adjusted to maintain a slight vacuum (~30 mm Hg). The bottles containing surface water were placed on an orbital shaker. After about 24 hours of equilibration, they were dosed with $^{14}$C BIT.<br><br>Sterile samples were prepared in an identical manner except that after the 24 h equilibration, 100 ppm $HgCl_2$ was added, mixed thoroughly, and then $^{14}$C-BIT added. Duplicate samples were taken at 24 hours and 120 hours.<br><br>To assist with metabolite identification, 500 mL of surface water was placed into a 1L glass bottle and equilibrated in the dark at 20°C. After about 24 hours of equilibration, the samples were dosed at either ~500 ppb or ~1000 ppb with $^{14}$C-BIT and placed on an orbital shaker.<br><br>To verify the biological activity of the test water, 250 mL glass bottles containing 100 mL of surface water were dosed with 100 ppb aniline and incubated at 20°C. | |
| 3.3.3 | Method of preparation of test solution | Initially, a stock solution was prepared by dissolving 10.34 mg of $^{14}$C-BIT in 2 ml of methanol. For the definitive study, a dosing solution was prepared by adding 100 µL of the above solution to 2 mL of methanol. Based on radioassay, the concentration of the dosing solution was 259µg/mL.<br><br><u>Nominal 20 ppb</u><br><br>To yield a final concentration of 20 ppb, 8 µL of the dosing solution was added to 100 mL of surface water. Radioassay yielded a final concentration of 25.6 ppb<br><br><u>Nominal 100 ppb</u><br><br>To yield a concentration of 100 ppb, 15 µL of the dosing solution was added to 50 mL of surface water. Radioassay yielded a final concentration of 105 ppb. | |
| 3.3.4 | Initial TS concentration | The initial test concentration for the definitive study was 25.6 ppb and 105 ppb. In addition, exaggerated doses of ~500 ppb and ~1000 ppb were employed to assist with metabolite identification. | |
| 3.3.5 | Duration of test | The duration of the definitive test dosed at 25.6 and 105 ppb was 216 hours. The duration of the bottles dosed at ~500 and ~1000 ppb to assist with metabolite identification was either 216 or 312 hours. | |
| 3.3.6 | Sampling | | |

| | | | |
|---|---|---|---|
| | | **Dosing Level** | **Sampling Intervals-Hours** |
| | | 25.6 ppb and 105 ppb | 0, 2, 6, 24, 48, 120, 216 |
| | | ~500 ppb metabolite identification (first dosing) | 48, 120,168, 192, 216 |
| | | ~500 ppb and ~1000 ppb metabolite identification (second dosing) | 48, 120, 192, 288, 312 |
| | | Sterile controls | 24, 120 |
| | | Aniline reference standards | 24, 48 |
| 3.3.7 | Replicates | Duplicate samples were taken at each interval for the 25.6 ppb and 105 ppb dosing level. Duplicate samples were also taken at each interval for the sterile system and aniline reference standards. | |
| 3.3.8 | Extraction and chromatography | At each sampling interval, duplicate samples were removed and radioassayed. The samples were applied to a preconditioned solid phase extraction cartridge (SPE; Max Oasis). The initial eluant was radioassayed and discarded. The cartridge was initially eluted with 10 ml of methanol followed by 10 mL of methanol:ethyl acetate:acetic acid (50:50:1) at a rate of about 2-3 drops/second and the eluants radioassayed. The 24 hour and later samples were additionally eluted with 10 mL of methanol:1N HCl and 10 mL of methanol:1N KOH and the eluants radioassayed. All the organic phases were concentrated to dryness with a stream of nitrogen, redissolved in methanol, and radioassayed. Aliquots of the concentrated extracts were applied to TLC plates for quantitation of parent and metabolite profiling/quantitation. | |
| 3.3.9 | Analysis of trapped volatiles | At 24, 48, 120, and 216 hours, duplicate 500 µL aliquots were removed from the ethylene glycol trap and from both KOH traps and radioassayed. | |
| 3.3.10 | Analytical Methods | Thin layer chromatography (TLC) was used to quantitate parent and metabolites. Extract aliquots were applied to silica gel TLC plates and eluted with ethyl acetate:acetonitrile:methanol:acetic acid (90:5:5:1, v/v/v/v). The location of radioactivity on the TLC plates was determined using a phosphorimager. Zones on the plate were demarcated and the silica gel scrapped and transferred to a liquid scintillation vial for radioassay. For metabolite isolation, plates were developed as above, and the silica from appropriate zone of radioactivity, identified by the phosphorimager, was scraped and transferred to a megabore Pasteur pipette containing a glass wool plug in the neck. The $^{14}C$-activity was eluted with methanol and concentrated | |

| | | |
|---|---|---|
| | | with a stream of nitrogen prior to LC-MS analysis.<br><br>Parent confirmation and metabolite identification was performed on an LC-ion trap-MS. A Metasil AQ-C18 column was employed using a gradient of 0.5% aqueous acetic acid and water and 0.5% acetic acid in methanol. An electrospray interface was used to introduce the LC flow into the MS and both positive and negative ionization were employed. A radioactivity detector was employed to locate the $^{14}$C-peaks. Additional spectrometry was performed using LC-TOF/MS which can supply exact mass data. Conditions were similar to the LC-ion trap-MS conditions.<br><br>Radioactivity from liquid fractions was measured by liquid scintillation counting/spectrometry. Samples were counted 3 times for 5 minutes each (total of 15 minutes) and counting efficiency was determined by an external $Ba^{133}$ standard. Data analysis was performed by validated Rohm and Haas developed software. |
| 3.3.11 | Degradation products | As described above, surface water was dosed at an exaggerated rate of ~500 ppb and ~1000 ppb to assist with metabolite isolation and identification. Samples were prepared as describe above using a SPE cartridge. SPE organic eluants were either applied to a TLC plate to isolate the metabolite or injected directly into the LC-MS. |
| | | **4 RESULTS** |
| **4.1 Range Finding Study** | | A preliminary experiment at 20.7 ppb and 104 ppb at 20°C was performed to estimate the half-life and determine the appropriate sampling intervals. The results are summarized in Table A7.1.2.2.1-2. Within 24 hour BIT had decreased to about 48% and 58% of the applied activity for surface water dosed at 20.7 ppb and 104 ppb, respectively. After 120 hours 6.4% and 9.0% of the applied dose was BIT at 20.7 ppb and 104 ppb, respectively. The half-life of BIT was 30.5 hours and 35.2 hours at 20.7 ppb and 104 ppb, respectively |
| **4.2 Definitive Experiment** | | |
| 4.2.1 | System feasibility | The results from sterile surface water describe the abiotic degradation of BIT. The results presented in Table A7.1.2.2.1-3 show that even after 120 hours of exposure BIT was very stable in a sterilized system as over 92% of the applied activity was BIT. As demonstrated below, biodegradation is the route of BIT dissipation in surface water.<br><br>Aniline degraded quickly in surface water at both 20 and 100 ppb dosing levels (Table A7.1.2.2.1-4). This indicates an acceptable microbial activity and the system is biologically viable.<br><br>The TLC limit of quantitation (LOQ) for $^{14}$C-BIT was determined as 4.68 ppb with an overall recovery of 95.7 ± 4.1% (n=8). Confirmation of BIT was performed by LC/MS. |
| 4.2.2 | Distribution and | Table A7.1.2.2.1-5 summarizes the distribution between the SPE |

| | | | |
|---|---|---|---|
| | recovery of $^{14}$C-activity | eluants and volatiles as well as the recovery of applied radioactivity. The amount of radioactivity in the initial SPE eluant from bottles dosed at 25.6 ppb decreased with time going from 99.4% at of the applied radioactivity at Time 0 to 62.2% at Time 216 hours. After 24 h of treatment it was deemed necessary to perform an additional elution of the SPE cartridges using acidic and basic methanol. The radioactivity in this fraction increased with time from 15.7% at Time 24 hours to 31.7% at 216 hours. Less than 1% of the applied activity was present as $^{14}CO_2$ after 216 hours and there was no detectable volatile organics in the ethylene glycol trap. Recovery of applied $^{14}$C-activity averaged 92.4 ± 5.6%.<br><br>For bottles dosed at 105 ppb $^{14}$C-BIT the percent of applied activity in the initial SPE eluant decreased from 112% at Time 0 to 82.2% at Time 216 hours. The activity in the additional SPE eluant fluctuated slightly between 24 and 216 hours, ranging from 13.1% of the applied activity to 20.1%. Similar to the lower dose, after 216 hours less than 1% of the applied dose was detected as $^{14}CO_2$ and there was no detectable volatile organics. Recovery of applied $^{14}$C-activity averaged 98.3 ± 7.5%. | |
| 4.2.3 | Quantitation of parent and metabolites | Quantitation of BIT and its metabolites is presented in Table A7.1.2.2.1-6. At 25.6 ppb, BIT decreased from 95.7% of the applied activity at time 0 to 0.6% at 216 hours. At 105 ppb, parent decreased from 107.7% at time 0 to 3.7% after 216 hours.<br><br>At 25.6 ppb there were 4 metabolites in surface water detected; M1, M2, M3 and Mx. Mx is nonspecific radioactivity representing all the areas on the TLC plate not corresponding to parent, M1, M2, and M3. Thus it is not a single compound but comprises multiple components. Mx was less than 7% of the applied activity at all sampling intervals. Metabolite M1 increased with time from 1.2% of the applied activity at Time 0 to 30.0% at 216 hours. Metabolite M2 also increased with time from 0.2% at Time 0 to 24.9% of the applied activity at 216 hours. Metabolite M3 increased from 0.1% of the applied activity at Time 0 to 48.3% after 48 hours and then decreased to 33.7% at 216 hours. Unlike the higher dose, metabolite M4 was less then the instrument level of detection for the 25.6 ppb dosing level.<br><br>At 105 ppb there were 5 metabolites detected: M1, M2, M3, M4, and Mx. Similar to the lower dose results, Mx was present at less than 7% of applied activity at all sampling intervals. Metabolite M1 was transient increasing from 1.4% of applied activity at Time 0 to 27.5% after 24 hours and then 2.3% at 216 hours. Metabolite M2 was less than the liquid scintillation counter's LOD through the first 6 hours and increased to 22.1% of the applied activity at 48 hours and remained fairly constant throughout the rest of the study. Similarly, M3 was less than the instrument LOD through the first 6 hours and increased to 54.0% of applied activity after 120 hours and then decreased to 43.8% at 216 hours. M4 was less than the instrument LOD through the first 120 hours and increased to 12.8% of applied activity at study termination, 216 hours.<br><br>The amount of $^{14}CO_2$ evolved (Table A7.1.2.2.1-5) was less than 1% for | |

| | | | |
|---|---|---|---|
| | | both dosing concentrations after 216 hours. This indicates that the benzene ring remained intact. | |
| 4.2.4 | Half-life | Quantitation of BIT at each sampling interval is presented in Table A7.1.2.2.1-6 and graphical presentations of the decline of BIT with time appear in Figures A7.1.2.2.1-1 and A7.1.2.2.1-2. The kinetic analysis of $^{14}$C-BIT in surface water is tabulated below:<br><br>**Parameter** \| 25.6 ppb \| 105 ppb<br>**k** \| 0.0224 \| 0.0165<br>**$r^2$** \| 0.9221 \| 0.7347<br>**$DT_{50}$** \| 30.8 hours \| 41.8 hours<br>**$DT_{90}$** \| 103 hours \| 139 hours<br><br>These results demonstrate that BIT biodegrades very quickly in surface water. The longer half-life observed at the higher concentration indicates that 105 ppb BIT may display some biocidal activity. Additionally, as noted in the OECD Guidelines, the more environmentally relevant concentration is 25.7 ppb. | |
| 4.2.5 | Identification of metabolites | Table A7.1.2.2.1-7 summarizes the metabolite identification. The metabolites were identified by mass spectroscopy as the following:<br><br>▪ M1, 2-sulfobenzamide,<br>▪ M2, 2-methylsuffinyl-benzamide,<br>▪ M3, 2-methylthio-benzamide,<br>▪ M4, 2-methylthio-benzoic acid methyl ester,<br><br>Structural identification was based on fragmentation and exact mass analysis.<br><br>The metabolite Mx is non-specific radioactivity representing all the areas on the TLC plate that are not components of parent or Metabolites M1, M2, M3, and M4. Thus it is not a single compound but comprises multiple components and was present at less than 7% of the applied activity. | |
| 4.2.6 | Metabolic pathway | A metabolic pathway is presented in Figure A7.1.2.2.1-3. | |
| | | **5 APPLICANT'S SUMMARY AND CONCLUSION** | |
| **5.1** | **Materials and methods** | The test guideline followed was the OECD Guideline for the Testing of Chemicals 309: Aerobic Mineralization in Surface Water -Simulation Biodegradation Test, April 13, 2004<br><br>Bottles containing 50 or 100 mL of estuarine surface water collected | |

from the Wissahickon Creek, Ambler Pennsylvania, USA and dosed at either 25.6 ppb or 105 ppb, respectively. The samples were placed on an orbital shaker in a dark incubator at 20°C. A vacuum was applied to maintain aerobic conditions and remove volatiles which were trapped in ethylene glycol and KOH. The flasks were incubated in the dark at a mean temperature of 20.69 ± 0.11°C. Sterile systems were prepared in a similar manner except $HgCl_2$ was added. Additional flasks were dosed with aniline to validate that there was satisfactory microbial activity.

Duplicate nonsterile samples were removed on Hours 0, 2, 6, 24, 48, 120, and 216 for both dosing levels. Sterile flasks were removed at Hours 24 and 120 and aniline dosed flasks at Hours 24 and 48. After radioassaying, the aqueous sample was applied to an SPE cartridge and eluted with methanol followed by methanol/ethyl acetate/acetic acid From 24 hours and later, an additional elution of the cartridge was performed using methanol:HCl followed by methanol:KOH. The organic phases were concentrated and chromatographed for quantitation of parent and metabolites and for characterization of metabolites. The ethylene glycol and KOH traps were also radioassayed. To aid in metabolite identification, additional samples of surface water were dosed at both 500 ppb and 1000 ppb $^{14}$C-BIT and processed with SPE cartridges as described above. Metabolites were identified by LC-MS.

**5.2 Results and discussion**

The amount of $^{14}$C activity initially eluted from the SPE cartridge decreased with time for both dosing levels. For samples dosed at 25.6 ppb the $^{14}$C-activity eluted with the additional solvents increased with time while for the 105 ppb it remained relatively constant. This indicates that BIT is being quickly degraded. Under sterile conditions, there was essentially no degradation of BIT.

BIT biodegrades very fast in the estuarine water studied. The half-lives at 20°C were 30.8 hours at 25.6 ppb and 41.8 hours at 105 ppb.

The table below provides the metabolites produced in surface water dosed with BIT and their maximum percentage. They were identified by mass spectroscopy.

| Metabolite | Maximum Percent of Applied Dose | |
|---|---|---|
| | 25.6 ppb | 105 ppb |
| 2-sulfobenzamide | 30 | 27.5 |
| 2-methylsulfinyl-benzamide | 24.9 | 22.1 |
| 2-methylthio-benzamide | 48.3 | 54.0 |
| 2-methylthio-benzoic acid methylester | Not present | 12.8 |

| | | | |
|---|---|---|---|
| | $^{14}CO_2$ | < 1 | < 1 |

.

| | | |
|---|---|---|
| **5.3 Conclusion** | | Similar to the results in other media (*e.g.* soil and STP), BIT quickly biodegrades in estuarine water. The half-life at 20°C was 30.8 hours at 25.6 ppb and 41.8 hours at 105 ppb. Sterile samples were essential stable. Metabolism involved cleavage of the isothiazolone ring, leading to the formation of benzamide and benzoic acid ester metabolites. . |
| 5.3.1 | Reliability | 1-valid without restrictions |
| 5.3.2 | Deficiencies | No |

| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *December 2010* |
| **Materials and Methods** | *2.3. Three GLP deviations:1) plate counts determining microbial activity were not conducted under GLP; 2) total organic carbon and nutrients of the surface water were analyzed after the kinetic test was completed; and 3) the temperature in the constant temperature room was out of the desired range, 20±2°C at four intervals (17.02, 17.86, 22.18, and 22.94°C).* |
| **Results and discussion** | *4.2.1. The TLC limit of quantitation (LOQ) for $^{14}$C-BIT was determined as 4.68 ppb with an overall recovery of 95.7 ± 4.1% (n = 8). In guidelines, it is recommended that the limit of quantification (LOQ) should be equal to or less than 10% of the applied concentration. In this case, LOQ exceed 10% of the concentration of 20 ppb.* |
| **Conclusion** | *According to the applicant the half-life at 20°C was 30.8 hours at 25.6 ppb and 41.8 hours at 105 ppb. These values did not match, however, with **Table A7.1.2.2.1-2: Range Finding Study—Quantitation of Parent**. Thus, eCA recalculated BIT DT50 for both concentrations tested using FOCUS Kinetic Guidance. Results show that BIT degraded very fast in water. Degradation involves the cleavage of the isothiazolone ring yielding 4 major metabolites. The half-life for water dosed at 25.6 µg/L was 0.505 days or 12.12 hours and at 105 µg/L, 0.654 days or 15.7 hours at 20°C. Degradation rates at 12°C were 22,9 and 29.7.* |
| **Reliability** | *1* |
| **Acceptability** | *Acceptable* |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.1** | **Fate and Behaviour in Water** |
| **Subsection A7.1.2.2** | **Aerobic aquatic degradation study** |
| **Subsection A7.1.2.2.1** | **Biodegradation in Estuarine Surface Water** |
| **Annex Point IIIA XII.2.1** | |

| | |
|---|---|
| **Remarks** | *Figure A7.1.2.2.1-3: "Metabolic Pathway of CMIT in Surface Water" should read "Proposed Metabolic Pathway of BIT in Surface Water".* |

**Table A7.1.2.2.1-1: Parameters of Test Water**

| Parameter | Method Development /Range Finding | 105 ppb Dosing | Metabolite Collection |
|---|---|---|---|
| pH | 7.4 | 7.6 | NA[1] |
| Temperature (°C)[2] | 18.7 | 12.1 | 13.2 |
| Calcium (ppm) | 58 | 55 | NA |
| Magnesium (ppm) | 14 | 16 | NA |
| Sodium (ppm) | 99 | 80 | NA |
| Hardness (mg equivalent $CaCO_3$/L) | 205 | 203 | NA |
| Conductivity (mmhos/cm) | 0.87 | 0.75 | NA |
| Sodium Adsorptin Ratio | 3.02 | 2.45 | NA |
| Total Dissolved Solids (ppm) | 534 | 428 | NA |
| Turbidity (NTU) | 0.81 | 0.86 | NA |
| Nitrogen/Phosphorus/ Potassium (mg/L) | NA | NA | 1.21/1.2/10.1 |
| Total Organic Carbon (mg/L) | NA | NA | 7.5 |
| Microbial Activity (cfu/ml) | $8.13 \times 10^3$ | $2.45 \times 10^4$ | $1.16 \times 10^5$ |

[1] N = nitrogen, P = phosphorus, K = potassium

[2] Temperature taken at sampling location

**Table A7.1.2.2.1-2: Range Finding Study—Quantitation of Parent**

| Sample Interval (hours) | Percent BIT[1] | |
|---|---|---|
| | 20.7 ppb | 104 ppb |
| 0 | 90.9 | 87.2 |
| 2 | 82.0 | 82.7 |
| 6 | 72.6 | 74.6 |
| 24 | 48.3 | 57.5 |
| 48 | 15.6 | 20.0 |
| 120 | 6.4 | 9.0 |

[1] Average of duplicate samples

**Table A7.1.2.2.1-3: tability of BIT in Sterile Water**

| Study Type | BIT in Sterile Water as Percent of Applied Activity[1] | |
|---|---|---|
| | 24 hours | 120 hours |
| 25.6 ppb Definitive | 92.1 | 93.1 |
| 105 ppb Definitive | 92.3 | 92.6[2] |

[1] Average of duplicate samples except for

[2] which is based on single sample

**Table A7.1.2.2.1-4: Dissipation of the System Reference Standard Aniline in Surface Water**

| Study Type | Aniline in Surface Water as Percent of Applied Concentration[1] | |
|---|---|---|
| | 24 hours | 48 hours |
| 20 ppb Definitive | 69.0 | 61.0 |
| 100 ppb Definitive | 64.2 | 52.2 |

**Table A7.1.2.2.1-5: Distribution of $^{14}C$-Activity in Surface Water**

| Sample Time (h) | Percent of Applied $^{14}C$-Activity (average of duplicate samples) | | | | |
|---|---|---|---|---|---|
| | Initial SPE Eluant | Additional SPE Eluant | $CO_2$ | Organic Volatiles | Recovery |
| **25.6 ppb** | | | | | |
| 0 | 99.4 | NA[1] | NA | ND[2] | 99.4 |
| 2 | 85.9 | NA | 0.0 | ND | 85.9 |
| 6 | 87.9 | NA | 0.0 | ND | 87.9 |
| 24 | 79.7 | 15.7 | 0.0 | ND | 95.4 |
| 48 | 77.1 | 20.8 | 0.0 | ND | 97.9 |
| 120 | 60.6 | 26.0 | 0.0 | ND | 86.6 |
| 216 | 62.2 | 31.7 | <1 | ND | 93.9 |
| | | | Average Recovery: | | 92.4 ± 5.6% |
| **105 ppb** | | | | | |
| 0 | 112 | NA | NA | ND | 112 |
| 2 | 99.7 | NA | 0.0 | ND | 99.7 |
| 6 | 99.5 | NA | 0.0 | ND | 99.5 |
| 24 | 78.7 | 20.1 | 0.0 | ND | 98.9 |
| 48 | 82.4 | 13.1 | 0.0 | ND | 95.5 |
| 120 | 70.0 | 17.0 | 0.0 | ND | 87.0 |
| 216 | 82.2 | 13.3 | <1 | ND | 95.5 |
| | | | Average Recovery: | | 98.3 ± 7.5% |

[1] NA = not applicable

[2] ND = not detected

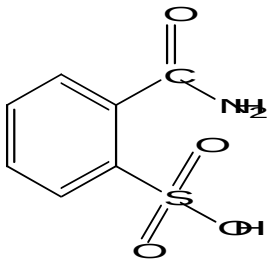
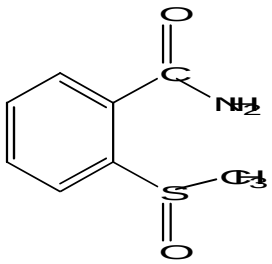
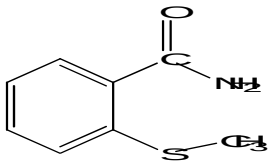
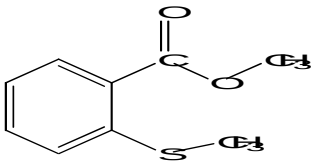
**Table A7.1.2.2.1-6: Quantitation of Parent and Metabolites**

| Sample Time (h) | Percent of Applied $^{14}C$-Activity (average of duplicate samples) | | | | | |
|---|---|---|---|---|---|---|
| | BIT | M1 | M2 | M3 | M4 | Mx[1] |
| **25.6 ppb** | | | | | | |
| 0 | 95.7 | 1.2 | 0.2 | 0.1 | NS[2] | 2.2 |
| 2 | 79.6 | 3.0 | 1.3 | 1.4 | NS | 0.8 |
| 6 | 67.2 | 8.0 | 3.7 | 2.2 | NS | 6.8 |
| 24 | 25.3 | 24.4 | 10.1 | 29.5 | NS | 6.0 |
| 48 | 7.1 | 24.2 | 11.8 | 48.3 | NS | 6.6 |
| 120 | 4.0 | 23.4 | 19.6 | 33.1 | NS | 6.6 |
| 216 | 0.6 | 30.0 | 24.9 | 33.7 | NS | 4.7 |
| **105 ppb** | | | | | | |
| 0 | 107.7 | 1.4 | NS | NS | NS | 2.7 |
| 2 | 93.3 | 2.8 | 0.0 | NS | NS | 3.6 |
| 6 | 86.2 | 9.9 | 0.0 | NS | NS | 3.4 |
| 24 | 35.5 | 27.5 | 11.9 | 17.3 | NS | 6.7 |
| 48 | 7.5 | 10.7 | 22.1 | 52.0 | NS | 3.3 |
| 120 | 3.4 | 4.3 | 19.1 | 54.0 | NS | 5.2 |
| 216 | 3.7 | 2.3 | 20.7 | 43.8 | 12.8 | 4.7 |

[1] Mx represents nonspecific radioactivity recovered from the TLC plate from areas other than the those of M1, M2, M3, M4, and BIT. Given the large area represented by Mx, it is nonspecific and is comprised of multiple compounds.

[2] NS = not significant or less than the liquid scintillation counter LOD.

**Table A7.1.2.2.1-7: Structure of Metabolites Produced in Surface Water Dosed with BIT**

| Component | Structure | Name | Maximum Percent of Applied $^{14}$C-Activity |
|---|---|---|---|
| M1 |  | 2-Sulfobenzamide | 30.0% at 216 hours (25.6 ppb)<br><br>27.5% at 24 hours (105 ppb) |
| M2 |  | 2-methylsulfinyl-benzamide | 24.9% at 216 hours (25.6 ppb)<br><br>22.1% at 48 hours (105 ppb) |
| M3 |  | 2-methylthio-benzamide | 48.3% at 48 hours (25.6 ppb)<br><br>54.0% at 120 hours (105 ppb) |
| M4 |  | 2-methylthiobenzoic acid methyl ester | Not present (25.6 ppb)<br><br>12.8% at 216 hours (105 ppb) |
| $CO_2$ | | Carbon Dioxide | <1%, at 25.6 ppb [a]<br><1%, at 105 ppb [a] |

[a] Total accumulation after 216 hours.

**Figure A7.1.2.2.1-1: Kinetic Analysis of BIT in Surface Water Dosed at 25.6 ppb**

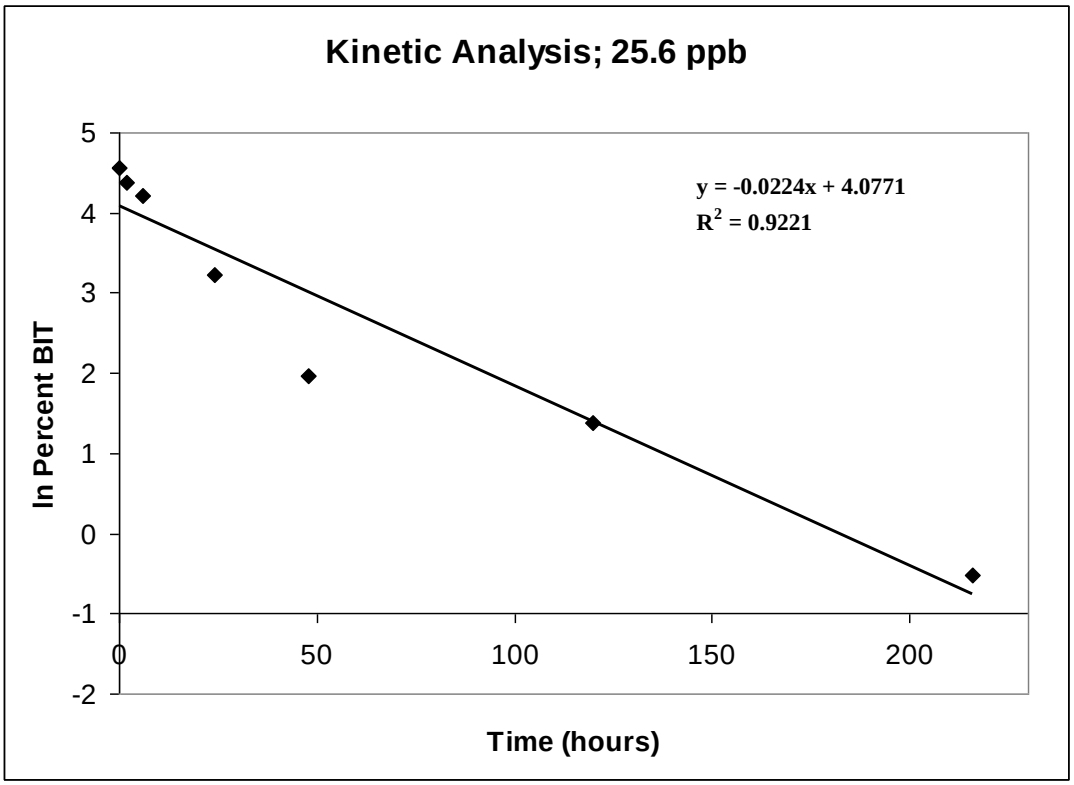



**Figure A7.1.2.2.1-2: Kinetic Analysis of BIT in Surface Water Dosed at 105 ppb.**

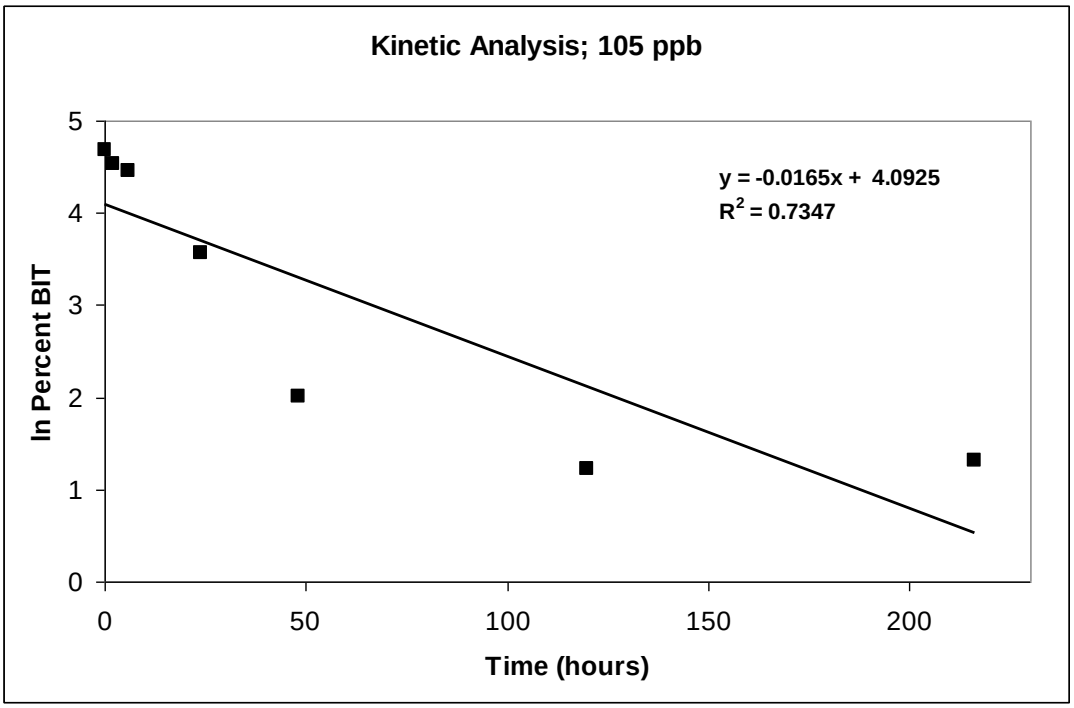
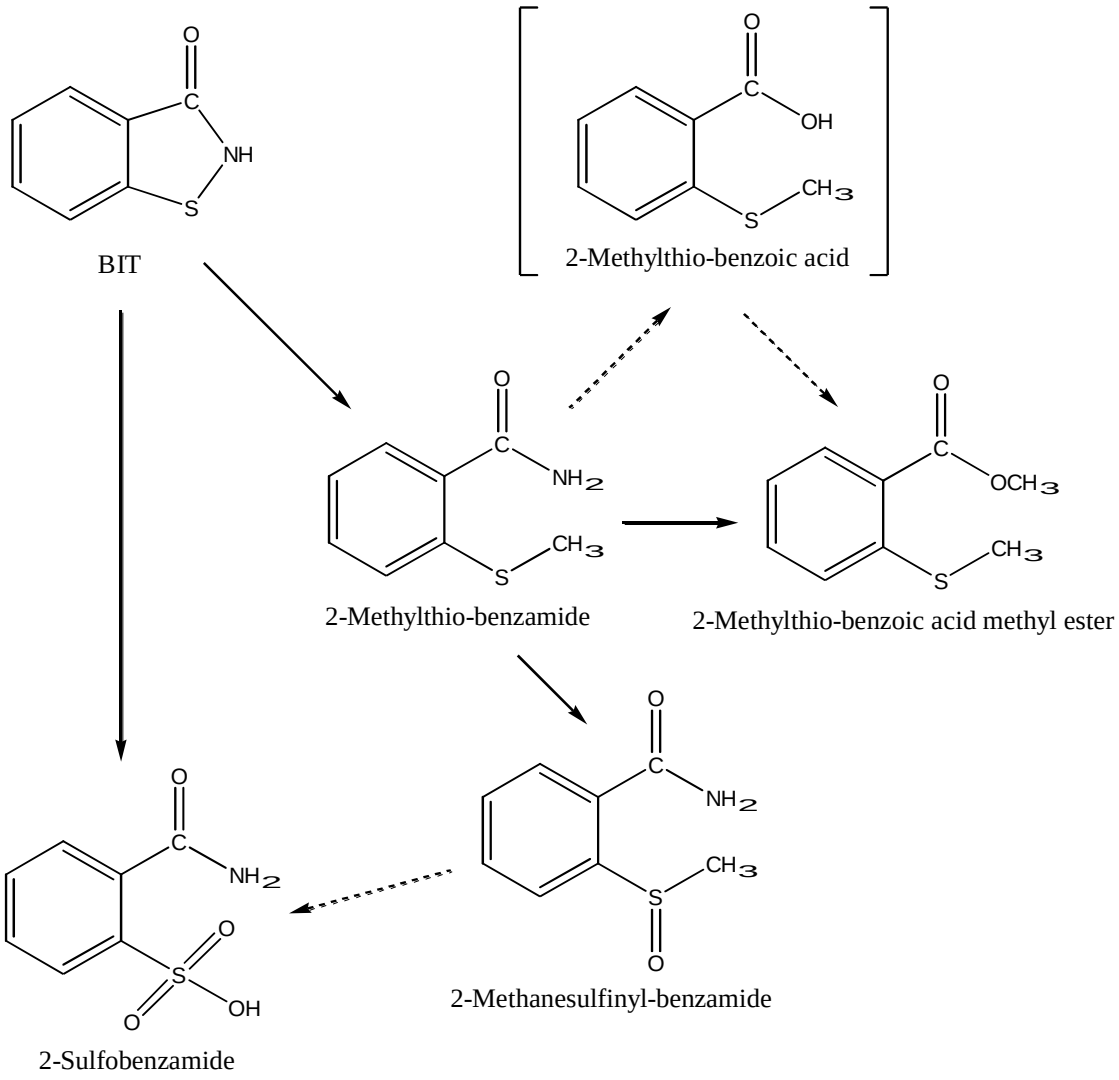
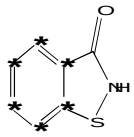


**Figure A7.1.2.2.1-3: Metabolic Pathway of CMIT in Surface Water**



| | | |
|---|---|---|
| **Section A7**<br><br>**Subsection A7.1**<br>**Subsection A7.1.2.2**<br>**Subsection A7.1.2.2.2**<br>**Annex Point IIIA XII.2.1** | **Ecotoxicological Profile Including Environmental Fate and Behaviour**<br>**Fate and Behaviour in Water**<br>**Water:Sediment Degradation Studies**<br>**Aerobic and Anaerobic** | |
| | **JUSTIFICATION FOR NON-SUBMISSION OF DATA** | **Official use only** |
| **Other existing data [X]** | **Technically not feasible [ ]** **Scientifically unjustified [ ]** | |
| **Limited exposure [ X]** | **Other justification [ ]** | |
| **Detailed justification:** | A waiver is requested from performing aerobic and anaerobic water:sediment studies (A7.1.2.2.2). As noted in Chapter 3, Section 7.0.2.3.2 (and Figure 1 in section 7) a sediment:water study is only required when the $K_p > 2000$. For BIT, the maximum measured $K_{oc}$, in a sediment is 35 (K = 0.67). In 4 soils the $K_{oc}$ ranged from 58 – 144. Therefore the $K_p$ will be significantly less than 2000. | |
| **Undertaking of intended data submission [ ]** | No studies are planned. | |
| | | |
| | | |
| | **Evaluation by Competent Authorities** | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | *December 2010* | |
| **Evaluation of applicant's justification** | *Applicant's justification is accepted* | |
| **Conclusion** | *Applicant's justification is accepted* | |
| **Remarks** | | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.1** | **Fate and Behaviour in Water** |
| **Subsection A7.1.3** | **Adsorption and Desorption Test - Soil and Sediment** |
| **Annex Point IIA VII.7.7.** | |

| | | | |
|---|---|---|---|
| | | **1 REFERENCE** | **Official use only** |
| **1.1** | **Reference** | **A7.1.3/01** [redacted] (2007). [$^{14}$C] BIT: Adsorption/Desorption in Soil and Sediment; [redacted] Rohm and Haas Technical Report N° TR-07-022. Unpublished. | |
| **1.2** | **Data protection** | Yes | |
| 1.2.1 | Data owner | Rohm and Haas Company | |
| 1.2.2 | Companies with letter of access | | |
| 1.2.3 | Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into Annex I/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **Guideline study** | Yes. OECD Guideline for testing chemicals 106: Adsorption-desorption using a batch equilibration method, adopted January 2006 and US EPA OPPTS 855.2210: Sediment and Soil Adsorption/Desorption Isotherm (January 1998) | |
| **2.2** | **GLP** | Yes | |
| **2.3** | **Deviations** | No claim of GLP compliance is made for soil sterilization or sterility testing. However these procedures were conducted in accordance with current GLP requirements. | |
| | | **3 MATERIALS AND METHODS** | |
| **3.1** | **Test material** | $^{14}$C-BIT<br>  <br>* site of $^{14}$C label | |
| 3.1.1 | Lot/Batch number | Lot 1069.00, sublot 1069.0005 | |
| 3.1.2 | Specification | As specified in the study guidelines, $^{14}$C-material was employed. Specifications for the $^{14}$C-material are listed below. | |

| | |
|---|---|
| 3.1.3 Radiopurity | Radiopurity : 98.3% |
| 3.1.4 Specific Activity | Specific activity: 53.57 mCi/g |
| 3.1.5 Further relevant properties | Water solubility is greater 0.7 g/L. |
| 3.1.6 Method of analysis | Adsorption and desorption was determined by radioassay of the two phases, soil and aqueous solution. Confirmation of parent stability examined by HPLC and LC-MS. |
| **3.2 Degradation products** | Degradation products were not tested in this study. Only the adsorption and desorption of parent was measured in this study. |
| 3.2.1 Method of analysis for degradation products | Not applicable |
| **3.3 Reference substance** | No system reference substance was employed. A BIT reference standard for chromatography was employed. |
| 3.3.1 Nature of reference substance | The chromatography reference standard employed was:<br>$^{12}$C-BIT, Lot MJB3787, Purity 100.1%. |
| **3.4 Soil types** | Four soils and one sediment were employed. The sample location, soil type, and physiochemical characteristics of the soils and sediment used in this study are presented in Table A7.1.3-1. Soils were obtained from the top 25 cm of agricultural land, were air dried, passed through a 2 mm sieve, and sterilized by gamma irradiated prior to use. |
| **3.5 Test Solutions** | |
| 3.5.1 BIT Test Solutions | The preparation of each dosing solution is described within the appropriate test performance section |
| 3.5.2 0.01M $CaCl_2$ | 0.01M $CaCl_2$ was prepared by dissolving either 1.11 g or 2.22 g of anhydrous $CaCl_2$ in 1L or 2L or water. Additionally it was prepared by dissolving 2.94 g of hydrated $CaCl_2$ in 2L of water. The solutions were sterilized by autoclaving |
| **3.6 Preliminary Investigations** | |
| 3.6.1 Solubility | Stock solutions were made by dissolving 4.560 mg $^{14}$C-BIT in 10 mL acetonitile and 138.272 mg $^{12}$C-BIT in 50 ml acetonitrile. 250 µL of the $^{14}$C-stock solution and 360 µL of the $^{12}$C stock solution were added to a centrifuge tube and taken to dryness. The BIT was reconstituted in 10 mL 0.01M $CaCl_2$ with the resulting concentration being 110 µg/mL. This is greater than twice the proposed highest application concentration. The solution was sonicated for 1 h, centrifuged and the supernatant |

| | |
|---|---|
| | radioassayed. The mean recovery was 97.4%.<br><br>A second solubility check was performed by adding 168 µL of the $^{14}$C-BIT stock solution and 244 µL of the $^{12}$C-BIT stock solution to a centrifuge tube and taking the sample to dryness. The was reconstituted in 15 mL $CaCl_2$ and the resulting 5 µg/mL solution sonicated for 10 minutes, centrifuged, and the supernatant radioassayed. The mean recovery was 100.8%. |
| 3.6.2 Adsorption to containers | 1.6 µL $^{14}$C-BIT stock solution and 2.4 µL $^{12}$C BIT stock solutions (stock solutions from solubility test) were added to a Teflon® centrifuge tube, taken to dryness, and reconstituted with 15 mL $CaCl_2$ (yielding a 0.5 µg/ml solution). Aliquots (2.5 mL) of the application solution were diluted with 22.5 mL of $CaCl_2$ (final concentration 0.05 µg/mL), shaken for 24 hours, and radioassayed. The mean recover was 102.6% demonstrating that there was no adherence of the test substance to the tube walls. |
| 3.6.3 Ratio of soil to solution | An application solution was prepared from the solubility test stock solutions. 340 µL $^{14}$C-BIT and 1750 µL $^{12}$C-BIT were added to a container, the acetonitrile evaporated, 100 mL of 0.01M $CaCl_2$ added, and the solution sonicated.<br><br>The testing scheme is tabulated below. |

| Soil:Solution Ratio | BIT (mL) | Soil (g) | 0.01M $CaCl_2$ (mL) |
|---|---|---|---|
| 1:1 | 1.0 | 10 | 9.0 |
| 1:2 | 2.0 | 10 | 18.0 |
| 1:5 | 2.5 | 5 | 22.5 |

| | |
|---|---|
| | The final BIT concentration was 5 µg/mL. The tubes were mixed for 24 hours, centrifuged, and the supernatant radioassayed. |
| 3.6.4 Equilibration time determination | An application solution was prepared by dissolving 5.718 mg $^{14}$C BIT in 114 mL of 0.01M $CaCl_2$ resulting in a nominal concentration of 50 µg/ml.<br><br>10 g of the four soils and one sediment were individually added to centrifuge tubes. Eight tubes per soil/sediment were prepared. To each tube, 18 mL of 0.01M $CaCl_2$ was added and the tubes shaken overnight. The next morning 2 mL of the BIT solution was added to give a concentration of 5 µg/mL and a soil:solution ratio of 1:2 (w/v). At Hours 1, 3, 6, and 24 duplicate tubes were removed for each soil/sediment, centrifuged, and the supernatant radioassayed. |
| 3.6.5 Stability test | The supernatants from the above equilibration determination were analyzed by HPLC. The soils were extracted three times by shaking (20 min) with methanol (20 mL) and centrifuged. They were further extracted an additional three times by shaking (20 min) with 0.1M |

| | |
|---|---|
| | NaOH:methanol (80:20; 20 mL) and centrifuged. The supernatant was radioassayed and then analyzed by HPLC.<br><br>The sterility of each soil was checked by plating aliquots on nutrient agar plates.<br><br>An additional test was performed using only the clay loam soil. Four samples were prepared as above except that that hydrogen peroxide was added at 1% and 3%. After shaking for 1 hour, the tubes were centrifuged, and the supernatant analyzed by HPLC. |
| **3.7 Definitive test (isotherm)** | A stock solution was prepared by dissolving 10.983 mg $^{14}$C BIT in 2 mL of acetonitrile. Three application solutions were prepared directly from the stock solution by taking to dryness 370 µL, 110 µL, and 36 µL and reconstituting in 40 mL of 0.01M $CaCl_2$ resulting in concentrations of 52µg/mL, 15 µg/mL, and 5 µg/mL. Two additional application concentrations were prepared by diluting 1240 µL and 400 µL of the 52 µg/mL solution in 40 mL $CaCl_2$ resulting in concentrations of 1.4 µg/mL and 0.5 µg/mL.<br><br>Ten samples were prepared for each soil/sediment so that five concentrations could be investigated in duplicate. 10 g of soil/sediment were added to a Teflon® centrifuge tube and mixed overnight with 18 ml of $CaCl_2$. The next day 2 mL of each application solution was added to duplicate tubes for each of the soil/sediment types. The resulting BIT concentration was 0.05, 0.15, 0.5, 1.5, and 5 µg/mL. After 1 h of mixing, the tubes were centrifuged, and the supernatants radioassayed and the pH measured. The supernatants were also analyzed by HPLC.<br><br>The soils dosed at 5 µg/mL were radioassayed. They were subsequently extracted as per the stability test (section 3.6.5) in the preliminary investigations. |
| 3.7.1 Analytical Procedures | Radioassay of liquid samples was performed using Packard liquid scintillation counter.<br><br>Radiopurity and aliquots from the buffer solutions were analyzed by HPLC using a modified C-18 column and a binary gradient composed of 0.5% aqueous formic acid and 0.5% methanolic formic acid. Detection employed a $^{14}$C-flow through monitor and/or UV detector (254 nm).<br><br>Thin layer chromatography (TLC) was used for radiopurity determination. Silica gel plates (250 µm thick) were developed with ethyl acetate:methanol:acetonitrile:acetic acid (90:5:5:1). Solutions were cochromatographed with non-radiolabeled BIT. Radiolabeled compounds were detected using a phophorimager while non-labeled compounds visualized with a UV lamp (254 nm).<br><br>Representative samples were analyzed by LC-MS (ion trap) to confirm the presence of parent. Analysis employed a modified C-18 column and a binary gradient composed of 0.5% aqueous formic acid and 0.5% methanolic formic acid. Detection was by a radioactivity flow monitor and the mass spectrometer. The LC effluent was split between the two detectors and introduction in to the MS via an API interface and positive and negative ionization was employed. |

**4 RESULTS**

**4.1 Preliminary Investigations**

Solubility

The solubility of BIT in 0.01M $CaCl_2$ was examined initially at 110 µg/mL, which was at least double the expected study concentration. Over 97% of the BIT was found soluble at this concentration. A second experiment was performed at the proposed final test concentration, 5 µg BIT/mL, and the solubility was 100%.

Adsorption to containers

BIT in 0.01M $CaCl_2$ was added to Teflon® centrifuge tubes without soil and shaken for 24 hours. The mean recovery of $^{14}$C-BIT was 103% demonstrating no adherence of the test compound to the test vessels.

Ratio of soil to solution

Soil:0.01M $CaCl_2$ ratios of 1:1, 1:2, and 1:5 and dosed at 0.5 µg $^{14}$C-BIT were examined. The results are summarized below.

| Soil | Percent $^{14}$C-BIT in Supernatant (0.01M $CaCl_2$) | | |
|---|---|---|---|
| | 1:1 Ratio | 1:2 Ratio | 1:5 Ratio |
| Clay Loam | 28.2 | 34.8 | 55.4 |
| Silt Loam | 12.1 | 22.2 | 44.1 |
| Loam/Silt Loam | 19.9 | 27.8 | 46.4 |
| Loamy Sand | 30.9 | 48.9 | 72.5 |
| Loamy Sand (sediment) | 14.8 | 25.7 | 53.1 |

Based on difference, this indicates that the following percentage ranges were adsorbed to the soil with the highest adsorption to the silt loam and the lowest to the loamy sand soil:

1:1—61.8% to 87.9%

1:2—51.1% to 74.3%

1:5—27.5% to 46.9%

The 1:2 soil:0.01M $CaCl_2$ ratio was chosen since the percent adsorption to soil and sediment was between 50% and 80%.

Equilibrium Time and Stability Tests

Distribution of radioactivity between soil and sediment is presented in Table A7.1.3-2. The average recovery of $^{14}$C-activity was 90.7 ± 10.6%. A graphical presentation of the equilibration results can be seen in Figure A7.1.3-1. The percent of applied radioactivity recovered as BIT is presented in Table A7.1.3-3. The average recovery of BIT for the 1 and 3

hour equilibration time intervals was 55.0 ± 11.0%.

The results indicate that the adsorption of BIT to soil did not fully reach equilibration in the 24 hour period. This is probably due to the dissipation of BIT during the study period. Examination of the soil sterility showed that it was sterile (no colony forming units observed on agar plates) and degradation was the result of abiotic activity.

Nucleophiles are known to cleave the isothiazolone ring. To examine if this was the cause for degradation, hydrogen peroxide was added to the soil:$CaCl_2$ mixture. The results showed that degradation was greater in the presence of peroxide than in its absence.

**4.2 Definitive test (isotherm)**

Based on the preliminary test a soil:0.01M $CaCl_2$ ratio of 1:2 and a 1 hour equilibration time were used for the isotherm test.

The pH of adsorption supernatants are presented in Table A7.1.3-4.

The mean percent of adsorption for the four soils and 1 sediment are presented in Table A7.1.3-4. For clay loam, silt loam, loam/silt loam, loamy sand soil, and loamy sand sediment the adsorption ranged from 44.5% to 65.7%, 63.2% to 77.4%, 49.7% to 65.8%, 23.1% to 37.8%, and 24.0 to 48.5%, respectively. The adsorption coefficients ($K_d$, $K_{doc}$, and $K_{dom}$) determined at each dosing concentration is presented in Table A7.1.3-5.

Fruedlich adsorption coefficients and linearity values, 1/n and $r^2$, are presented in Table A7.1.3-6. The $K_{oc}$ values range from 35-144 mL/g. A summary of these results are presented below.

| Soil | Adsorption Range (%) | $K_d$ | $K_{oc}$ | $r^2$ |
|---|---|---|---|---|
| Clay Loam | 45 - 66 | 1.98 | 41 | 0.9966 |
| Silt Loam | 63 - 77 | 3.88 | 144 | 0.9985 |
| Loam/Silt Loam | 50 – 66 | 2.27 | 58 | 0.9987 |
| Loamy Sand Soil | 23 – 38 | 0.75 | 94 | 0.9958 |
| Loamy Sand Sediment | 24 - 49 | 0.67 | 35 | 0.9764 |

The $r^2$ values demonstrate there is a good correlation between the log of the concentration adsorbed and the log of the dosing concentrations.

The mobility class of BIT in soil is high mobility.

**4.3 Desorption test**

No desorption test was performed due to the degradation of BIT during the adsorption phase.

**4.4 Mass balance**

Material balance was determined for the 4 soils and 1 sediment from the isotherm test at an application rate of 5 µg/ml. The results are presented in Table A7.1.3-7. Recoveries ranged from 96.8% to 98.4% with a mean

of 97.5 ± 0.9%.

**5 APPLICANT'S SUMMARY AND CONCLUSION**

**5.1 Materials and methods**

The test guideline followed was OECD 106 and US EPA OPPTS 855.2210. There were no deviations from this test guideline. The four soils and 1 sediment were gamma irradiated prior to dosing to enhance sterility and prevent biodegradation of BIT during the course of the experiment.

Initially the solubility of BIT in 0.01M $CaCl_2$ and the potential to adsorb to the test vessel were examined. Both tests were performed in the absence of soil. $^{14}$C-BIT was added to Teflon® centrifuge tubes and the supernatant radioassayed.

The effect of the ratio of soil to 0.01M $CaCl_2$ solution was examined. Soil:$CaCl_2$ solutions ratios of 1:1, 1:2, and 1:5 were examined. Soil and $CaCl_2$ were equilibrated by shaking overnight and the next morning $^{14}$C-BIT was added. The mixture was shaken for 24 hours, centrifuged, and the supernatant radioassayed.

A study to determine the time necessary to reach equilibration was performed by adding soil and 0.01M $CaCl_2$ in a 1:2 ratio and mixing overnight. $^{14}$C-BIT was added at 5 µg/ml and duplicate tubes removed and radioassayed at 1, 3, 6, and 24 hours. The supernatants were also chromatographed (HPLC). The soils from the 1 and 3 hours intervals were extracted with methanol and NaOH:methanol and the extracts chromatographed (HPLC).

The definitive adsorption isotherm study was performed with a soil:0.01M $CaCl_2$ solution ratio of 1:2 and $^{14}$C-BIT concentrations of 0, 0.05, 0.15, 0.5, 1.5, and 5 µg/ml. The soil and $CaCl_2$ solution were added to Teflon® centrifuged tubes, mixed overnight, and then the $^{14}$C-BIT added. Tubes were shaken for 1 hour, centrifuged, and the supernatant radioassayed and chromatographed. The soils dosed at 5 µg/ml were extracted with methanol and NaOH:methanol in order to obtain a material balance.

**5.2 Results and discussion**

BIT showed a small adsorption to the 5 soils/sediment examined. There was abiotic degradation of BIT observed during the preliminary investigations and thus a 1 hour equilibration time was chosen for the isotherm test. Due to the degradation of BIT no desorption study was performed. Where examined, the recovery of applied $^{14}$C-activity was greater than 96%.

5.2.1 Adsorbed a.s. [%]

The percent of $^{14}$C-adsorption for the 5 soils/sediment after a 1 hour equilibrium is tabulated below.

| Clay Loam | Silt Loam | Loam/Silt Loam | Loamy Sand Soil | Loamy Sand Sediment |
|---|---|---|---|---|
| 45-66 | 63- 77 | 50-66 | 23-38 | 24-49 |

| | | |
|---|---|---|
| 5.2.2 | $K_d$ (adsorption) | The adsorption coefficients (Kd) from the isotherm test are tabulated below. |

| Clay Loam | Silt Loam | Loam/Silt Loam | Loamy Sand Soil | Loamy Sand Sediment |
|---|---|---|---|---|
| 1.98 | 3.88 | 2.27 | 0.94 | 0.67 |

| | | |
|---|---|---|
| 5.2.3 | $K_{oc}$ (adsorption) | The adsorption constants (Koc) are tabulated below. |

| Clay Loam | Silt Loam | Loam/Silt Loam | Loamy Sand Soil | Loamy Sand Sediment |
|---|---|---|---|---|
| 41 | 144 | 58 | 94 | 35 |

| | | |
|---|---|---|
| 5.2.4 | Degradation products | BIT degraded in the test system. Degradation was abiotic as the system was found to be sterile after a 24 hour equilibration period. The identity of the degradate(s) was not determined, however, it is probably an oxidation product such as hydroxylation of the benzene ring or oxidation of the sulfur moiety. |
| **5.3** | **Conclusion** | The study provided is satisfactory to describe the mobility of BIT in soil. According to the US EPA classification scheme, BIT is considered high to very highly mobile. While the compound did degrade during testing, the adsorption values obtained here are similar to those reported in the US. EPA Registration Eligibility Document (RED) and thus are probably representative of BIT adsorption. It is highly likely that BIT and its oxidized products are similar in adsorption/mobility. Additionally, a ready biodegradation study (A7.1.1.2.1) demonstrated that BIT rapidly biodegrades. In soil BIT is probably biodegraded before it can leach and be an environmental concern. |
| 5.3.1 | Reliability | 1-valid without restrictions |
| 5.3.2 | Deficiencies | No |

| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *March 2015.* |

**Section A7** **Ecotoxicological Profile Including Environmental Fate and Behaviour**

**Subsection A7.1** **Fate and Behaviour in Water**

**Subsection A7.1.3** **Adsorption and Desorption Test - Soil and Sediment**

**Annex Point IIA VII.7.7.**

| | |
|---|---|
| **Materials and Methods** | *Applicant's version is accepted with the following remarks:*<br>▪ *3.2. Degradation products are not tested.*<br>▪ *The pH of the aqueous phase should be measured before and after contact with the soil, since it plays an important role in the adsorption process, especially for ionisable substances such as BIT. Nevertheless, in this report, applicant only provides the value of pH of the supernatant after the performance of the test.*<br>*According to OECD guidelines, the detection limits of the analytical method should be at least two orders of magnitude below the nominal concentration. In this test, the applicant does not provide the limit of detection of BIT with the analytical method.* |
| **Results and discussion** | *Applicant's version is accepted with the following remarks:*<br>*Table A7.1.3-6: Frundlich Coefficients for $^{14}$C-BIT 1/n (linearity term of the equation) and Kd values Table A7.1.3- show that the sorption of BIT is concentration dependent. Therefore the Freundlich K parameter is underestimating the sorption of BIT at environmentally relevant concentrations (corresponding to the low part of the isotherm). In the absence of a risk exposure assessment depending on the adsorbed concentration, an average value of the single $K_d$ measure is more representative than the $K_{Feundlich}$ value.*<br>*The final $K_d$ and $K_{oc}$ table should be:* |
| **Conclusion** | *Applicant's version is accepted with minor changes:*<br>*While determining the equilibration time it was discovered that BIT was degrading. This degradation was due to an abiotic process (oxidation) because the soils were sterile. It was necessary to use a short equilibration time (1 h) to reduce the effect of degradation on the study even though BIT had not come to a complete equilibrium. Yet, according to the water solubility and Kd values published in the US EPA Registration Eligibility Document (RED) for BIT, $K_d$ values ranged between 1.24 and 9.56 L/kg. Therefore the adsorption values obtained in this study are reasonable and BIT can be considered as a highly mobile compound. A mean $K_{oc}$ value of 114 L/kg for soils and 64 L/kg for sediments is used for the risk assessment.* |

| ***Soil Class*** | ***Percent AS Adsorbed*** | ***$K_d$*** | ***$K_{oc}$*** |
|---|---|---|---|
| *Clay Loam* | *45 - 66* | *2.85* | *59* |
| *Silt Loam* | *63 - 77* | *5.41* | *200* |
| *Loam/Silt Loam* | *50 - 66* | *3.01* | *79* |
| *Loamy Sand Soil* | *23 - 38* | *0.94* | *117* |
| *Loamy Sand Sediment* | *24 - 49* | *1.22* | *64* |

## Section A7 Ecotoxicological Profile Including Environmental Fate and Behaviour

## Subsection A7.1 Fate and Behaviour in Water

## Subsection A7.1.3 Adsorption and Desorption Test - Soil and Sediment

**Annex Point IIA VII.7.7.**

| | |
|---|---|
| **Reliability** | *2* |
| **Acceptability** | *Acceptable* |
| **Remarks** | |

**Table A7.1.3-1: Classification and Physiochemical Characteristics of Soils and Sediment Used as Absorbents**

| Parameter | Soil Type | | | | |
|---|---|---|---|---|---|
| | Clay Loam | Silt Loam | Loam/Silt Loam | Loamy Sand Soil | Loamy Sand Sediment |
| Sampling Location | Chapel Hill Farm, Empingham, Rutland UK | Chelmorton, Derbyshire UK | Kenslow Farm Middleton Derbyshire UK | Worsop Nottinghamshire UK | The Lake, Fountains Abbey UK |
| Percent Sand[1] | 40 | 23 | 32 | 87 | 76 |
| Percent Silt[1] | 32 | 61 | 50 | 4 | 20 |
| Percent Clay[1] | 28 | 16 | 18 | 9 | 4 |
| Organic Matter (%) | 8.3 | 4.7 | 6.7 | 1.4 | 3.3 |
| Organic Carbon (%) | 4.8 | 2.7 | 3.9 | 0.8 | 1.9 |
| pH | 8.0 | 7.0 | 5.3 | 5.1 | 7.3 |
| CEC[2] (meq/100g) | 41.6 | 26.7 | 23.3 | 11.4 | 12.1 |
| Water Holding Capacity (0.33 bar) | 31.7 | 26.3 | 15.5 | 7.1 | 11.2 |
| Nitrogen content (%) | 0.50 | 0.34 | 0.30 | 0.12 | 0.17 |

[1] USDA particle size distribution

[2] CEC = Cation Exchange Capacity

**Table A7.1.3-2: Distribution and Recovery of $^{14}C$-Activity During Equilibration Time Determination**

| Soil | Sampling Interval (h) | Supernatant | Methanol Soil Extract | NaOH/Methanol Soil Extract | Recovery |
|---|---|---|---|---|---|
| Clay Loam | 1 | 33.5 | 30.9 | 24.4 | 88.8 |
| | 3 | 31.7 | 28.3 | 24.9 | 84.9 |
| | 6 | 29.2 | 27.1 | 24.2 | 80.5 |
| | 24 | 26.1 | 16.2 | 19.4 | 61.7 |
| Silt Loam | 1 | 24.7 | 40.9 | 33.9 | 99.5 |
| | 3 | 21.6 | 40.4 | 35.9 | 97.9 |
| | 6 | 19.1 | 34.6 | 39.0 | 92.7 |
| | 24 | 16.3 | 34.5 | 38.8 | 89.6 |
| Loam/Silt Loam | 1 | 27.2 | 36.9 | 27.1 | 91.2 |
| | 3 | 24.7 | 35.5 | 27.8 | 88.0 |
| | 6 | 22.0 | 34.7 | 28.5 | 85.2 |
| | 24 | 19.4 | 23.0 | 26.1 | 68.5 |
| Loamy Sand Soil | 1 | 57.2 | 40.3 | 5.0 | 102.5 |
| | 3 | 54.6 | 41.5 | 6.3 | 102.4 |
| | 6 | 52.8 | 43.6 | 6.4 | 102.8 |
| | 24 | 45.0 | 44.6 | 9.6 | 99.2 |
| Loamy Sand Sediment | 1 | 36.2 | 34.7 | 27.5 | 98.4 |
| | 3 | 27.2 | 35.0 | 33.9 | 96.1 |
| | 6 | 26.9 | 34.5 | 33.9 | 95.3 |
| | 24 | 20.3 | 30.5 | 38.2 | 89.0 |

**Table A7.1.3-3: Distribution and Recovery of $^{14}$C-BIT**

| Soil | Sampling Interval (hrs) | BIT as a Percent of Applied Radioactivity | | |
|---|---|---|---|---|
| | | Supernatant | Total Soil Extract | Recovery |
| Clay Loam | 1 | 44.5 | 7.7 | 52.2 |
| | 3 | 37.5 | 8.3 | 45.8 |
| Silt Loam | 1 | 33.0 | 30.7 | 63.7 |
| | 3 | 28.5 | 27.7 | 56.2 |
| Loam/Silt Loam | 1 | 35.9 | 14.7 | 50.6 |
| | 3 | 29.9 | 22.1 | 52.0 |
| Loamy Sand Soil | 1 | 68.6 | 3.3 | 71.9 |
| | 3 | 65.6 | 7.5 | 73.1 |
| Loamy Sand Sediment | 1 | 46.2 | 1.2 | 47.4 |
| | 3 | 36.6 | 0.1 | 36.7 |

**Table A7.1.3-4: Adsorption of $^{14}C$ BIT to Soil During the Isotherm Test**

| Soil | Nominal Dose (µg/ml) | Percent of $^{14}C$ BIT Applied[1] | | pH |
|---|---|---|---|---|
| | | Adsorbed to Soil | Supernatant | |
| Clay Loam | 5 | 44.5 | 55.5 | 7.37 |
| | 1.5 | 52.3 | 47.7 | 7.49 |
| | 0.5 | 59.5 | 40.5 | 7.65 |
| | 0.15 | 65.7 | 34.3 | 7.69 |
| | 0.05 | 64.5 | 35.5 | 7.77 |
| Silt Loam | 5 | 63.2 | 36.8 | 6.49 |
| | 1.5 | 70.2 | 29.8 | 6.87 |
| | 0.5 | 73.0 | 27.0 | 6.71 |
| | 0.15 | 77.3 | 22.7 | 6.66 |
| | 0.05 | 77.4 | 22.6 | 6.68 |
| Loam/Silt Loam | 5 | 49.7 | 50.3 | 4.27 |
| | 1.5 | 56.7 | 43.3 | 5.25 |
| | 0.5 | 60.3 | 39.7 | 5.22 |
| | 0.15 | 65.4 | 34.6 | 5.22 |
| | 0.05 | 65.8 | 34.2 | 5.21 |
| Loamy Sand Soil | 5 | 23.1 | 76.9 | 4.33 |
| | 1.5 | 29.0 | 71.0 | 4.36 |
| | 0.5 | 31.3 | 68.7 | 4.33 |
| | 0.15 | 37.5 | 62.5 | 4.33 |
| | 0.05 | 37.8 | 62.2 | 4.32 |
| Loamy Sand Sediment | 5 | 24.4 | 75.6 | 6.85 |
| | 1.5 | 24.0 | 76.0 | 6.58 |
| | 0.5 | 36.8 | 63.2 | 7.09 |
| | 0.15 | 48.4 | 51.6 | 7.22 |
| | 0.05 | 48.5 | 51.5 | 7.65 |

[1] Average of duplicate samples

**Table A7.1.3-5: Adsorption Coefficients Resulting from the Isotherm Test**

| Soil | Nominal Dose (µg/mL) | Adsorption Coefficients (mL/g)[1] | | |
|---|---|---|---|---|
| | | $K_d$ | $K_{doc}$ | $K_{dom}$ |
| Clay Loam | 5 | 1.63 | 34 | 20 |
| | 1.5 | 2.19 | 46 | 26 |
| | 0.5 | 2.97 | 62 | 36 |
| | 0.15 | 3.81 | 79 | 46 |
| | 0.05 | 3.65 | 76 | 44 |
| Silt Loam | 5 | 3.41 | 126 | 72 |
| | 1.5 | 4.64 | 172 | 99 |
| | 0.5 | 5.39 | 200 | 115 |
| | 0.15 | 6.79 | 251 | 144 |
| | 0.05 | 6.84 | 253 | 146 |
| Loam/Silt Loam | 5 | 1.98 | 51 | 30 |
| | 1.5 | 2.64 | 68 | 39 |
| | 0.5 | 3.02 | 78 | 45 |
| | 0.15 | 3.81 | 98 | 57 |
| | 0.05 | 3.87 | 99 | 58 |
| Loamy Sand Soil | 5 | 0.60 | 74 | 43 |
| | 1.5 | 0.81 | 101 | 58 |
| | 0.5 | 0.89 | 111 | 64 |
| | 0.15 | 1.18 | 147 | 84 |
| | 0.05 | 1.20 | 150 | 85 |
| Loamy Sand Sediment | 5 | 0.65 | 34 | 20 |
| | 1.5 | 0.62 | 33 | 19 |
| | 0.5 | 1.14 | 60 | 35 |
| | 0.15 | 1.83 | 96 | 55 |
| | 0.05 | 1.86 | 98 | 56 |

[1] Average of duplicate samples

**Table A7.1.3-6: Frundlich Coefficients for $^{14}$C-BIT**

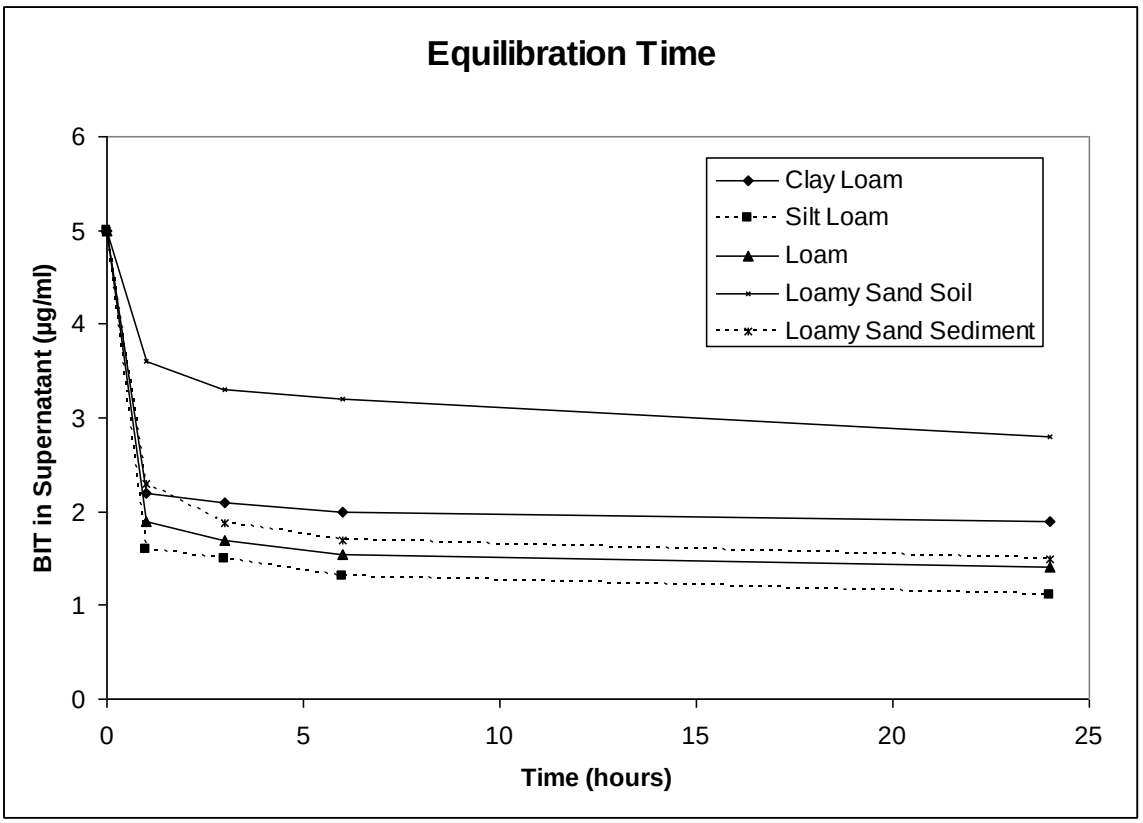
| Soil | Adsorption Coefficients (mL/g) | | | 1/n | $r^2$ |
|---|---|---|---|---|---|
| | K | $K_{oc}$ | $K_{om}$ | | |
| Clay Loam | 1.98 | 41 | 24 | 0.8319 | 0.9966 |
| Silt Loam | 3.88 | 144 | 83 | 0.8629 | 0.9985 |
| Loam/Silt Loam | 2.27 | 58 | 34 | 0.8654 | 0.9987 |
| Loamy Sand Soil | 0.75 | 94 | 54 | 0.8538 | 0.9958 |
| Loamy Sand Sediment | 0.67 | 35 | 20 | 0.7463 | 0.9794 |

**Table A7.1.3-7: Material Balance of Applied Radioactivity from Soils Treated at 5 µg/ml**

| Soil | Percent of Applied $^{14}$C-Activity[1] | | | | | |
|---|---|---|---|---|---|---|
| | Supernatant | Methanol Soil Extract | NaOH/Methanol Soil Extrtact | Acetone Soil Wash | Combusted Residues | Recovery |
| Clay Loam | 31.0 | 31.6 | 23.7 | 0.6 | 10.1 | 96.8 |
| Silt Loam | 23.1 | 39.9 | 31.9 | 0.7 | 2.9 | 98.4 |
| Silt/Silt Loam | 26.2 | 38.8 | 25.0 | 1.0 | 6.5 | 97.4 |
| Loamy Sand Soil | 56.0 | 36.5 | 3.9 | 0.1 | 0.5 | 96.9 |
| Loamy Sand Sediment | 27.3 | 33.7 | 30.7 | 0.9 | 5.4 | 97.9 |
| Mean | | | | | | 97.5 ± 0.9 |

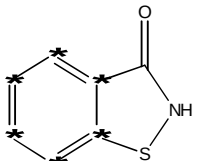
[1] Average of duplicate samples

**Table A7.1.3.b-1: Adsorption Equilibration**



| | | |
|---|---|---|
| **Section A7**<br>**Subsection A7.1**<br>**Subsection A7.1.4**<br>**Annex point IIIA XII.2.1** | **Ecotoxicological Profile Including Environmental Fate and Behaviour**<br>**Fate and Behaviour in Water**<br>**Field Study on Accumulation in Sediment** | |
| | **JUSTIFICATION FOR NON-SUBMISSION OF DATA** | **Official use only** |
| **Other existing data [ ]** | **Technically not feasible [ ] Scientifically unjustified [ ]** | |
| **Limited exposure [ X]** | **Other justification [ ]** | |
| **Detailed justification:** | A waiver for performing Field Studies on the accumulation of BIT in sediment is requested. A waiver has been requested for performing water:sediment studies (A7.1.2.2.2) based on the limited adsorption of BIT to sediment. According to Chapter 3, Section 7.0.2.3.2 (and Figure 1 in section 7) a sediment:water study is only required when the $K_p$ > 2000. As this is not the case, field studies on sediment are not applicable.<br>Additionally, based on the use pattern, there should be limited exposure to sediment. Thus this study will have no impact on the environmental risk assessment. | |
| **Undertaking of intended data submission [ ]** | No studies are planned. | |
| | | |
| | | |
| | **Evaluation by Competent Authorities** | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | *January 2011* | |
| **Evaluation of applicant's justification** | *Applicant's justification is accepted.* | |
| **Conclusion** | *Applicant's justification is accepted.* | |
| **Remarks** | | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.2** | **Fate and Behaviour in Soil** |
| **Subsection A7.2.1** | **Aerobic Degradation in soil including extent and nature of bound residues** |
| **Annex Point IIIA, VII.4** | |
| **XII.1.1** | |

| | | | Official use only |
|---|---|---|---|
| | | **1 REFERENCE** | |
| **1.1** | **Reference** | **A7.2.1/01** [redacted] (2008) $^{14}$C-BIT: Aerobic Soil Metabolism and Degradation. [redacted] Rohm and Haas Technical Report No. TR-08-011 (15 September 2008), Unpublished. | |
| **1.2** | **Data protection** | Yes | |
| 1.2.1. | Data owner | Rohm and Haas Company | |
| 1.2.2. | Companies with letter of access | | |
| 1.2.3. | Criteria for data protection | Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I/IA.<br>Data protection claimed in accordance with Article 12.1(c)(ii), as data generated after the entry into force of the Directive. | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **Guideline Study** | Yes. OECD 307: Aerobic Soil Metabolism and Degradation (April 2002). | |
| **2.2** | **GLP** | Yes | |
| **2.3** | **Deviations** | Two minor protocol deviations: 1) it was stated that the soil biomass would be measured during the study. This was not performed but it was determined at the beginning and end of the study and 2) it was stated that the soil organic carbon would be between 0.5% and 2.5% however soil analysis showed that it was 2.9%. Neither of these deviations has any impact on the integrity or quality of the study. | |
| | | **3 MATERIAL AND METHODS** | |
| **3.1** | **Test Material** | $^{14}$C-BIT<br>  <br><br>* position of the $^{14}$C-label | |
| 3.1.1 | Lot/Batch number | 1069.00 (sublot 1069.0005) and 1077.00 (sublot 1077.0002) | |
| 3.1.2 | Purity | Radiopurity > 98% | |

| | |
|---|---|
| 3.1.3 Further relevant properties | • Soil adsorption $K_f$ = 55.6<br>• Water solubility (deionized water) > 0.7 g/L<br>• Half-life in aerobic surface water simulation study is 30.8 hours at 20°C and 25.6 µg/L BIT |
| **3.2 Reference substances** | No reference substances were employed to validate the study. The following compounds were used as chromatography standards.<br>2,3-dihydroxybenzoic acid, Lot 09026KB, Purity: 99.9%<br>Benzene sulfonamide, Lot 14024BB, Purity: 99.0%<br>Catechol, Lot 03812AD, Purity: 99.2%<br>2-sulfobenzoic acid, Lot 15101MB, Purity: 75.4%<br>Saccharin, Lot 11330EA-385, Purity: 99.9% |
| **3.3 Soil types** | The soil used for this study was a sandy loam obtained from Woolverstone, Ipswich, UK. The physical and chemical characteristics of the soils appear in Table A7.2.1-1. |
| **3.4 Test procedures** | |
| 3.4.1 Test system | The test system consisted of a test flask and a series of trap flasks. Moistened air was drawn into the test flask which contained 50 g dry weight soil which had been adjusted to 24.5% of the maximum water holding capacity (moisture loss replaced by adding water). Connected to the output side of the soil flask were 4 traps; 1) ethanediol (traps polar volatiles), 2) 2% paraffin in xylene (traps nonpolar volatiles), 3) 2M NaOH (traps $CO_2$), and 4) 2M NaOH. The system was equilibrated for 2 days prior to dosing.<br>$^{14}$C-BIT was applied dropwise on the soil surface, the solvent allowed to evaporate, and then mixed.<br>The dosing concentration and number of flask employed for the method development studies, the preliminary study, and the main study are tabulated below. |

| Test | Concentration (µg/g dry wt) | Sample Type | Number of Units |
|---|---|---|---|
| Method Development Test 1 | 0.5, 2, 10 | Non-sterile | 3 |
| Method Development Test 2 | 0.5 | Non-sterile | 4 |
| Method Development Test 3 | 10 | Non-sterile | 6 |

| | | | | |
|---|---|---|---|---|
| | Preliminary Test: Estimation of Rate of Degradation | 5, 10, 20 | Non-sterile | 12 |
| | Main Test | 5 | Non-sterile | 22 |
| | Main Test | 5 | Sterile | 6 |

3.4.2 Preparation of test solution

Method Development Test 1

A stock solution for the 0.5 and 2 µg/g dosing levels were prepared by dissolving 9.5 mg of $^{14}$C-BIT (lot 1069.00) into 3.8 mL of ACN and the concentration based on radioassayed was 2.356 mg/mL. A dosing solution was prepared from the stock solution by removing 100 µL and 500µL and combining with 1.9 and 2 mL of ACN, respectively. Based on radioassay the solution concentration was 0.117 mg/mL and 0.466 mg/mL, respectively. The 10µg/g application solution was prepared by dissolving 6.5 mg $^{14}$C-BIT (lot 1069.00) with 2.6 mL of ACN and the radioassay yielded a solution concentration of 2.328 mg/mL.

Method Development Test 2

The 0.5 µg/g dosing solution was prepared by removing 140 µL of the Test 1 2.356 mg/mL solution and dissolving with 1.86 mL of ACN. Based on radioassay the solution concentration was 0.156 mg/mL.

Method Development Test 3

The 2.328 mg/mL dosing solution from Test 1 was used in this study.

Preliminary Test: Rate of Degradation Estimation Test

The 5 µ/g and 10 µ/g dosing solution was prepared by combining the remaining solutions from Test 1 (2.326 mg/mL and 2.328 mg/mL) and the radioassay yielded 2.276 mg/mL. The 20 µg/g dosing solution was prepared by dissolving 5.6 mg $^{14}$C-BIT (lot 1069.00) and 6.8 mg $^{12}$C-BIT with 1.1 mL ACN and 1.36 mL ACN, respectively. From each solution, 960 µL were combined and based on radioassay the resulting concentration was 5.006 mg/mL.

Main Test

The 5 µg/g soil dosing solution was prepared by dissolving 14.5 mg $^{14}$C-BIT (lot 1077.00) with 11.6 mL acetonitrile and based on radioassay the concentration was 1.21 mg/mL.

The doing regime is described in Table A7.2.1-2.

3.4.3 Duration of test and sampling intervals

Preliminary Test: Rate of Degradation Estimation duration was 48 hours with sampling at 0, 8, 24, 48 hours.

Main Test duration was 100 days with sampling at 0, 2, 4, 8, and 24 hours and 14, 30, 61, and 100 days. Sterile flask at 1 and 100 days

3.4.4 Replicates

Method Development Test 1: One flask at each of the three test

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| | concentrations.<br><br>Method Development Test 2: One flask for each of the 4 extraction solvents examined.<br><br>Method Development Test 3: One flask for each of the 6 extraction solvents examined.<br><br>Preliminary Test: Rate of Degradation Estimation: One flask at each of the three concentrations and at each of the 4 sample intervals.<br><br>Main Test: Duplicate flasks at each sample interval for both the sterile and non-sterile systems. |
| 3.4.5 Sampling and extraction details | All flasks were dosed by adding BIT dropwise to the soil surface and the solvent allowed to evaporate. The test substance was mixed with the soil by rotating the flask by hand.<br><br>Method Development Test 1<br><br>Soils were dosed at 0.5, 2, and 10 µg/g soil and immediately after dosing extracted successively with methanol:0.1% ammonia solution (4:1), methanol:1% ammonia solution (4:1), acetonitrile:1% ammonia solution (1:1), 0.1% ammonia solution, 1% ammonia solution, 5% ammonia solution and acetonitrile:1% ammonia solution (4:1). Each sample was extracted by shaking up to three times (*ca* 15 minutes) and centrifuged.<br><br>Method Development Test 2<br><br>Four flasks with 0.5 µg/g soil and immediately extracted as listed below.<br><br>methanol (1x) + methanol:1% ammonia, 1:1 (4x)<br><br>ACN (1x) + ACN:1% ammonia, 1:1 (4x)<br><br>Methanol:1% ammonia, 1:1 (4x)<br><br>ACN:1% ammonia, 1:1 (4x)<br><br>Method Development Test 3<br><br>The stability of BIT in soils was tested. Six flasks were dosed at 10 µg/g soil. Immediately after dosing five of the flask were extracted with one of the following solvents: acetonitrile:1% ammonia (1:1), 1% ammonia in acetonitrile, 1% DMSO in acetonitrile, 0.1% Cu (II) nitrate in acetonitrile:1% ammonia (1:1), and 0.015 $HgCl_2$ in acetonitrile:1% ammonia (1:1). The sixth flask was extracted with acetonitrile:1% ammonia prior to dosing and the extract dosed. Soils were extracted up to 4 times with the respective solvent and the headspace sparged before and after extraction with nitrogen to reduce oxidation of BIT. The extracts were centrifuged and aliquots removed for chromatography. Additional aliquots were obtained, stored overnight in a freezer and at room temperature and subsequently chromatographed. |

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|---|---|
| | Preliminary Test: Rate of Degradation Estimation<br><br>Four flasks were dosed at each 5, 10, and 20 µg/g soil. Single flasks were removed at 0, 8, 24, and 48 hours for each dosing concentration. The soils were extracted with 3 times with 100 mL of acetonitrile:1% ammonia (1:1, v/v) for 15 minutes, the extract centrifuged, radioassayed, and the aliquots of the extract chromatographed immediately. The headspace was sparged during and after extraction with nitrogen to minimize oxidation of BIT.<br><br>Main Test<br><br>Twenty-two non-sterile and six sterilized (autoclave) flask containing 50 g dry wt of soil were dosed at 5 µg/g of soil. At 2, 4, 8, and 24 hours and at 14, 30, 61, and 100 days duplicate non-sterile flasks were removed. On days 1 and 100 duplicate sterile flasks were removed. Soils were immediately extracted 3 times with 100 mL of acetontirle:1% ammonia (1:1, v/v), centrifuged, and radioassayed. The headspace was sparged during and after extraction with nitrogen. An aliquot was immediately chromatographed. Day 14 and 100 non-sterile soils and Day 100 sterile soil were additionally extracted with 100 mL acetonitrile:1% ammonia (1:3, v/v), centrifuged and radioassayed. A subsample was removed and chromatographed immediately. A Day 61 sample was additionally Soxhlet extracted overnight with acetonitrile:1% ammonia (1:3)<br><br>All extracts containing 5% or more of the applied activity were analyzed by HPLC. TLC was used for confirmation.<br><br>Volatile traps were sampled at the same time both sterile and non-sterile flasks were taken for analysis. In addition, the non-sterile traps from Day 30 onward were sampled every 30 to 40 days as necessary. The presence of $CO_2$ in the NaOH traps was confirmed in the Day 100 samples by precipitation with $BaCl_2$. |
| 3.4.6 Bound residues-extent and nature | The two Day 100 soil residues samples that remained after extraction with acetonitrile:1% ammonia were further extracted for bound residues using 0.5M NaOH. After 24 h the sample was centrifuged and the solid humin fraction was washed with an additional 25 mL of NaOH. The combined supernatant fraction was acidified with HCl (~pH1). The resulting supernatant, fulvic acid fraction, was radioassayed. The precipitate resulting from acidification, humic acid fraction, was redissolved in 0.5M NaOH and radioassayed. The remaining insoluble matrix, humin, was radioassayed by combustion. |
| 3.4.7 Analytical methods | Soil extracts were chromatographed by reversed phase HPLC using a Restek Ultra Aqueous C-18 column. The mobile phase consisted of a gradient of 0.5% Formic acid in water and 0.5% formic acid in methanol. Radioactivity was monitored with a flow through radioactivity monitor and UV at 254 nm.<br><br>TLC was performed on silica gel plates using ethyl acetate: methanol:acetonitrile:acetic acid (90:5:5:1, v/v/v/v) as the eluant. Radioactivity was imaged using a phosphorimager and non-radioactive |

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| | standards using a 254 nm UV light.<br><br>Liquid scintillation spectrometry was performed using Packard liquid scintillation spectrometers. Radiocombustion was performed in a Harvey Biological Sample Oxidizer and subsequently quantitated by liquid scintillation spectrometry.<br><br>For metabolite identification, accurate masses were obtained using an LC-Fourier Transform MS. A modified C-18 column was employed with a gradient consisting of 0.5% aqueous formic acid and 0.5% formic acid in methanol. The LC effluent was introduced into the MS via an API interface and both positive and negative ionization was employed. |
| 3.4.9 Degradation products | Degradation products were quantitated by HPLC. They were identified and in some instances additional quantitation was performed by LC-MS. |
| 3.4.10 Calculations | The half-life was calculated employing first degree kinetics and the equation, $y = C_0 \times e^{-kt}$ where y is the percent of the test substance at time t, $C_0$ is the initial BIT concentration, and k is the rate constant. |
| | **4 RESULTS** |
| **4.1 Method Development** | <u>Method Development Test 1</u><br><br>A single flask dosed at either 0.5, 2, or 10 µg $^{14}$C-BIT/g soil was extracted successively with 8 different solvent systems. The results were inclusive due to low extraction efficiency and possible instability of BIT. Thus a second test was performed.<br><br><u>Method Development Test 2</u><br><br>Soil was dosed 0.5 µg $^{14}$C-BIT/g soil and extracted immediately with several solvent systems. Acetonitrile:1% ammonia (1:1) and methanol:1% ammonia (1:1) yielded the best extraction efficiency. BIT stored for 2 days at either room temperature or < -10°C was stable in these two solvent systems.<br><br><u>Method Development Test 3</u><br><br>Flasks were extracted immediately after dosing with one of 5 solvent systems. Aliquots of the extracts were chromatographed (HPLC) either immediately after extraction or after overnight storage at room temperature or <-10°C. In addition, one blank sample was extracted and the extract was dosed with $^{14}$C-BIT. It was found that $^{14}$C-BIT degraded in all the overnight extracts except the blank sample extract.<br><br>As a result of these test it was decided to initially extract the soils with acetonitrile:1% ammonia (1:1, v/v) and that the extracts should be analyzed immediately and not stored. In addition, the headspace would be sparged with nitrogen during and after extraction to reduce oxidation of BIT. |
| **4.2 Preliminary Test: Rate of** | To estimate the rate of degradation, soils were dosed at 5, 10, and 20 µg $^{14}$C-BIT/g soil and analyzed at 0, 8, 24, and 48 hours. Recovery of applied $^{14}$C ranged from 101% to 104% (Table A7.2.1-3). For all three |

| | | |
|---|---|---|
| **degradation estimation** | dosing concentration, the percent of solvent extractable $^{14}C$-activity decreased from about 100% at Hour 0 to about 63% at Hour 48 while the percent unextractable increased from about 2% at Hour 0 to about 38% at Hour 48. There was no activity detected in the volatile organic traps. However in the $CO_2$ traps (NaOH) there was about 1% after 48 hours.<br><br>Parent decreased from about 94% at Hour 0 to less than 5% after 48 hours (Table A7.2.1-4). Using first order kinetics, the half-life for the dosing concentrations 5, 10, 20 µg/g were 6.5, 7.9, and 8.9 hours, respectively. Four metabolites were detected; Unknown A, Unknown B, Unknown C, and Polar Material. Unknown A and C and Polar Material were detected at greater than 10% while Unknown B was less than 10%.<br><br>Based on these results it was decided to dose the main test at 5 µg/g. This concentration was high enough so that the samples could be chromatographed immediately after extraction without the need to concentrate the samples. | |
| **4.3 Main Test** | | |
| 4.3.1 Distribution and recovery of radioactivity | The distribution of $^{14}C$-activity in non-sterilized and sterilized soil dosed with $^{14}C$-BIT is summarized in Table A7.2.1-5. For non-sterilized soil samples, the percent of applied radioactivity extractable with acetonitrile:1% ammonia (1:1) decreased in time from 99.4% at Time 0 to 67.5% after 1 day and 8.3% after 100 days. A secondary extraction with acetonitrile:1% ammonia (1:3) was initiated starting Day 14 but this yielded less than 7% of the applied activity at any one interval. Concurrent with the decrease in extractability was an increase in the unextractable (bound) residue remaining in soil. The unextractable residue increased from 2.5% of the applied activity on Day 0 to 41.5% on Day 14 and 45.5% on Day 100. The evolved $^{14}CO_2$ trapped in the NaOH traps reached 40.2% by Day 100. This indicates that there was cleavage and extensive oxidation of the benzene ring in soil. There was no activity detected in the traps for volatile organics. Recovery ranged from 97.9% (Day 100) to 106.3% (Hour 4) of the applied activity. The average recovery was 101.5 ± 4.8%.<br><br>The distribution and recovery from sterilized soils appears in Table A7.2.1-5. Similar to the non-sterile soil there was a decrease in solvent extractability with incubation time and a correlating increase in non-extractable soil bound residue. There was no detectable activity in the volatile organic traps and less than 0.5% of the applied activity was present as evolved $^{14}CO_2$ after 100 days. Recovery of applied activity was about 103%. | |
| 4.3.2 Characterization of radioactivity | Quantitation of parent and significant metabolites is presented in Table A7.2.1-6 and Figure A7.2.1-1. In non-sterilized soils parent decreased from 92% of applied activity at Time 0 to 0.5% on Day 30 with none being detected at Day 61 and 100. In sterile soils, BIT declined from 88.1% of the applied activity on Day 1 to 1.3% on Day 100. Abiotic degradation of BIT under sterile conditions was slower and less | |

extensive (see $CO_2$ evolution in Table A7.2.1-5) then biodegradation. It is possible that a significant portion of abiotic degradation was due to aerobic oxidation of BIT.

In non-sterile soil, Unknown A and the Polar Material where the only two metabolites to exceed 10% of applied material. Unknown A reached a maximum of about 23% after 8 hours and then decreased to 1.5% by Day 100. The Polar Material reached a maximum of 28% on Day 30 and then decreased to 4.2 percent. Thus these two major metabolites are transient. Their dissipation correlates with the increase in $^{14}CO_2$ indicating that they are being oxidized to $CO_2$. Of the remaining 3 detectable metabolites, Unknown B reached at maximum of 4.6% on Day 14, Unknown C, 8.1% on Day 1, and Unknown D, 6.3% on Day 14. Similar to Unknown A and the Polar Material, the three minor metabolites were also transient (Figure A7.2.1-1). A secondary extraction of the Day 30 and 61 soils yielded only small additional amounts of these 5 metabolites. There were other very minor metabolites detected but they were present at a maximum of 1.4% or less.

In the sterile soils, Unknown A, B, and C were present at 7.4% or less (Unknown D was not detected). The Polar Material was present at 14.4% on Day 100.

4.3.3 Half-life

The results in Table A7.2.1-6 demonstrate that BIT quickly biodegrades in soil. The kinetic end points are tabulated below.

| End Point | Results (h) |
|---|---|
| $DT_{50}$ | 5.6 |
| $DT_{75}$ | 11.2 |
| $DT_{90}$ | 18.6 |

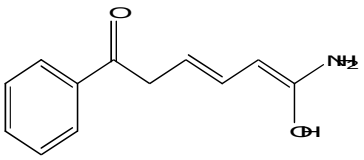
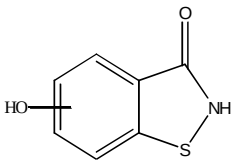
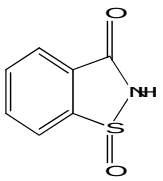
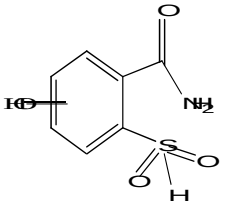
4.3.4 Extend and nature of bound residues

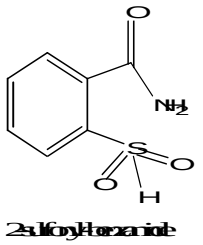
The bound $^{14}C$-residue remaining on Day 100 after extensive extraction with acetonitrile:1% ammonia was subjected a 24 hour extraction with NaOH. The results are presented in Table A7.2.1-7. After extraction with NaOH, the acid soluble fraction, the fulvic acid fraction, comprised about 17% of the applied radioactivity while the acid insoluble fraction, the humic acid fraction, comprised about 12%. The base insoluble fraction (humin), which is essentially the inorganic soil lattice, comprised 16.8% of the applied activity.

**4.4 Metabolite Identification**

The two detected metabolites, Unknown A and Polar Material (included some Unknown C due to HPLC separation), present at greater than 10% of the applied activity were further analyzed by LC-MS to determine their structure. Both metabolites consisted of two primary metabolites. The structure, name, and approximate percentage of the metabolites observed are presented in the table below. Mass spectroscopy could not determine the site of oxidation for Unknown A, m/z 168 so both possibilities are presented (though it is present at less than 10% of the

applied activity). Finally, a metabolic pathway is presented in Figure A7.2.1-2.

| Designation | Structure/Name | Approx. % [1] |
|---|---|---|
| Unknown A | | |
| m/z 205 |  N-(4-amino-4-hydroxybuta-1,3-dienyl)benzamide | 20.7 |
| m/z168 |  Hydroxy-1,2-benzisothiazolin-3-one | 2.5 |
| | OR | |
| |  1,2-benzisothiazolin-3-one-1-oxide | |
| Polar Material/Unknown C | | |
| m/z 200 |  4-hydroxy-2-sulfobenzamide | 6.6 |

| | | |
|---|---|---|
| m/z 184 |  2-sulfobenzamide | 8.1 |

[1] Percent estimated from HPLC and LC-MS analysis. These are estimates and assume equal ionization for each component.

**5 APPLICANT'S SUMMARY AND CONCLUSION**

**5.1 Materials and methods**

The test guidelines were OECD Guideline 307, Aerobic Soil Metabolism and Degradation.

A stream of moisten air was passed through a flask containing 50 g (dry weight basis) of moist soil and the exhaled air was passed through a series of traps containing ethylenediol and paraffin in xylene to trap volatile organics and NaOH to trap evolved $CO_2$. The system was allowed to equilibrate and then $^{14}$C-BIT was added dropwise to the soil surface, the solvent allowed to evaporate, the flask contents mixed, and then the system reconnected to the traps. For the main test 22 flask containing non-sterile soil and 6 sterile flasks containing autoclaved sterilized soil were dosed at 5 µg/g soil.

Duplicate flasks were removed at 0, 2, 4, 8, and 24 hours and 14, 30, 61, and 100 days. Soils were extracted with acetonitrile:1% ammonia. The resulting extract was radioassayed and chromatographed using HPLC immediately after extraction. The traps were radioassayed periodically also. The presence of $CO_2$ in the NaOH traps was confirmed by precipitation with $BaCl_2$.

Quantitation of parent and metabolites was done primarily by HPLC though some quantitation of metabolites was performed by LC-MS. Structure determination of the applicable metabolites was performed using LC-MS.

The nature and extent of the bound residues remaining after extraction with acetonitrile:1% ammonia was determined by a 24 hour extraction with 0.5N NaOH. The extract was acidified resulting in a soluble fulvic acid fraction and the precipitated humic acid fraction. The solid residue remaining after NaOH extraction was the humin. All three phases were radioaasyed.

**5.2 Results and Discussion**

5.2.1 Method development and preliminary tests

Preliminary studies discovered that acetonitrile:1% ammonia was the preferred extraction solvent and that extracts needed to be chromatographed immediately since there was a stability issue with BIT

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| | in the extracts. Kinetic analysis indicated the half-life would be less than 12 hours. |
| 5.2.2 Main Test | |
| 5.2.2.1 Distribution and recovery | The percent of applied radioactivity that was solvent extractable from the soil decreased with time; from 99% at Time 0 to 8% at Day 100. Concurrently, the percent of non-extractable activity remaining bound to the soil increased from 2.5% on Day 0 to 45.5% on Day 100. Evolved $CO_2$ increased with time accounting for 40% of the applied radioactivity by Day 100. No volatile organic activity was detected. The recovery of applied activity ranged from 97.9% to 106.3% with an average of 101.5 ± 4.8% |
| 5.2.2.2 Kinetics | $DT_{50}$ 5.6<br>$DT_{75}$ 11.2<br>$DT_{90}$ 18.6<br><br>Thus, BIT quickly biodegrades in soil. By Day 14 less than 1% of the applied activity was parent in the non-sterile soils. In the sterile soils, by Day 100 about 1% of the activity was BIT. Thus there is an abiotic component of BIT degradation in soil, probably attributable to oxidation, but this is much smaller then biodegradation. |
| 5.2.2.3 Nature and extent of bound residues | By Day 100 about 45% of the applied $^{14}C$-activity was incorporated into the bound residues. In the preliminary studies and Time 0 samples it was shown that parent can be quantitatively extracted from soil and therefore the bound residues must be metabolites. NaOH extraction released about 66% of the bound residue. The humic acid and humin comprised about 17% each of the applied activity and the fulvic acid, about 12%. |
| 5.2.3.4 Metabolites | $CO_2$ was the major metabolite at the end of the study comprising about 40% of the applied activity. This demonstrates that there was extensive metabolism occurring involving cleavage of the isothiazolone and benzene rings. There were two metabolites that were present at greater than 10%; Unknown A and the Polar Material/Unknown C. Both were transient and both appear to oxidize to $^{14}CO_2$. Analysis by LC-MS demonstrated that both metabolites were comprised of two major components. The two identified Unknown A metabolites were: |

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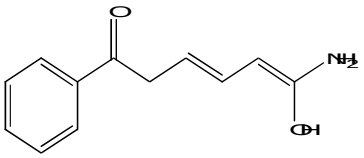
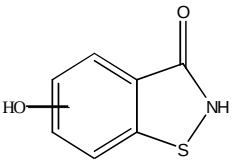
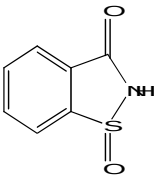
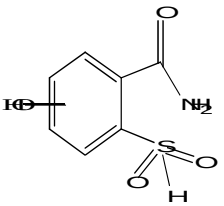
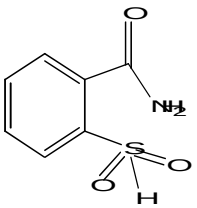
|---|---|
| m/z 205 |  N-(4-amino-4-hydroxybut-1,3-dienyl)benzamide |
| m/z168 |  Hydroxy-1,2-benzisothiazolin-3-one |
| | OR |
| |  1,2-benzisothiazolin-3-one-1-oxide |

and for the Polar Material:

| | |
|---|---|
| m/z 200 |  4-hydroxy-2-sulfoylbenzamide |
| m/z 184 |  2-sulfoylbenzamide |

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| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.2** | **Fate and Behaviour in Soil** |
| **Subsection A7.2.1**<br>**Annex Point IIIA, VII.4**<br>**XII.1.1** | **Aerobic Degradation in soil including extent and nature of bound residues** |

| | | |
|---|---|---|
| **5.3** | **Conclusion** | BIT quickly biodegrades in soil with a half-life of 5.6 hours in soils incubated at 20°C (extrapolated to about 9.3 hours at 12°C). As demonstrated by the presence of significant percentage of evolved $^{14}CO_2$, metabolism was extensive and involved the cleavage of the isothiazolone and benzene rings. There were two metabolite fractions present at greater than 10% and both of these fractions were transient and contained two major components. These four metabolites were subsequently identified by LC-MS. About 45% of the applied activity was non-solvent extractable bound residue at study termination (Day 100). None of the bound residue was parent. |
| 5.3.1 | Reliability | 1-valid without restrictions |
| 5.3.2 | Deficiencies | None |

| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *May 2013.* |
| **Materials and Methods** | *Applicant's version is accepted with the following remarks:*<br>▪ *Two protocol deviations: 1) It was stated that the soil biomass would be measured during the study. This was not performed but it was determined at the beginning and end of the study and 2) it was stated that the soil organic carbon would be between 0.5% and 2.5% however soil analysis showed that it was 2.9%.*<br>▪ *3.3. Only one soil sample is employed. According to OECD guidelines, for transformation rate studies at least three additional soils should be used representing a range of relevant soils.*<br>*Regression calculation used: Curves were constructed through appropriate data points using nonlinear regression analysis to give lines of best fit. The degradation rate of BIT was determined using the first order kinetics equation.* |
| **Results and discussion** | *Applicant's version is accepted* |
| **Conclusion** | *BIT quickly biodegrades in soil with a half-life of 5.6 hours in soils incubated at 20°C (extrapolated to about 9.3 hours at 12°C). As demonstrated by the presence of significant percentage of evolved $^{14}CO_2$, metabolism was extensive and involved the cleavage of the isothiazolone and benzene rings. There were two metabolite fractions present at greater than 10% and both of these fractions were transient and contained two major components. These four metabolites were subsequently identified by LC-MS. About 45% of the applied activity was non-solvent extractable bound residue at study termination (Day 100). None of the bound residue was* |

| | |
|---|---|
| | *parent.*<br>*Sterilized soil (abiotic control) showed a high degradation (88.1% of parent compound the first day vs. 1.3 % after 100 days). This could be due to an abiotic degradation of BIT at the soil matrix or to an uncompleted sterilization of the soil matrix.* |
| **Reliability** | *2* |
| **Acceptability** | *Acceptable.* |
| **Remarks** | |

**Table A7.2.1-1: Physical and Chemical Properties of Silt Loam and Loamy Sand**

| Characteristic | Silt Loam |
|---|---|
| Percent Sand | 62 |
| Percent Silt | 32 |
| Percent Clay | 6 |
| Percent Organic Matter | 5.0 |
| Cation Exchange Capacity (meq/100 g) | 21.6 |
| pH | 7.4 |
| Percent Moisture (1/3 bar) | 19.0 |
| Bulk Density (g/cc) | 1.1 |

**Table A7.2.1: Summary of Dosing Regime**

| Test Type | Application Rate (µg/g dry weight equivalent) | No. of Flask | Concentration of application solution applied (mg/mL) | Volume of application solution applied (µL) | Weight of test substance applied (µg) |
|---|---|---|---|---|---|
| Method Development Test 1 | 0.5 | 1 | 0.117 | 210 | 25 |
| | 2 | 1 | 0.466 | 215 | 100 |
| | 10 | 1 | 2.328 | 215 | 501 |
| Method Development Test 2 | 0.5 | 4 | 0.156 | 160 | 25 |

| | | | | | |
|---|---|---|---|---|---|
| Method Development Test 3 | 10 | 6 | 2.356 | 215 | 507 |
| Preliminary Test: Rate of Degradation Estimation Test | 5 | 4 | 2.276 | 110 | 250 |
| | 10 | 4 | 2.276 | 215 | 489 |
| | 20 | 4 | 5.006 | 200 | 1001 |
| Main Test | | | | | |
| Sterile | 5 | 22 | 1.21 | 210 | 254 |
| Non-Sterile | 5 | 6 | 1.21 | 210 | 254 |

**Table A7.2.1-3: Distribution of applied radioactivity from $^{14}$C-BIT Treated Soils—Preliminary Test**

| Dose/Sample Interval | Percent of Applied | | | | | |
|---|---|---|---|---|---|---|
| | Soil Extract | Unextracted from Soil | Ethylenediol Trap | Paraffin/ Xylene Trap | NaOH Trap | Recovery |
| 5 µg/g | | | | | | |
| 0 h | 99.0 | 2.2 | NA[1] | NA | NA | 101.2 |
| 8 h | 78.6 | 23.7 | ND[2] | ND | 0.1 | 102.4 |
| 24 h | 63.0 | 40.1 | ND | ND | 0.8 | 103.9 |
| 48 h | 62.5 | 37.7 | ND | ND | 1.2 | 101.4 |
| 10 µg/g | | | | | | |
| 0 h | 102.1 | 1.8 | NA | NA | NA | 103.9 |
| 8 h | 75.1 | 27.8 | ND | ND | 0.1 | 103.0 |
| 24 h | 67.3 | 34.5 | ND | ND | 0.4 | 102.2 |
| 48 h | 62.7 | 39.2 | ND | ND | 0.9 | 102.8 |
| 20 µg/g | | | | | | |
| 0 h | 98.9 | 1.8 | NA | NA | NA | 100.7 |
| 8 h | 85 | 18.1 | ND | ND | ND | 103.1 |
| 24 h | 72.6 | 28.9 | ND | ND | 0.3 | 101.8 |
| 48 h | 65.1 | 36.8 | ND | ND | 0.6 | 102.5 |

[1] NA = Not Applicable [2] ND = Not Detected (<0.1%)

**Table A7.2.1-4: Percent of Parent and Metabolites Detected in the Soil Extracts—Preliminary Study**

| Dose/Sample Interval | Percent of Applied | | | | |
|---|---|---|---|---|---|
| | Parent | Unknown A | Unknown B | Unknown C | Polar Material |
| 5 µg/g | | | | | |
| 0 h | 91.1 | 7.8 | ND | ND | ND |
| 8 h | 39.7 | 21.9 | ND | 4.8 | 10.1 |
| 24 h | 4.7 | 12.0 | 5.3 | 10.2 | 23.6 |
| 48 h | 3.5 | 11.0 | 8.4 | 14.7 | 22.5 |
| 10 µg/g | | | | | |
| 0 h | 94.8 | 6.6 | ND | ND | ND |
| 8 h | 27.3 | 19.3 | 3.1 | 5.9 | 12.8 |
| 24 h | 11.1 | 19.5 | 4.5 | 9.3 | 19.7 |
| 48 h | 3.1 | 12.4 | 7.3 | 11.7 | 25.8 |
| 20 µg/g | | | | | |
| 0 h | 94.4 | 4.2 | ND | ND | ND |
| 8 h | 48.3 | 16.6 | ND | 4.3 | 8.9 |
| 24 h | 15.8 | 22.9 | 3.2 | 8.3 | 17.2 |
| 48 h | 4.3 | 15.9 | 5.2 | 11.8 | 23.6 |

**Table A7.2.1-5: Distribution of Applied Radioactivity from $^{14}$C-BIT Treated Non-Sterile and Sterilized Soils—Main Test**

| Sample Interval | Percent of Applied Activity[1] | | | | | |
|---|---|---|---|---|---|---|
| | Primary Soil Extract | Secondary Soil Extract | Unextracted From Soil | Volatile Organic[2] Traps | NaOH Trap | Recovery |
| **Non-Sterile Soil** | | | | | | |
| 0 hours | 99.4 | NA[3] | 2.5 | NA | NA | 101.8 |
| 2 hours | 88.2 | NA | 14.8 | ND[3] | ND | 103.0 |
| 4 hours | 84.6 | NA | 21.7 | ND | ND | 106.3 |
| 8 hours | 75.3 | NA | 25.8 | ND | 0.1 | 101.2 |
| 1 day | 67.5 | NA | 32.8 | ND | 0.4 | 100.7 |
| 14 days | 48.3 | 3.3 | 41.1 | ND | 9.4 | 102.1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 30 days | 43.4 | 4.8 | 41.2 | ND | 12.3 | 101.7 |
| 61 days | 15.6 | 6.8 | 44.3 | ND | 32.8 | 99.4 |
| 100 days | 8.3 | 4.0 | 45.5 | ND | 40.2 | 97.9 |
| **Sterilized Soil** | | | | | | |
| 1 Day | 96.7 | NA | 6.3 | ND | ND | 103.0 |
| 100 days | 32.3 | 3.7 | 67.1 | ND | 0.3 | 103.3 |

[1] Average of duplicate samples

[2] Paraffin in xylene and ethanediol traps combined.

[3] NA = not applicable; ND = not detected

**Table A7.2.1-6: Percent of Parent and Metabolites Detected in Non-Sterile and Sterile Soil Extracts—Main Study.**

| Sample Interval | Percent of Applied $^{14}$C-Activity[1] | | | | | |
|---|---|---|---|---|---|---|
| | **Parent** | **Unknown A** | **Unknown B** | **Unknown C** | **Unknown D** | **Polar Material** |
| **Non-Sterile Soils** | | | | | | |
| 0 hours | 92.0 | 6.1 | ND[2] | ND | ND | ND |
| 2 hours | 60.3 | 17.0 | ND | 2.2 | ND | 6.3 |
| 4 hours | 51.6 | 18.7 | 0.5 | 2.7 | ND | 7.9 |
| 8 hours | 33.5 | 22.8 | 2.1 | 4.6 | ND | 9.8 |
| 1 day | 12.6 | 22.6 | 4.1 | 8.1 | ND | 17.5 |
| 14 days | 0.4 | 1.6 | 4.6 | 5.4 | 6.3 | 25.8 |
| 30 days | 0.5 | 0.8 (0.8)[3] | 3.7 (0.9) | 6.2 (0.2) | 4.2 (0.6) | 26.4 (1.6) |
| 61 days | ND | ND (1.0) | 1.8 (1.5) | 1.4 (0.3) | 2.9 (0.6) | 7.1 (2.5) |
| 100 days | ND | 1.6 | 1.1 | ND | 0.9 | 4.2 |
| **Sterilized Soil** | | | | | | |
| **Sample Interval** | **Percent of Applied $^{14}$C-Activity[1]** | | | | | |
| | **Parent** | **Unknown A** | **Unknown B** | **Unknown C** | **Polar Material** | |
| 1 day | 88.1 | 7.4 | ND | ND | ND | |
| 100 days | 1.3 | 4.2 | 3.0 | 4.7 | 14.4 | |

[1] Average of duplicate samples

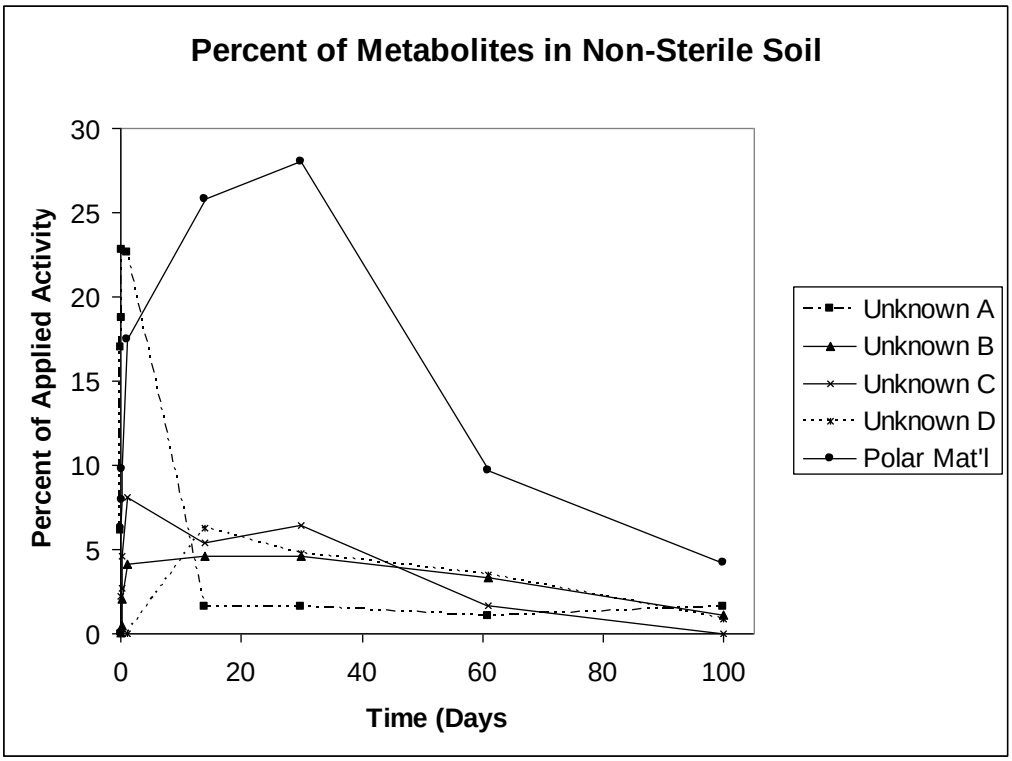
[2] ND = not detected

[3] Values in parenthesis are quantitation from a second extraction procedure (acetonitrile:1% ammonia (1:3).

**Table A7.2.1-7: Nature and Extent of Bound Residues from Day 100 Non-Sterile Soil Samples**

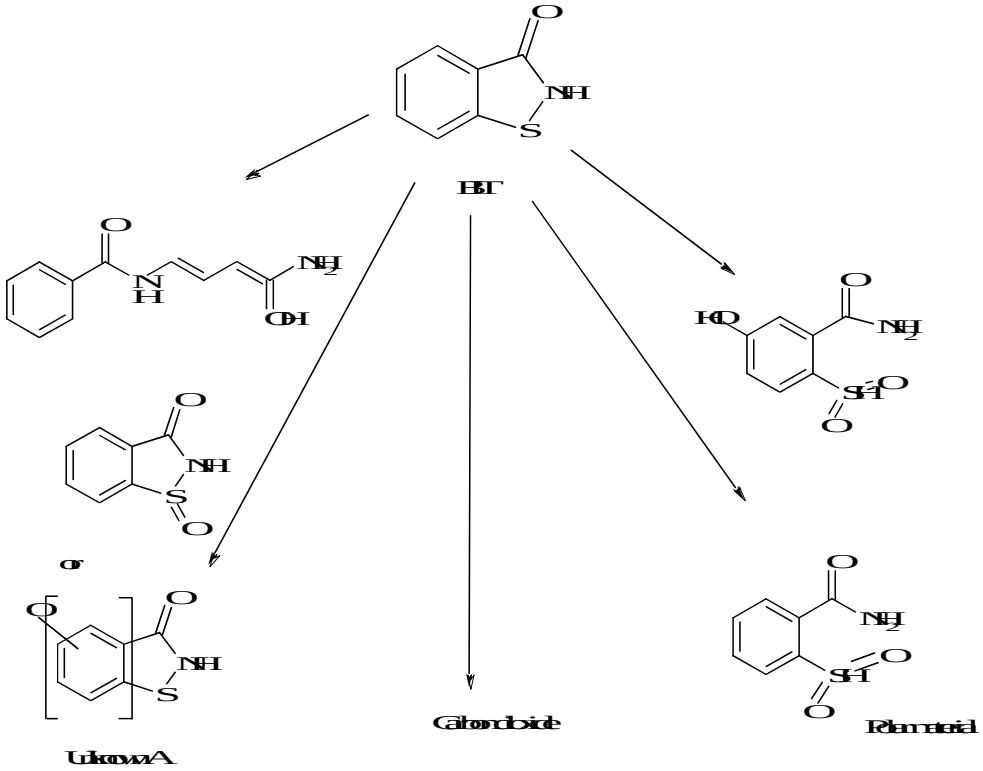
| Fraction | Percent of Applied Activity | | |
|---|---|---|---|
| | Flask D17 | Flask D18 | Average |
| Original Residue | 43.1 | 47.8 | 45.5 |
| NaOH Hydrolysis | 29.4 | 30.8 | 30.1 |
| Fulvic Acid (acid soluble) | 16.2 | 17.7 | 17.0 |
| Humic Acid (acid insoluble) | 11.8 | 11.9 | 11.9 |
| Humin (NaOH insoluble) | 16.6 | 16.9 | 16.8 |
| Total Extracted Bound Residue | 44.6 | 46.5 | 45.6 |

**Figure A7.2.1-1: Metabolites in Non-Sterile Soil as a Percentage of Applied Activity[1]**




[1] The percent applied is the sum of both the primary extraction (acetonitrile:1% ammonia (1:1)) and secondary extraction (acetonitrile:1% ammonia (1:3)).

**Figure A7.2.1-2: Metabolic Pathway for BIT in Soil**



Official use only

- **REFERENCE**

| | |
|---|---|
| **Reference** | [redacted] (2020a) 1,2-Benzisothiazol-3(2H)-one – Route and Rate of Degradation of [14C]-1,2-Benzisothiazol-3(2H)-one in Four Soils under Aerobic Conditions. [redacted] GLP, non-published, 20 December 2018, Amended 28 January 2020. |
| **Data protection** | Yes |
| • Data owner | Laboratorios Miret, S.A., LANXESS Deutschland GmbH, Lonza Ltd, Specialty Electronic Materials Switzerland GmbH (former The Dow Chemical Company), Thor GmbH, Troy Chemical Company BV |
| • Criteria for data protection | Data on existing a.s. submitted for first entry into the European list of approved biocidal active substance |

- **GUIDELINES AND QUALITY ASSURANCE**

| | |
|---|---|
| **Guideline study** | Yes<br>(OECD Guideline 307 (Adopted 24th April 2002)<br>OPPTS 835.4100, US EPA, October 2008) |
| **GLP** | Yes |
| **Deviations** | No |

- **MATERIALS AND METHODS**

| | |
|---|---|
| **Test material** | Test substance details are summarised below |
| • General information | 1,2-Benzisothiazol-3(2H)-one; CAS number: 2634-33-5; Molecular formula: $C_7H_5NOS$; Molecular weight: 151.19 g/mol |
| • Labelled test material (Lot/Batch number; purity) | 1,2-[ring-U-$^{14}$C]Benzisothiazol-3(2H)-one (thereafter referred to as [$^{14}$C]Benzisothiazolone); (CFQ43104; radiochemical purity: 96.6%) |
| • Unlabelled test material (Lot/Batch number; purity, description) | 1,2-Benzisothiazol-3(2H)-one; (MKBZ4459V; purity: 99.3%; white to yellow and faint beige to beige powder) |
| • Reference items | MET1 (R1): Hydroxy-1,2-benzisothiazolin-3-one<br>MET2 (R2): 1,2-Benzisothiazolin-3-one-1-oxide<br>MET3 (R3): Dihydroxy-1,2-benzisothiazolin-3-one<br>MET4 (R4): o-Sulphobenzamide (sodium salt)<br>MET7 (R7): N-(4-amino-4-hydroxy-buta-1,3-dienyl)-benzamide<br>Saccharin (R8): 1,2-Benzisothiazolin-3-one-1-dioxide<br>2-Sulphanylbenzamide (R9)<br>2-Sulphobenzoic acid hydrate (R11) |

| | |
|---|---|
| • Stability | Stability was determined before and after application. Test substance was stable during the application procedure. |
| • Composition of Product | Not relevant as active substance was tested |
| **Test system** | Laboratory test |
| • Selection of test system | Four field fresh soil types were selected to evaluate the route and rate of degradation of the test substance in the environment. |
| • Soil type and preparation | Four standard representative fresh field soils with a wide range of soil properties were used: Soil I: Speyer 2.4 (loam), Soil II: Speyer 5M (sandy loam), Soil III: RefeSol 02-A (silt loam) and Soil IV: RefeSol 04-A (loamy sand). Soils were characterised for particle size distribution, moisture content at water holding capacity and pF 2, pH, % organic matter and cation exchange capacity. Details are given in Table A7.2.1/01-1. Bioactive soils were conditioned to room temperature for approx. 6-8 days prior to application. Sterile soils were sterilised by gamma radiation. Moisture content was adjusted to pF 2, controlled during incubation and adjusted if necessary. |
| • Determination microbial biomass | For bioactive soil the microbial biomass was determined before during and at the end of incubation according to the fumigation extraction method by Vance, Brookes and Jenkinson. |
| • Experimental conditions | The test was performed under aerobic conditions in the dark in an air-conditioned room at a temperature of 20.8 ± 0.2°C and 20.9 ± 0.2°C and a soil moisture content of pF 2. Samples are equipped with a trapping system including a safety trap and two absorption traps for organic volatiles and $CO_2$. |
| **Treatment and sampling** | Soil samples of 100 g (equivalent dry weight) were treated with 50 µg test substance which is equivalent to an initial concentration of 0.5 mg per kg dry soil equivalent. Duplicate samples were taken for extraction and analysed after 0.00, 0.04, 0.08, 0.17, 0.33, 1.0, 2.1, 4, 7, 14, 28, 56, 91 (Soils I-III only) and 120 (Soil IV only) days of incubation for bioactive soils and after 0.00, ~1, 13, 28, 91 (Soils I-III only) and 120 (Soil IV only) days of incubation for sterile soils. |
| **Extraction** | Soils were extracted four times with acetonitrile, acetonitrile/water (4:1, v:v), acetonitrile/water (1:1, v:v) and acetonitrile/0.1 hydrochloric acid (1:1, v:v). Soxhlet extraction using acetonitrile/water/32% hydrochloric acid (80:20:0.1, v:v:v) was performed if >10% AR remained non-extracted in the samples after the first four extraction steps. If non-extractable radioactivity is still > 10% AR harsh extraction under reflux conditions followed by organic matter fractionation was performed. |
| **Analytical method** | Radioactivity contained in solutions was measured by liquid scintillation counting (LSC). Volumes of extracts were determined and dispensed aliquots were assayed in duplicate. The quantity of radioactivity was determined using a calibrated Packard liquid scintillation counter equipped with DPM and luminescence options. Non-extractable radioactivity remaining within the soils was determined after combustion by LSC and volatile radioactivity in the trapping solutions were also analysed by LSC. For identification radioactive components were compared with reference standards by co-chromatography. Aliquots from extracts were mixed with solutions of reference items and the mixtures injected to the HPLC system. Mass spectrometry (MS) was used to confirm the identification of major metabolites performed by |

co-chromatography with reference standards and to identify metabolite(s) for which no reference standard was available.

- **RESULTS**

**Analytical results**

Total mean recovery of radioactivity during the incubation period accounted for 97.6 ± 2.9, 96.8 ± 3.4, 96.7 ± 3.4 and 94.9 ± 3.0% AR for four bioactive soils respectively. The corresponding values for the sterile soils were 98.0 ± 0.8, 97.7 ± 1.0, 97.4 ± 1.1 and 97.1 ± 3.4% AR. The mean amount of extractable radioactivity at room temperature at 0.00 DAT was 66.7, 83.1, 79.6 and 90.3% AR in the bioactive soils respectively, and 70.9, 91.6, 88.0 and 91.9% AR in the sterile soils, respectively. Thereafter, it decreased to 2.8, 2.4, 5.7 and 11.4% AR in the bioactive soils, respectively, and to 59.1, 62.5, 61.3 and 43.4% AR in the sterile soils, respectively. Soxhlet extraction was performed for all soil samples except 3 samples where the extractable radioactivity was below >90% AR after extractions at room temperature. The mean amount of radioactivity extractable with Soxhlet extraction reached a maximum of 5.6, 5.9, 7.5 and 6.0% AR for bioactive soils, and a maximum of 7.2, 5.6, 5.5 and 7.8% AR for sterile soils, respectively. Non-extractable residues increased from 27.6, 10.3, 13.0 and 6.9% AR on 0.00 DAT to maximum levels of 52.0, 42.9, 44.6 and 45.6% AR on 56 DAT respectively for bioactive soils tested, and from 20.3, 5.8, 8.1 and 4.8% AR on 0.00 DAT to maximum levels of 40.7, 37.7, 36.4 and 47.0% AR on 13-28 DAT, respectively, for the four sterile soils tested. At the end of incubation, amounts were 48.6, 39.9, 43.2 and 41.9% AR respectively for the four bioactive soils and 36.2, 33.1, 31.3 and 41.8% AR, respectively, for the four sterile soils tested. The mineralisation of [14C]Benzisothiazolone was extensive and carbon dioxide reached a maximum of 47.9, 56.2, 46.1 and 39.9% AR at the end of incubation in bioactive soils. Harsh extraction of bioactive soil samples from 56 DAT under reflux conditions further released 5.7, 3.7, 7.3 and 5.7 % AR, proving that only small amounts might become bioavailable in addition. Mineralisation in sterile soils was negligible. No other organic volatiles exceed 0.1% AR over the study duration. Determination of the microbial biomass showed that the soils were viable throughout the incubation period.

**Degradation and transformations**

In the bioactive soils, up to six major degradation products were detected with maximum occurrences of 29.4 (MET2), 8.2 (M5), 16.9 (M8), 45.0 (M6 and M6b; could not sufficiently separated by HPLC), and 21.1% (M9) AR. MET 2, M5, M8 and M6b were confirmed to be 1,2-Benzisothiazolin-3-one-1-oxide, Saccharin, 2-Sulphanyl benzamide and 2-Sulphobenzoic acid. M6 was proposed to be 2-Sulpha-moylbenzoic acid and M9 to be 2-Aminosulphinylbenzoic acid. [$^{14}$C]Benzisothiazolone degraded in the bioactive soils with $DT_{50}$ values between 0.02 and 0.24 days, and $DT_{90}$ values ≤0.80 days based on the SFO kinetic model (please refer to Table A7.2.1/01-1). In the sterile soils, the degradation was only slightly slower with $DT_{50}$ values of 0.4 to 0.7 days, and $DT_{90}$ values ≤2.45 days.

- **APPLICANT'S SUMMARY AND CONCLUSION**

**Materials and methods**

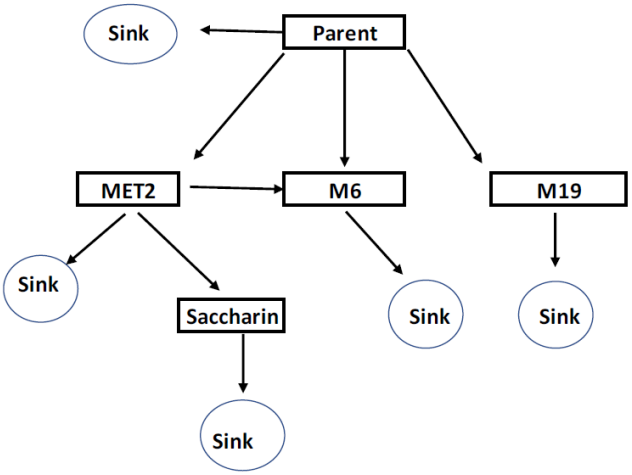
The degradation of [$^{14}$C]Benzisothiazolone was performed according to the Regulation (EU) No 528/2012 and the OECD Guideline 307 (2002)

| | |
|---|---|
| | and the US EPA Guideline OPPTS 835.4100 (2008).<br>[$^{14}$C]Benzisothiazolone was applied to four soils and incubated under aerobic conditions at a temperature of 20.8 ± 0.2°C and 20.9 ± 0.2°C and a soil moisture content of pF2 in the dark for up to 120 days. |
| **Results and discussion** | Mineralization of [$^{14}$C]Benzisothiazolone was extensive in bioactive soils and carbon dioxide released reached a maximum of 47.9, 56.2, 46.1 and 39.9% AR at the end of incubation in four soils tested, respectively. Mineralization of [$^{14}$C]Benzisothiazolone in sterile soils was negligible and did not exceed 0.4% AR. [$^{14}$C]Benzisothiazolone degraded via oxidation to 1,2-Benzisothiazolin-3-one-1-oxide (MET2) and further to Saccharin (M5). Two other degradation products M6 and M9 were observed, which were proposed to be 2-Sulphamoylbenzoic acid and 2-Aminosulphinyl-benzoic acid. M6 and M9 were presumably formed by opening of the thiazolinone ring. Further oxidation or hydrolysis formed 2-Sulphobenzoic acid (M6b). Additionally, the transient metabolite 2-Sulphanyl benzamide (M8) was quickly oxidised to 2-Sulphobenzoic acid. Non-extractable residues increased to maximum levels of 52.0, 42.9, 44.6 and 45.6% AR on 56 DAT respectively for four soils tested. |
| **Conclusion** | [$^{14}$C]Benzisothiazolone degraded in soil with half-lives ranging from 0.02 to 0.24 days, and $DT_{90}$ values ≤ 0.80 days. [$^{14}$C]Benzisothiazolone degrades under formation of 1,2-Benzisothiazolin-3-one-1-oxide (MET2), Saccharin (M5), M6, M9, 2-Sulphobenzoic acid (M6b), and the transient metabolite 2-Sulphanyl benzamide (M8) with ultimate formation of bound residues and $CO_2$. |
| • Reliability | 1 |
| • Deficiencies | No |

| EVALUATION BY COMPETENT AUTHORITIES | |
|---|---|
| | |
| **Date** | *19/08/2021* |
| **Materials and Methods** | Applicant's version is adopted. The degradation of [14C]Benzisothiazolone was performed according to the Regulation (EU) No 528/2012 and the OECD Guideline 307 (2002) and the US EPA Guideline OPPTS 835.4100 (2008).<br>Four standard representative fresh field soils with a wide range of soil properties were used: Soil I: Speyer 2.4 (loam), Soil II: Speyer 5M (sandy loam), Soil III: RefeSol 02-A (silt loam) and Soil IV: RefeSol 04-A (loamy sand).<br>Sampling was done after 0.00, 0.04, 0.08, 0.17, 0.33, 1.0, 2.1, 4, 7, 14, 28, 56, 91 (Soils I-III only) and 120 (Soil IV only) days of incubation for bioactive soils and after 0.00, ~1, 13, 28, 91 (Soils I-III only) and 120 (Soil IV only) days of incubation for sterile soils.<br>The soils were applied at three time-points with application solution #1, #2 and #3 on March 20, 2018, March 22, 2018 and April 26, 2018 respectively. On each application day, prior to, during and after application, identical aliquots (i.e. 1000 µl) of the used application solution were diluted to 20 mL with water.<br>The required recovery of radioactivity (90-110% AR) was achieved for all samples with an exception of four replicates from bioactive soils (intervals of 7, 14 and 28 DAT; Table 3 to Table 6). For these four replicates, it can be assumed that the loss of radioactivity occurred in trapping of radiolabelled carbon dioxide, as might be noted from the lower levels of 14CO2 found in these samples in comparison to corresponding other replicates, and intervals before and after. Therefore, the results obtained from HPLC analysis of these replicates are considered acceptable, and have not been excluded from the kinetic evaluation. |

| | |
|---|---|
| **Results and discussion** | The applicant's version is acceptable with the following remarks:<br>Total mean recovery of radioactivity during the incubation period accounted for 97.6 ± 2.9, 96.8 ± 3.4, 96.7 ± 3.4 and 94.9 ± 3.0% of applied radioactivity (AR) for four bioactive soils respectively. The corresponding values for the sterile soils were 98.0 ± 0.8, 97.7 ± 1.0, 97.4 ± 1.1 and 97.1 ± 3.4% AR. The required recovery of radioactivity (90-110% AR) was achieved for all samples with an exception of four replicates from bioactive soils (intervals of 7, 14 and 28 DAT. For these four replicates, it can be assumed that the loss of radioactivity occurred in trapping of radiolabelled carbon dioxide, as might be noted from the lower levels of 14CO2 found in these samples in comparison to corresponding other replicates, and intervals before and after.<br>BIT disappears very fast in every soil and the number of data points before the DT50 is limited. In addition DT50 values presented in this summary are not adequate because:<br>• Values presented in table Table A7.2.1/01- 18 correspond only to parent. Metabolites were not considered in the parent's DT50 calculation and they should be considered as indicated Generic guidance for Estimating Persistence and Degradation Kinetics from Environmental Fate Studies on Pesticides in EU Registration (FOCUS Kinetics Guidance)<br>• Data issues such as time zero samples or values below the quantification and detection limit were not adequately considered for DT50 calculations.<br>Nevertheless, the applicants have presented a document: "Determination of rates of decline for 1,2-Benzisothiazol-3(2H)-one and its metabolites in soil according to FOCUS Kinetics Guidance" written by Dr. A. Mamouni, Dr. T. Jarvis & V. Montesano where all these aspects were adequately considered.<br>The procedure followed for kinetic assessment has been the following:<br>The data were fitted directly using CAKE v. 3.3 using the Application Preferences FOCUS Guideline and the Iteratively Reweighted Least Squares (IRLS) fitting option. The optimisation was conducted as follows:<br>• First, the parent compartment was fitted, without any reference to the metabolite.<br>• Then the metabolite compartment was fitted, with the parameters for the parent calculated in the first step fixed (and therefore not increasing the complexity of the optimisation).<br>• Finally, both compartments were fitted, using the results of step 2 as a starting point. This step is complex (with all parameters free) but started from near the optimum.<br>Metabolites were fitted in the stepwise procedure indicated by the guidance (FOCUS, 2014). Parent data were fitted with the parent best-fit model, the parameters were fixed for the metabolite fitting step and, finally, the parameters were un-fixed for a re-fit. For the kinetic fit, parent BIT was assumed to degrade according to the metabolism scheme as presented in Figure 1 and 2, next. This pathway showed to give the best fit for the metabolites in all soils. |

**Figure 2. Simplified metabolic pathway used for metabolite kinetics**




The first step of degradation of the parent compound was observed to be the oxidation of sulphur to form the 1,2-benzisothiazolin-3-one-1-oxide metabolite (MET2), followed by a further oxidation step to form saccharin (M5) and/or opening of the thiazolinone ring leading to several metabolites such as the 2-sulphamoylbenzoic acid metabolite (M6), and the transient 2-aminosulphinyl-benzoic acid metabolite (M9). The ultimate oxidation/hydrolysis products were identified as 2-sulphobenzoic acid (M6b), which is rapidly mineralized, and the minor metabolite o-sulphobenzamide MET4 (detected in sterile soils only). Additionally, the transient 2-sulphanyl benzamide metabolite (M8) was observed, and it was quickly oxidised under the incubation conditions to 2-sulphobenzoic acid.

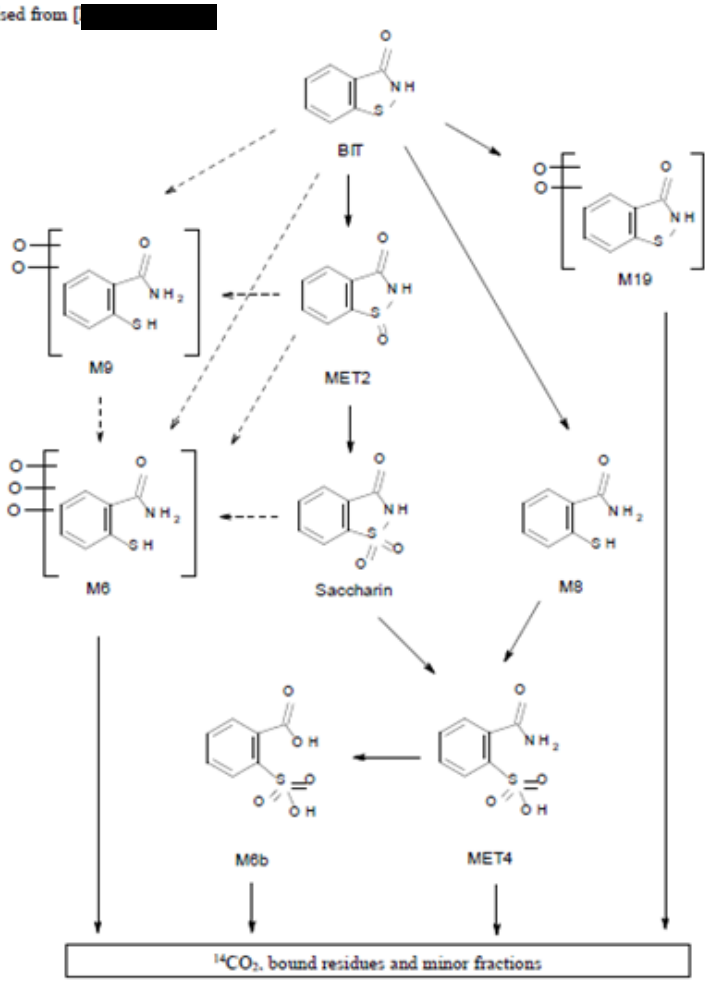
Major degradants include 1,2-benzisothiazolin-3-one-1-oxide (met 2, max average 23.1% of AR across the 4 soils). MET-2 is an intermediate metabolite with unclear structure, but it degrades rapidly to saccharin. Saccharin (7.8% AR across the three soils were it was found)%), 2-sulphanyl benzamide (M8) (10.52%), 2-aminosulphinylbenzoic acid (M9) (14.1%), Metabolite 6 (whose chemical structure could not be identified, 40.55% including M6b). Metabolite M19 did not exceed 5% in the non-sterile soils and reached the maximum of 4.9% AR. M9 is a transient metabolite which is further rapidly degraded to M6. M8 also degraded very fast, as well as saccharin and 1,2-benzisothiazolin-3-one-1-oxide.

Formation fractions of the different metabolites were: 0.31 for metabolite 2 (from parent), 0.88 for metabolite 6 (including M6b) (from parent and from met 2), 0.366 for met 5 or saccharin (from met 2) and 0.046 for M19 (see also the transformation pathway above).

Several other unidentified metabolites were found in bioactive soils, but none of them at levels >10% AR at a single sampling event, or ≥5% AR at two consecutive sampling intervals

**Figure 1. Proposed metabolic pathway ([redacted])**

Revised from [redacted]




All metabolites but MET4 were found in bioactive and sterile soils; MET4 was found in sterile soils only [Piskorski, 2020a].
Structures of metabolites M6, M9 and M19 were tentatively proposed based on the LC-MS structure elucidation and chromatographic behaviour only [Piskorski, 2020a]; likely structures of M6 and M9 are given on page 9.

For determining the best model aspects such as visual fit, chi square and t-test were considered for goodness of fit.

The values reported for parent alone are:

Table 6: Summary of BIT kinetics in soil calculated with parent only under aerobic soil conditions

**Laboratory study: Parent (non-sterile conditions) / Trigger (T) and modelling (M) endpoints**

| Soil | Kinetic model | Mo | Parameter (K, K1, k2, g, tb, α, β) | χ2 %-error & visual fit | Prob>t | Lower CI | Upper CI | DT50 [days] | DT90 [days] |
|---|---|---|---|---|---|---|---|---|---|
| Soil I | **SFO (T & M)** | 94.3 | k=63.97 | 5.2 Very good | 1.8E-09 | 56.5 | 71.5 | 0.01 | 0.04 |
| | FOMC | 94.3 | α=1.192 β=0.004875 | 3.5 Very good | n.r. n.r. | 0.48 -0.003 | 1.91 0.013 | 0.004/0.009** not reliable | 0.029 not reliable |
| | DFOP | 94.3 | K1=70.9 K2=0.3004 g =0.9823 | 1.1 Very good | 1.8E-09 0.27 n.r. | 64.9 -0.80 0.97 | 76.97 1.4 0.99 | nd not reliable | nd not reliable |
| Soil II | **SFO (M)** | 93.8 | k=32.12 | 9.9 Very good | 1.4E-10 | 28.4 | 35.8 | 0.02 | 0.07 |
| | **FOMC (T)** | 94.1 | α=1.545 β=0.02729 | 3.2 Very good | n.r. n.r. | 1.09 0.014 | 2.0 0.04 | 0.02/0.03** | 0.09 |
| | DFOP | 94.1 | K1=45.44 K2=6.311 g =0.8532 | 4.3 Very good | 1.9E-05 0.039 n.r. | 30.96 -0.86 0.69 | 59.9 13.48 1.02 | 0.02/0.11* not reliable | 0.09 not reliable |
| Soil III | **SFO (M)** | 92.4 | k=45.75 | 8.1 Very good | 3.1E-09 | 40.07 | 51.44 | 0.02 | 0.05 |
| | **FOMC (T)** | 92.5 | α=1.315 β=0.01197 | 3.6 Very good | n.r. n.r. | 0.84 0.003 | 1.79 0.02 | 0.01/0.02** | 0.06 |
| | DFOP | 92.5 | K1=53.64 K2=1.344 g =0.9588 | 3.2 Very good | 6.6E-09 0.06 n.r. | 48.28 -0.48 0.94 | 59.0 3.17 0.98 | 0.01/0.52* not reliable | nd not reliable |
| Soil IV | SFO | 84.5 | k=6.67 | 17.3 Acceptable | 1.5E-05 | 4.43 | 8.91 | 0.10 | 0.35 |
| | FOMC | 93.5 | α=0.7476 β=0.04234 | 6.3 Very good | n.r. n.r. | 0.51 0.02 | 0.98 0.07 | 0.06/0.27** | 0.88 |
| | **DFOP (T&M)** | 94.2 | K1=42.53 K2=2.731 g =0.4576 | 3.5 Very good | 0.004 1.1E-04 n.r. | 13.39 1.65 0.33 | 71.66 3.81 0.59 | 0.05/0.25* | 0.02 |

* slow phase
** DT90/3.32
n.r. = not relevant.
nd:= not determined
Bold: optimum fit/ T= Trigger / M = Modelling
Prob >t: P value from the t-test (acceptability criteria P ≤ 0.05)
CI: confidence interval (95%)

Once the best model for parent was determined, metabolites fitting was done starting from the best parent fit. SFO was considered enough for metabolites fitting.

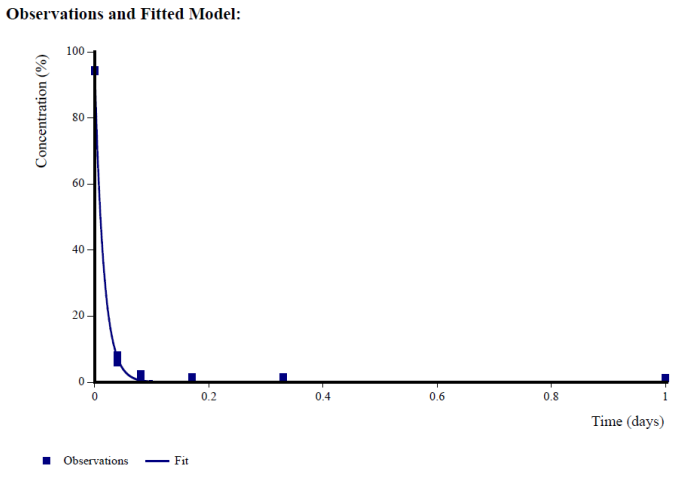
The next table shows parent results when all metabolites are included in Cake iteration process.

| Soil | Kinetic model for parent | Parameter (k, k1,k2, k3, g | Chi-square | T test | DT50 | DT90 |
|---|---|---|---|---|---|---|
| I | SFO | 62.89 | 5.26 | 1.38E-29 | 0.01 | 0.004 |
| II | FOMC | Alpha =1.452 Beta: 0.025 | 3.65 | N/A | 0.0157 0.0993/3.32 = 0.03 | 0.09 |
| III | FOMC | Alpha: 1.308 Beta: 0.01178 | 3.56 | N/A | 0.00823 0.0567/3.32 = 0.017 | 0.06 |
| IV | DFOP | K1: 41.23 K2: 2.5 | 3.64 | 8.93E-6 8.1E-10 | Overall: 0.056 DT50k1: 0.0168 DT50k2: 0.27 | 0.656 |

The results are similar to the DT50s obtained with parent alone, eCA considers this is a good indication of good adjustment.

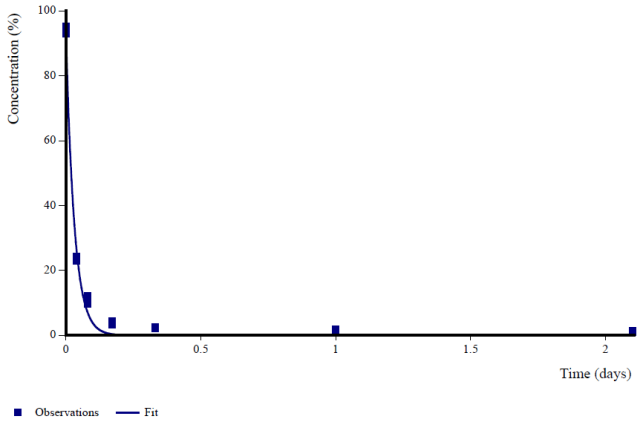
eCA notes that that due to the rapid disappearance of BIT, the number of data points before the DT50 occurs is limited in three of the soils, in fact only the initial value was measured as the following graphs show.

Soil I



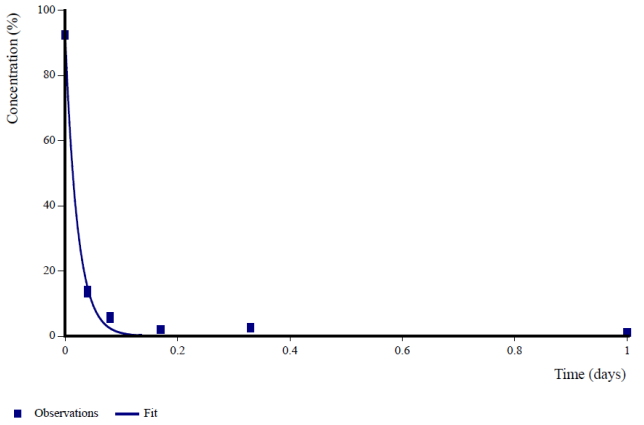
Soil II

**Observations and Fitted Model:**



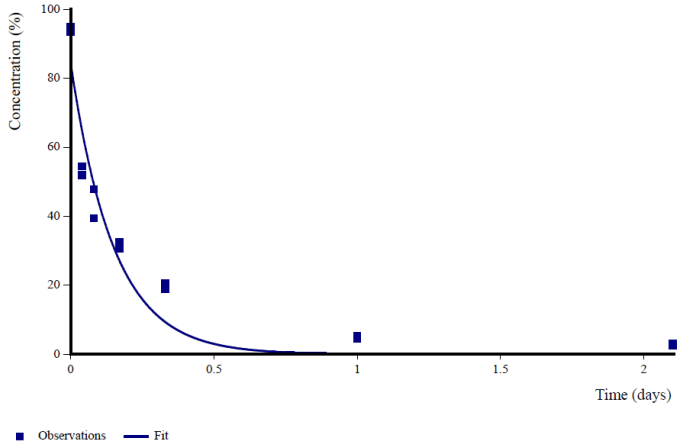

Soil III

**Observations and Fitted Model:**




*Soil IV*

**Observations and Fitted Model:**



| | |
|---|---|
| | This was considered as an uncertainty to the calculated DT50s for soils I, II and III. For this reason, eCA considered it adequate to use the worst case (loamy sand) DT50 = 0.27 days or 0.54 at 12ºC for risk assessment, also because soil IV is the case where more data points (3) exist before the DT50. This DT50 is the result of modelling the best parent fit for soil IV Refe Sol 04-A (loamy sand) which is DFOP, with the metabolites found in this soil.<br>In soil, among the relevant metabolites, the highest DT50 corresponds to metabolite M6. The rate of degradation of M6 metabolite (including M6b fraction and the transient metabolite M9) was much slower when compared to the parent compound. DT50 values ranging from 21.5 to 46.3 days were calculated (43.8 and 94 days at 12ºC and 62.14 geomean at 12ºC). MET2 metabolite, which was shown to be rapidly formed from the parent compound, was very rapidly degraded in all soils with DT50 values ranging from 0.3 to maximum 2.3 (slow phase) days. Saccharin and M19 metabolites showed also acceptable fits and were degraded with DT50 values ranging from 6.3 (12.6) to 10.3 (20.6), and 2.0 (4) to 23.2 (46.4 at 12ºC) days, respectively. Due to the rapid degradation and the lack of sufficient data points, no kinetics can be calculated for metabolites M8 and M9.<br>For metabolites risk assessment eCA considers it relevant to assess metabolite 6. This metabolite has a DT50 in soil of 62.14 at 12ºC (geomean) and a predicted koc = 10 L/kg and is a concern in case of direct releases to soil, which occur in the paint and coatings scenario. The other metabolites of BIT are less toxic than the parent substance and show a potential for rapid degradation in the environment. In addition, they do not show a potential for bioaccumulation.<br>Mineralization of [14]Benzisothiazolinone was extensive and carbon dioxide released reached a maximum of 47.9, 56.2, 46.1 and 39.9% AR at the end of incubation in four soils tested, respectively. In the sterile soils, the mineralization of BIT was negligible and did not exceed 0.4% AR in all soils tested. For the bioactive soils, the mean amount of non-extractable residues increased from 27.6, 10.3, 13.0 and 6.9% AR on 0 DAT to maximum levels of 52.0, 42.9, 44.6 and 45.6% AR on 56 DAT respectively for four soils tested. At the end of incubation, the amounts were 48.6, 39.9, 43.2 and 41.9% AR respectively for four soils tested. |
| | |
| | |
| **Conclusion** | eCA considers the study and analysis provided by the applicant valid. The test was done according to Guidelines. The required recovery of radioactivity (90-110% AR) was achieved for all samples with an exception of four replicates from bioactive soils (intervals of 7, 14 and 28 DAT). For these four replicates, it can be assumed that the loss of radioactivity occurred in trapping of radiolabelled carbon dioxide, as might be noted from the lower levels of 14CO2 found in these samples in comparison to corresponding other replicates, and intervals before and after.<br>Due to the rapid disappearance of BIT, the number of data points before the DT50 occurs is limited in three of the soils, in fact only the initial value was measured. This adds uncertainty to the calculated DT50s for these three soils (soil I, II and III). For this reason, eCAs considers it adequate to use the worst case (loamy sand) DT50 = 0.27 days or 0.54 at 12ºC for risk assessment also because soil IV is the case where more data points (3) exist before the DT50. A DT50 = 62.14 d will be considered for metabolite 6. |
| **Reliability** | *1* |

| | |
|---|---|
| **Acceptability** | acceptable |

**Table A7.2.1/01-2: Test soils used**

| Parameters | Soil I | Soil II | Soil III | Soil IV |
|---|---|---|---|---|
| | Speyer 2.4 | Speyer 5M | RefeSol 02-A | RefeSol 04-A |
| | | | | |
| **Site location** | [redacted] | [redacted] | [redacted] | [redacted] |
| **Batch** | [redacted] | [redacted] | [redacted] | [redacted] |
| **Sampling date** | 19.01.2018 | 19.01.2018 | 11.01.2018 | 11.01.2018 |
| **Sampling depth (cm)** | Approx. 0-20 | Approx. 0-20 | 0-25 | 0-25 |
| **Soil characteristics*** | | | | |
| - pH (0.01 M $CaCl_2$) | 7.4 ± 0.1 | 7.3 ± 0.1 | 6.54 | 5.11 |
| - Organic carbon (%) | 2.04 ± 0.17 | 1.01 ± 0.09 | 1.04 | 3.04 |
| - Nitrogen content (%) | 0.22 ± 0.01 | 0.13 ± 0.01 | 1.20 | 1.76 |
| - Cation exchange capacity (meq/100 g soil) | 26.5 ± 15.5 | 15.7 ± 5.3 | 40.60 | 41.20 |
| - C/N Ratio** | 9.3 | 7.77 | 0.87 | 1.73 |
| - Organic matter (OM %)*** | 3.52 | 1.74 | 1.79 | 5.24 |
| - Weight per volume (g/l)* | 1251 ± 39 | 1221 ± 72 | Not available | Not available |
| | | | | |
| **Soil type (USDA [7])*** | Loam | Sandy loam | Silt loam | Loamy sand |
| **Particle size analysis (mm)*** | | | | |
| < 0.002 (clay) % | 26.6 ± 0.7 | 11.2 ± 0.8 | 15.8 | 6.5 |
| 0.002-0.05 (silt) % | 41.2 ± 1.3 | 29.8 ± 1.2 | 80.1 | 12.2 |
| > 0.05 (sand) % | 32.3 ± 1.4 | 59.0 ± 1.6 | 4.1 | 81.2 |
| | | | | |
| **Soil water content (g water/100 g soil)** | | | | |
| at pF 1.0 (WHC)* | 44.6 ± 2.2 | 41.6 ± 2.6 | 47.1 | 34.6 |
| at pF 2.0**** | 28.1 | 19.6 | 35.8 | 7.7 |
| | | | | |
| **Biomass** | | | | |
| Start of incubation (mg C/100 g dry soil) | 74.28 | 22.52 | 26.57 | 17.69 |
| Start of incubation (% OC) | 3.6 | 2.2 | 2.6 | 0.6 |
| During incubation (mg C/100 g dry soil) | 71.20 | 30.17 | 20.27 | 10.92 |
| During incubation (% OC) | 3.5 | 3.0 | 1.9 | 0.4 |
| End of incubation (mg C/100 g dry soil) | 60.46 | 20.94 | 15.68 | 14.39 |

| Parameters | Soil I | Soil II | Soil III | Soil IV |
|---|---|---|---|---|
| | Speyer 2.4 | Speyer 5M | RefeSol 02-A | RefeSol 04-A |
| End of incubation (% OC) | 3.0 | 2.1 | 1.5 | 0.5 |

* Mean values of different batch analyses ± standard deviations given by LUFA, 67346 Speyer, Germany (Soil I and II; GLP) or by the [redacted]
** C/N ratio = % organic carbon / % nitrogen content
*** %OM = 1.724 x % organic carbon
**** Determined under GLP by [redacted]
OC: Organic carbon
WHC: water holding capacity

**Table A7.2.1/01-3: Material balance in Soil I (Speyer 2.4); bioactive soil incubated at 20°C**

| Sampling times (days) | Extractables (RT) | Extractables (Soxhlet) | Total Extractables | $^{14}CO_2$ | Other organic volatiles | Bound residues | Material balance |
|---|---|---|---|---|---|---|---|
| | [% applied radioactivity] | | | | | | |
| 0.00 | 66.7 | 3.0 | 69.7 | na | na | 27.6 | 97.3 |
| 0.04 | 54.2 | 5.6 | 59.8 | <0.1 | <0.1 | 37.6 | 97.4 |
| 0.08 | 53.0 | 4.4 | 57.4 | <0.1 | <0.1 | 38.8 | 96.2 |
| 0.17 | 53.4 | 3.4 | 56.8 | <0.1 | <0.1 | 41.1 | 97.9 |
| 0.33 | 53.6 | 3.2 | 56.8 | 0.2 | <0.1 | 37.3 | 94.3 |
| 1.0 | 53.3 | 3.5 | 56.8 | 1.8 | <0.1 | 40.2 | 98.8 |
| 2.1 | 49.3 | 2.3 | 51.6 | 4.0 | <0.1 | 43.9 | 99.4 |
| 4 | 47.6 | 2.4 | 50.0 | 6.7 | <0.1 | 41.4 | 98.1 |
| 7 | 42.9 | 2.3 | 45.2 | 9.2 | <0.1 | 42.9 | 97.4 |
| 14 | 34.2 | 2.3 | 36.6 | 16.7 | <0.1 | 45.3 | 98.6 |
| 28 | 19.5 | 1.3 | 20.8 | 23.0 | <0.1 | 48.7 | 92.5 |
| 56 | 5.2 | 1.4 | 6.6 | 42.8 | <0.1 | 52.0 | 101.4 |
| 91 | 2.8 | 0.8 | 3.6 | 47.9 | <0.1 | 48.6 | 100.1 |

na: not analysed

**Table A7.2.1/01-4: Material balance in Soil II (Speyer 5M); bioactive soil incubated at 20°C**

| Sampling times (days) | Extractables (RT) | Extractables (Soxhlet) | Total Extractables | $^{14}CO_2$ | Other organic volatiles | Bound residues | Material balance |
|---|---|---|---|---|---|---|---|
| | [% applied radioactivity] | | | | | | |
| 0.00 | 83.0 | 3.8 | 86.8 | na | na | 10.3 | 97.1 |
| 0.04 | 61.6 | 5.6 | 67.2 | <0.1 | <0.1 | 28.4 | 95.6 |
| 0.08 | 57.6 | 5.9 | 63.6 | <0.1 | <0.1 | 33.4 | 97.0 |
| 0.17 | 55.8 | 4.6 | 60.4 | <0.1 | <0.1 | 37.4 | 97.9 |
| 0.33 | 57.5 | 4.1 | 61.6 | 0.4 | <0.1 | 34.7 | 96.7 |
| 1.0 | 59.0 | 3.3 | 62.3 | 1.1 | <0.1 | 31.6 | 94.9 |
| 2.1 | 57.7 | 2.7 | 60.4 | 5.7 | <0.1 | 34.2 | 100.3 |
| 4 | 53.9 | 2.2 | 56.1 | 7.6 | <0.1 | 31.8 | 95.5 |
| 7 | 49.7 | 2.4 | 52.1 | 5.6 | <0.1 | 33.7 | 91.4 |
| 14 | 37.4 | 2.2 | 39.6 | 18.9 | <0.1 | 36.9 | 95.4 |
| 28 | 24.7 | 1.6 | 26.3 | 34.9 | <0.1 | 39.3 | 100.5 |
| 56 | 7.5 | 1.6 | 9.0 | 44.7 | <0.1 | 42.9 | 96.7 |
| 91 | 2.4 | 1.2 | 3.6 | 56.2 | <0.1 | 39.9 | 99.6 |

na: not analysed

**Table A7.2.1/01-5: Material balance in Soil III (RefeSol 02-A); bioactive soil incubated at 20°C**

| Sampling times (days) | Extractables (RT) | Extractables (Soxhlet) | Total Extractables | $^{14}CO_2$ | Other organic volatiles | Bound residues | Material balance |
|---|---|---|---|---|---|---|---|
| | [% applied radioactivity] | | | | | | |
| 0.00 | 79.6 | 2.9 | 82.5 | na | na | 13.0 | 95.5 |
| 0.04 | 59.5 | 6.4 | 66.0 | <0.1 | <0.1 | 32.0 | 98.0 |
| 0.08 | 56.5 | 7.5 | 64.1 | <0.1 | <0.1 | 32.9 | 97.0 |
| 0.17 | 56.7 | 4.9 | 61.6 | <0.1 | <0.1 | 36.4 | 98.0 |
| 0.33 | 56.3 | 6.3 | 62.6 | 0.4 | <0.1 | 34.1 | 97.1 |
| 1.0 | 53.8 | 4.4 | 58.2 | 2.4 | <0.1 | 37.0 | 97.7 |
| 2.1 | 51.0 | 4.0 | 55.0 | 4.5 | <0.1 | 38.9 | 98.4 |
| 4 | 48.6 | 4.1 | 52.7 | 5.9 | <0.1 | 38.4 | 97.0 |
| 7 | 45.7 | 4.0 | 49.6 | 8.1 | <0.1 | 40.1 | 97.8 |
| 14 | 37.7 | 4.3 | 42.0 | 11.6 | <0.1 | 40.3 | 93.9 |
| 28 | 26.7 | 4.6 | 31.3 | 19.9 | <0.1 | 37.2 | 88.4 |
| 56 | 12.6 | 3.6 | 16.2 | 39.2 | <0.1 | 44.6 | 100.0 |
| 91 | 5.7 | 3.0 | 8.7 | 46.1 | <0.1 | 43.2 | 98.0 |

na: not analysed

**Table A7.2.1/01-6: Material balance in Soil IV (RefeSol 04-A); bioactive soil incubated at 20°C**

| Sampling times (days) | Extractables (RT) | Extractables (Soxhlet) | Total Extractables | $^{14}CO_2$ | Other organic volatiles | Bound residues | Material balance |
|---|---|---|---|---|---|---|---|
| | [% applied radioactivity] | | | | | | |
| 0.00 | 90.3 | na | 90.3 | na | na | 6.9 | 97.2 |
| 0.04 | 78.6 | 2.2 | 80.8 | <0.1 | <0.1 | 16.0 | 96.9 |
| 0.08 | 74.3 | 4.7 | 79.0 | <0.1 | <0.1 | 16.7 | 95.7 |
| 0.17 | 68.4 | 5.8 | 74.2 | <0.1 | <0.1 | 22.2 | 96.5 |
| 0.33 | 59.2 | 6.0 | 65.2 | <0.1 | <0.1 | 28.5 | 93.8 |
| 1.0 | 54.8 | 4.3 | 59.2 | 0.4 | <0.1 | 34.2 | 93.9 |
| 2.1 | 53.7 | 5.0 | 58.7 | 1.0 | <0.1 | 35.0 | 94.7 |
| 4 | 52.5 | 5.8 | 58.3 | 1.8 | <0.1 | 31.0 | 91.1 |
| 7 | 48.3 | 2.7 | 51.1 | 3.4 | <0.1 | 40.2 | 94.6 |
| 14 | 45.6 | 5.5 | 51.2 | 4.2 | <0.1 | 35.5 | 90.8 |
| 28 | 37.3 | 4.9 | 42.3 | 13.8 | <0.1 | 34.9 | 91.0 |
| 56 | 25.1 | 5.5 | 30.6 | 24.1 | <0.1 | 45.6 | 100.2 |
| 91 | 11.4 | 4.6 | 16.0 | 39.9 | <0.1 | 41.9 | 97.7 |

na: not analysed

**Table A7.2.1/01-7: Material balance in Soil I (Speyer 2.4); sterile soil incubated at 20°C**

| Sampling times (days) | Extractables (RT) | Extractables (Soxhlet) | Total Extractables | $^{14}CO_2$ | Other organic volatiles | Bound residues | Material balance |
|---|---|---|---|---|---|---|---|
| | [% applied radioactivity] | | | | | | |
| 0.00 | 90.3 | na | 90.3 | na | na | 6.9 | 97.2 |
| 0.91 | 78.6 | 2.2 | 80.8 | <0.1 | <0.1 | 16.0 | 96.9 |
| 13 | 74.3 | 4.7 | 79.0 | <0.1 | <0.1 | 16.7 | 95.7 |
| 28 | 68.4 | 5.8 | 74.2 | <0.1 | <0.1 | 22.2 | 96.5 |
| 91 | 59.2 | 6.0 | 65.2 | <0.1 | <0.1 | 28.5 | 93.8 |

na: not analysed

**Table A7.2.1/01-8: Material balance in Soil II (Speyer 5M); sterile soil incubated at 20°C**

| Sampling times (days) | Extractables (RT) | Extractables (Soxhlet) | Total Extractables | $^{14}CO_2$ | Other organic volatiles | Bound residues | Material balance |
|---|---|---|---|---|---|---|---|
| | [% applied radioactivity] | | | | | | |
| 0.00 | 91.6 | na | 91.6 | na | na | 5.8 | 97.4 |
| 0.88 | 61.3 | 5.6 | 66.8 | <0.1 | <0.1 | 29.8 | 96.6 |
| 13 | 55.0 | 4.2 | 59.3 | <0.1 | <0.1 | 37.7 | 97.1 |
| 28 | 58.4 | 3.3 | 61.7 | 0.2 | <0.1 | 36.8 | 98.6 |
| 91 | 62.5 | 2.6 | 65.1 | 0.4 | <0.1 | 33.1 | 98.6 |

na: not analysed

**Table A7.2.1/01-9: Material balance in Soil III (RefeSol 02-A) sterile soil incubated at 20°C**

| Sampling times (days) | Extractables (RT) | Extractables (Soxhlet) | Total Extractables | $^{14}CO_2$ | Other organic volatiles | Bound residues | Material balance |
|---|---|---|---|---|---|---|---|
| | [% applied radioactivity] | | | | | | |
| 0.00 | 88.0 | 1.4 | 89.4 | na | na | 8.1 | 97.5 |
| 0.83 | 62.5 | 5.5 | 68.0 | <0.1 | <0.1 | 28.6 | 96.6 |
| 13 | 55.6 | 5.2 | 60.9 | <0.1 | <0.1 | 35.9 | 96.8 |
| 28 | 57.4 | 4.7 | 62.1 | 0.2 | <0.1 | 36.4 | 98.7 |
| 91 | 61.3 | 4.5 | 65.8 | 0.4 | <0.1 | 31.3 | 97.5 |

na: not analysed

**Table A7.2.1/01-10: Material balance in Soil IV (RefeSol 04-A); sterile soil incubated at 20°C**

| Sampling times (days) | Extractables (RT) | Extractables (Soxhlet) | Total Extractables | $^{14}CO_2$ | Other organic volatiles | Bound residues | Material balance |
|---|---|---|---|---|---|---|---|
| | [% applied radioactivity] | | | | | | |
| 0.00 | 91.9 | na | 91.9 | na | na | 4.8 | 96.7 |
| 0.83 | 67.5 | 3.1 | 70.5 | <0.1 | <0.1 | 24.5 | 95.1 |
| 13 | 47.2 | 6.4 | 53.7 | <0.1 | <0.1 | 46.5 | 100.3 |
| 28 | 45.8 | 7.5 | 53.3 | <0.1 | <0.1 | 47.0 | 100.4 |
| 91 | 43.4 | 7.8 | 51.3 | 0.2 | <0.1 | 41.8 | 93.3 |

na: not analysed

**Table A7.2.1/01-11: Degradation of [$^{14}$C]Benzisothiazolone and formation of major metabolites in extracts of bioactive soil samples (Soil I; Speyer 2.4) incubated at 20°C**

| Sampling times (days) | Benzisothiazolone | MET2 | M5 | M6 (incl. M6b) | M8 | M9 |
|---|---|---|---|---|---|---|
| | [% applied radioactivity] | | | | | |
| 0.00 | 46.5 | 14.9 | nd | 3.7 | 4.5 | nd |
| 0.04 | 7.0 | 19.3 | nd | 28.1 | 2.0 | nd |
| 0.08 | 2.1 | 8.7 | nd | 17.4 | 1.7 | 21.1 |
| 0.17 | 1.3 | 12.9 | nd | 35.1 | 1.4 | nd |
| 0.33 | 1.6 | 11.6 | 2.3 | 33.3 | 1.5 | nd |
| 1.0 | 1.2 | 2.8 | 4.9 | 38.3 | 2.1 | nd |
| 2.1 | 0.4 | 1.3 | 7.3 | 36.9 | 2.0 | nd |
| 4 | nd | nd | 6.8 | 39.0 | nd | nd |
| 7 | 0.3 | 0.4 | 4.8 | 37.5 | nd | nd |
| 14 | nd | nd | 2.1 | 30.0 | nd | nd |
| 28 | nd | nd | nd | 17.9 | nd | nd |
| 56 | 0.3 | 0.2 | nd | 3.0 | nd | nd |
| 91 | 0.2 | <LOD | nd | 0.4 | <LOD | nd |

nd: not detected

**Table A7.2.1/01-12: Degradation of [$^{14}$C]Benzisothiazolone and formation of major metabolites in extracts of bioactive soil samples (Soil II; Speyer 5M) incubated at 20°C**

| Sampling times (days) | Benzisothiazolone | MET2 | M5 | M6 (incl. M6b) | M8 | M9 |
|---|---|---|---|---|---|---|
| | [% applied radioactivity] | | | | | |
| 0.00 | 54.6 | 18.9 | nd | 2.4 | 10.9 | nd |
| 0.04 | 23.6 | 22.7 | nd | 14.0 | 2.0 | nd |
| 0.08 | 11.0 | 17.9 | nd | 18.8 | 2.9 | 7.1 |
| 0.17 | 3.9 | 21.0 | nd | 26.7 | 2.7 | nd |
| 0.33 | 2.3 | 16.4 | 2.1 | 31.8 | 2.1 | nd |
| 1.0 | 1.7 | 6.9 | 3.3 | 40.9 | 2.0 | nd |
| 2.1 | 1.2 | 2.1 | 7.6 | 41.5 | nd | nd |
| 4 | nd | nd | 8.2 | 41.4 | nd | nd |
| 7 | 0.6 | nd | 6.4 | 42.0 | 0.6 | nd |
| 14 | nd | nd | 2.0 | 35.1 | nd | nd |
| 28 | nd | nd | nd | 25.6 | nd | nd |
| 56 | 0.4 | nd | nd | 7.3 | nd | nd |
| 91 | 0.4 | 0.3 | nd | 0.4 | 0.3 | nd |

nd: not detected

**Table A7.2.1/01-13: Degradation of [$^{14}$C]Benzisothiazolone and formation of major metabolites in extracts of bioactive soil samples (Soil III; RefeSol 02-A) incubated at 20°C**

| Sampling times (days) | Benzisothiazolone | MET2 | M5 | M6 (incl. M6b) | M8 | M9 |
|---|---|---|---|---|---|---|
| | [% applied radioactivity] | | | | | |
| 0.00 | 47.2 | 15.4 | nd | 2.4 | 16.9 | nd |
| 0.04 | 13.7 | 21.0 | nd | 22.1 | 2.0 | nd |
| 0.08 | 5.6 | 21.5 | nd | 28.5 | 2.1 | nd |
| 0.17 | 2.0 | 19.4 | nd | 31.6 | 2.2 | nd |
| 0.33 | 2.5 | 15.6 | 1.9 | 32.5 | 1.7 | nd |
| 1.0 | 1.2 | 7.0 | 4.3 | 35.0 | 2.4 | nd |
| 2.1 | 1.0 | 1.8 | 6.3 | 35.4 | nd | nd |
| 4 | 0.4 | nd | 7.9 | 36.2 | nd | nd |
| 7 | nd | nd | 6.0 | 35.9 | nd | nd |
| 14 | 1.0 | nd | 2.7 | 31.5 | nd | nd |
| 28 | 0.8 | nd | nd | 26.4 | nd | nd |
| 56 | 0.5 | nd | nd | 11.0 | nd | nd |
| 91 | 0.4 | 0.4 | nd | 2.1 | nd | nd |

nd: not detected

**Table A7.2.1/01-14: Degradation of [$^{14}$C]Benzisothiazolone and formation of major metabolites in extracts of bioactive soil samples (Soil IV; RefeSol 04-A) incubated at 20°C**

| Sampling times (days) | Benzisothiazolone | MET2 | M5 | M6 (incl. M6b) | M8 | M9 |
|---|---|---|---|---|---|---|
| | [% applied radioactivity] | | | | | |
| 0.00 | 51.1 | 29.4 | nd | nd | 9.8 | nd |
| 0.04 | 53.2 | 22.3 | nd | 5.3 | nd | nd |
| 0.08 | 43.6 | 21.5 | nd | 8.1 | 5.9 | nd |
| 0.17 | 31.7 | 21.3 | nd | 18.8 | nd | nd |
| 0.33 | 19.8 | 15.0 | nd | 25.4 | 1.8 | nd |
| 1.0 | 4.9 | 10.4 | nd | 37.5 | 3.2 | nd |
| 2.1 | 2.8 | 7.4 | nd | 45.0 | 2.1 | nd |
| 4 | 2.5 | 4.1 | nd | 43.5 | 0.5 | nd |
| 7 | 1.3 | 1.9 | nd | 41.7 | 2.7 | nd |
| 14 | 1.4 | 1.1 | nd | 39.5 | nd | nd |
| 28 | 1.0 | 0.4 | nd | 35.0 | nd | nd |
| 56 | 1.1 | nd | nd | 22.4 | nd | nd |
| 91 | 1.2 | nd | nd | 2.6 | 0.7 | nd |

nd: not detected

**Table A7.2.1/01-15: Degradation of [$^{14}$C]Benzisothiazolone and formation of major metabolites in extracts of sterile soil samples (Soil I; Speyer 2.4) incubated at 20°C**

| Sampling times (days) | Benzisothiazolone | MET2 | M5 | M6 (incl. M6b) | M8 | M9 | M19 |
|---|---|---|---|---|---|---|---|
| | [% applied radioactivity] | | | | | | |
| 0.00 | 62.7 | 9.4 | nd | 1.9 | nd | nd | nd |
| 0.91 | 17.1 | 28.0 | 4.8 | 8.5 | nd | 1.0 | 2.7 |
| 13 | 1.4 | nd | 20.5 | 26.2 | nd | 1.4 | 7.2 |
| 28 | 0.4 | nd | 22.7 | 28.1 | nd | 0.7 | 6.4 |
| 91 | nd | nd | 20.8 | 29.4 | nd | 2.4 | 7.1 |

nd: not detected

**Table A7.2.1/01-16: Degradation of [$^{14}$C]Benzisothiazolone and formation of major metabolites in extracts of sterile soil samples (Soil II; Speyer 5M) incubated at 20°C**

| Sampling times (days) | Benzisothiazolone | MET2 | M5 | M6 (incl. M6b) | M8 | M9 | M19 |
|---|---|---|---|---|---|---|---|
| | [% applied radioactivity] | | | | | | |
| 0.00 | 73.7 | 12.7 | nd | nd | nd | 1.6 | nd |
| 0.91 | 19.7 | 35.6 | 2.4 | 4.8 | nd | 1.0 | 2.0 |
| 13 | 1.8 | 1.6 | 7.3 | 36.9 | nd | 2.2 | 6.2 |
| 28 | 1.0 | nd | 8.7 | 39.7 | nd | 1.0 | 6.9 |
| 91 | 1.0 | 0.5 | 9.5 | 38.6 | nd | 2.4 | 6.8 |

nd: not detected

**Table A7.2.1/01-17: Degradation of Name [$^{14}$C]Benzisothiazolone and formation of major metabolites in extracts of sterile soil samples (Soil III; RefeSol 02-A) incubated at 20°C**

| Sampling times (days) | Benzisothiazolone | MET2 | M5 | M6 (incl. M6b) | M8 | M9 | M19 |
|---|---|---|---|---|---|---|---|
| | [% applied radioactivity] | | | | | | |
| 0.00 | 77.4 | 12 | nd | nd | nd | nd | nd |
| 0.91 | 19.4 | 33.8 | 1.9 | 7.0 | 1.7 | nd | 3.1 |
| 13 | 1.8 | 2.0 | 11.4 | 30.8 | 1.3 | nd | 12.0 |
| 28 | 1.4 | nd | 12.3 | 33.5 | nd | 0.9 | 12.0 |
| 91 | 1.3 | 0.7 | 14.2 | 33.2 | 0.5 | 1.3 | 12.3 |

nd: not detected

**Table A7.2.1/01-18: Degradation of [$^{14}$C]Benzisothiazolone and formation of major metabolites in extracts of sterile soil samples (Soil IV; RefeSol 04-A) incubated at 20°C**

| Sampling times (days) | Benzisothiazolone | MET2 | M5 | M6 (incl. M6b) | M8 | M9 | M19 |
|---|---|---|---|---|---|---|---|
| | [% applied radioactivity] | | | | | | |
| 0.00 | 77.1 | 14.8 | nd | nd | nd | nd | nd |
| 0.91 | 36.0 | 23.3 | nd | 5.1 | 1.1 | nd | 0.9 |
| 13 | 2.8 | 15.0 | 1.5 | 25.5 | 0.5 | 0.5 | 5.5 |
| 28 | 2.6 | 11.4 | 1.5 | 28.0 | 0.6 | nd | 6.6 |
| 91 | 2.4 | 3.3 | 2.2 | 32.4 | 1.1 | nd | 6.9 |

nd: not detected

**Table A7.2.1/01-19: $DT_{50}$ and $DT_{90}$ values of [$^{14}$C]Benzisothiazolone in soil**

| | Degradation Kinetics for Bioactive Soils | | | | | |
|---|---|---|---|---|---|---|
| | **$DT_{50}$ [days]** | **$DT_{90}$ [days]** | **Parameter** | **$\chi^2$ error %** | **$r^2$** | **Prob > t** |
| | **Soil Speyer 2.4** | | | | | |
| Parent (SFO) | 0.0151 | 0.05 | k = 46.02 | 11.2 | 0.9955 | 7.91E-013 |
| Parent (FOMC) | 0.00763 | 0.0568 | $\alpha$ = 1.199<br>$\beta$ = 0.009743 | 8.35 | 0.9955 | n/a |
| Parent (DFOP) | 0.0139 | 0.0509 | k1 = 52.76<br>k2 = 0.4687 | 2.67 | 0.997 | 1.82E-011<br>0.1136 |
| | **Soil Speyer 5M** | | | | | |
| Parent (SFO) | 0.0346 | 0.115 | k = 20.06 | 9.92 | 0.9963 | 1.20E-015 |
| Parent (FOMC) | 0.0307 | 0.143 | $\alpha$ = 2.582<br>$\beta$ = 0.09963 | 7.11 | 0.9972 | n/a |
| Parent (DFOP) | 0.0328 | 0.128 | k1 = 22.82<br>k2 = 0.4671 | 2.14 | 0.9988 | 4.88E-017<br>0.01204 |
| | **Soil RefeSol 02-A** | | | | | |
| Parent (SFO) | 0.0237 | 0.0787 | k = 29.25 | 15.3 | 0.9941 | 4.95E-017 |
| Parent (FOMC) | 0.0176 | 0.107 | $\alpha$ = 1.539<br>$\beta$ = 0.03093 | 11.2 | 0.9949 | n/a |
| Parent (DFOP) | nd | 0.0867 | k1 = 34<br>k2 = 0.4603 | 6.74 | 0.9965 | 1.16E-016<br>0.03544 |
| | **Soil RefeSol 04-A** | | | | | |
| Parent (SFO) | 0.24 | 0.797 | k = 2.89 | 10.8 | 0.9803 | 1.24E-010 |
| Parent (FOMC) | 0.233 | 0.947 | $\alpha$ = 4.252<br>$\beta$ = 1.318 | 10.8 | 0.9796 | n/a |
| Parent (DFOP) | nd | 0.871 | k1 = 3.15<br>k2 = 0.009803 | 9.35 | 0.9809 | 1.26E-009<br>0.3306 |

Official use only

## 1 REFERENCE

| | |
|---|---|
| **Reference** | [redacted] (2020b): 1,2-Benzisothiazol-3(2H)-one: Confirmation of Identification of Metabolites from Soil Degradation Study [redacted] 20170175. [redacted] GLP, non-published, 29 January 2020. |
| **Data protection** | Yes |
| 1.1.1 Data owner | Laboratorios Miret, S.A., LANXESS Deutschland GmbH, Lonza Ltd, Specialty Electronic Materials Switzerland GmbH (former The Dow Chemical Company), Thor GmbH, Troy Chemical Company BV |
| 1.1.2 Criteria for data protection | Data on existing a.s. submitted for first entry into the European list of approved biocidal active substance |

## 2 GUIDELINES AND QUALITY ASSURANCE

| | |
|---|---|
| **Guideline study** | Yes<br>(OECD Guideline 307 (Adopted 24th April 2002)<br>OPPTS 835.4100, US EPA, October 2008) |
| **GLP** | Yes |
| **Deviations** | No |

## 3 MATERIALS AND METHODS

**Test material**

| | |
|---|---|
| 3.1.1 General information | 1,2-Benzisothiazol-3(2H)-one; CAS number: 2634-33-5; Molecular formula: $C_7H_5NOS$; Molecular weight: 151.19 g/mol |
| 3.1.2 Labelled test material (Lot/Batch number; purity) | 1,2-[ring-U-$^{14}$C]Benzisothiazol-3(2H)-one (thereafter referred to as [$^{14}$C]Benzisothiazolone); (CFQ43104; radiochemical purity: 96.6%) |
| 3.1.3 Unlabelled test material (Lot/Batch number; purity, description) | 1,2-Benzisothiazol-3(2H)-one; (MKBZ4459V; purity: 99.3%; white to yellow and faint beige to beige powder) |
| 3.1.4 Reference items | 1,2-Benzisothiazol-3(2H)-one (R0)<br>MET1 (R1): Hydroxy-1,2-benzisothiazolin-3-one<br>MET2 (R2): 1,2-Benzisothiazolin-3-one-1-oxide<br>MET3 (R3): Dihydroxy-1,2-benzisothiazolin-3-one<br>MET4 (R4): o-Sulphobenzamide (sodium salt)<br>MET7 (R7): N-(4-amino-4-hydroxy-buta-1,3-dienyl)-benzamide<br>Saccharin (R8): 1,2-Benzisothiazolin-3-one-1-dioxide<br>2-Sulphanylbenzamide (R9)<br>2-Sulphobenzoic acid hydrate (R11) |

| | |
|---|---|
| | 2-Sulphamoylbenzoic acid (R12) |
| 3.1.5 Stability | Concentrated soil extracts, generated in the IES study # 20170175 and treated with [14C]-1,2-Benzisothiazol-3(2H)-one were used for analysis. Extracts (stored at -20°C) were thawed, centrifuged, and measured by LSC to determine the radioactive residues content. Storage recovery was between 85.3 and 102.5 %. |
| **Study conduct** | Concentrated soil extracts were measured by LSC to determine the radioactive residues content, and then analysed by HPLC to confirm the presence of the radioactive fractions to be confirmed. Afterwards, the samples were re-analysed as applicable by co-chromatography with the reference item(s) with HPLC-RAD and HPLC-UV, and/or TLC with phosphorimaging, and/or LC-MS. Nine soil extracts were used for HPLC co-chromatography with reference item R12 and two soil extracts were taken for TLC co-chromatography. |
| **Analytical method** | Volumes of extracts were determined and dispensed aliquots were assayed for radioactivity in duplicate. The aliquots were added directly to a known volume of scintillant and assayed by liquid scintillation counting (LSC). The quantity of radioactivity was determined using a calibrated Packard liquid scintillation counter equipped with DPM and luminescence options. Reversed-phase HPLC (RP-HPLC) was used for chromatographic profiling of the soil extracts. For identification, radioactive components were compared with reference standards by co-chromatography. Aliquots from extracts were mixed with solutions of reference items and the mixtures injected to the HPLC system. Additionally, Normal-phase TLC (NP-TLC) was used to confirm the HPLC chromatographic profile of sample extracts. Radioactive components were compared with reference standards by co-chromatography for their identification. The radiolabelled test item and metabolites were detected using a phosphorimager, and unlabelled test item and the reference items were detected using a UV lamp (254 nm). Mass spectrometry (MS) was used to confirm the identity of reference standards. |
| | **4 RESULTS** |
| **Storage stability** | Concentrated soil extracts, generated in the IES study # 20170175 and treated with [$^{14}$C]-1,2-Benzisothiazol-3(2H)-one were analysed after a storage period of approximately 1 year. HPLC profiles were compared to corresponding profiles in the IES study report # 20170175 or the study raw data. Sufficient stability during storage and presence of metabolite M6 could be confirmed. |
| **Analytical results** | The reference standard of 2-sulphamoylbenzoic acid (R12) was analysed by HPLC in water/MeCN (95/5) and in DMSO with three HPLC methods as well as two LC-MS methods. All HPLC and LC-MS analyses of both batches of the reference item R12 showed multiple peaks, irrespective of the solvent used for the solution, and of the HPLC method (mobile and stationary phase) used. Two peaks detected by LC-MS corresponded to the m/z value expected for 2-sulphamoylbenzoic acid, and one of them matched the retention time of M6 as well, however, this peak was found only in one of the reference standards R12 and showed the lowest intensity. The other two peaks, not matching m/z of R12, correspond to 2-sulphobenzoic acid and saccharin, the latter at ~70% ROI, both of which are possible products of hydrolysis of 2-sulphamoylbenzoic acid. Results suggest either instability during |

chromatographic analysis or instability during storage. Additionally, the R12 reference solutions when directly introduced into the ion source without chromatography showed the presence of the same components as observed with LC-MS. Nevertheless, selected soil samples were analysed with HPLC with co-chromatography with the reference standard R12. The results for all samples showed presence of M6 with the retention time observed analyses in the IES study # 20170175. To corroborate the presence of metabolite M6 in the soil samples, a selected extract was subjected to TLC co-chromatography with the reference standards, including 2-sulphamoylbenzoic acid.
The TLC analysis confirmed presence of an abundant, corresponding to the abundance of M6 that did not co-chromatograph with any of the available reference standards.

## 5 APPLICANT'S SUMMARY AND CONCLUSION

**Materials and methods**

Concentrated soil extracts, generated in the IES study # 20170175 were used for further analytical work. Sufficient stability was verified by comparison of HPLC profiles obtained in study # 20170175 with new profiles. Soil extract samples were re-analysed as applicable by co-chromatography with the reference item(s) with HPLC-RAD and HPLC-UV, and/or TLC with phosphorimaging, and/or LC-MS.

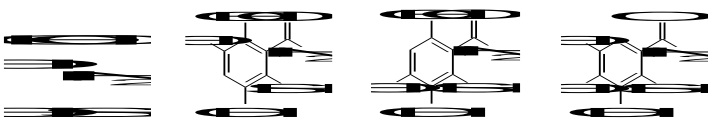
**Results and discussion**

All HPLC and LC-MS analyses of both batches of the reference item R12 showed multiple peaks, irrespective of the solvent used for the solution, and of the HPLC method used. Two peaks correspond to the m/z value expected for 2-sulphamoylbenzoic acid and one matched the retention time of M6 but was only found in at a very low intensity and only in one of the references for R12. Other peaks correspond to 2-sulphobenzoic acid and saccharin. This would suggest instability of the substance either during chromatographic analysis, or during storage. Nevertheless, soil extract samples were analysed with HPLC with co-chromatography with the reference standard R12. To corroborate the presence of metabolite M6 in the soil samples, a selected soil extract was subjected to TLC co-chromatography with the reference standards, including 2-sulphamoylbenzoic acid. The TLC analysis confirmed presence of an abundant metabolite, corresponding to the abundance of M6 that did not co-chromatograph with any of the available reference standards. In conclusion, following HPLC, TLC and LC-MS co-chromatography it could not be confirmed that metabolite M6 was 2-sulphamoylbenzoic acid.

**Conclusion**

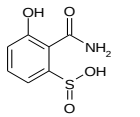
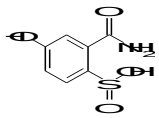
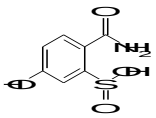
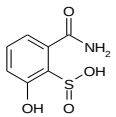
Results of HPLC, TLC and LC-MS co-chromatography of selected soil samples with reference standards including R12 (2-sulphamoylbenzoic acid) and additional MS experiments showed, that metabolite M6 could not be confirmed to be 2-sulphamoylbenzoic acid.

Within the original study (IES study # 20170175) the molecular weights and molecular formulae of M6 and M9 (probably transient metabolite of M6) were reported although the positions of oxidations could not be determined. However, based on the reported results, the likely structures of M6 are:



M6.1 M6.2 M6.3

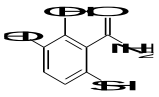
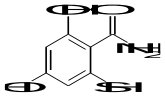
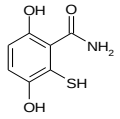
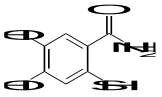
M6.4

M6.5 M6.6 M6.7

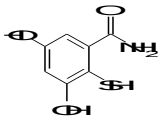
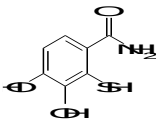
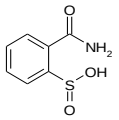
M6.8

Similarly, based on the total information available of M6 likely structures, the likely structures of M9 are:

M9.1 M9.2 M9.3

M9.4

M9.5 M9.6 M9.7

5.1.1 Reliability 1

5.1.2 Deficiencies No

| EVALUATION BY COMPETENT AUTHORITIES | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *17/02/20* |
| **Materials and Methods** | *Adopt applicant's version* |
| **Results and discussion** | *Adopt applicant's version* |
| **Conclusion** | *Adopt applicant's version* |
| **Reliability** | *1* |
| **Acceptability** | acceptable |

| | | |
|---|---|---|
| **Section A7**<br><br>**Subsection A7.2**<br>**Subsection A7.2.2**<br>**Annex point IIIA, XII.1.1** | **Ecotoxicological Profile Including Environmental Fate and Behaviour**<br>**Fate and Behaviour in Soil**<br>**Aerobic degradation in soil, further studies** | |
| | **JUSTIFICATION FOR NON-SUBMISSION OF DATA** | **Official use only** |
| **Other existing data [X]** | **Technically not feasible [ ] Scientifically unjustified [ ]** | |
| **Limited exposure [ X]** | **Other justification [ ]** | |
| **Detailed justification:** | The data from aqueous photolysis (7.1.1.1.2) and ready biodegradation (7.1.1.2.1) are sufficient to drive and as a result not critical to the risk assessment.<br>7.2.2.1: Aerobic degradation in soil, further studies<br>Based on the use pattern there is limited exposure to the soil. Thus this study will have a negligible impact on the environmental risk assessment. Also, based on available data, the half-life in soil will be significantly less than 21 days. Thus kinetics in additional soils will not be necessary.<br>7.2.2.2: Field soil dissipation and accumulation<br>Based on the use pattern there is limited exposure to the soil. Thus this study will have a negligible impact on the environmental risk assessment.<br>7.2.2.3: Extent and nature of bound residues.<br>Based on the use pattern there is limited exposure to the soil. Thus this study will have a negligible impact on the environmental risk assessment.<br>This data was collected in the soil metabolism that has been recently conducted.<br>7.2.2.4: Other soil degradation studies<br>Based on the use pattern there is limited exposure to the soil. Thus this study will have a negligible impact on the environmental risk assessment. | |
| **Undertaking of intended data submission []** | No study planned. | |
| | | |
| | | |
| | | |
| | **Evaluation by Competent Authorities** | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | *January 2011* | |

| | | |
|---|---|---|
| **Section A7**<br><br>**Subsection A7.2**<br>**Subsection A7.2.2**<br>**Annex point IIIA, XII.1.1** | **Ecotoxicological Profile Including Environmental Fate and Behaviour**<br>**Fate and Behaviour in Soil**<br>**Aerobic degradation in soil, further studies** | |
| **Evaluation of applicant's justification** | *Applicant's justification is accepted.* | |
| **Conclusion** | *Applicant's justification is accepted.* | |
| **Remarks** | | |

**Section A7.2.2.3/01** **Aerobic degradation in soil, further studies:**
**Annex Point IIIA, XII.1.4** **Extent and nature of bound residues**

| | |
|---|---|
| | • **REFERENCE** |
| **Reference** | [redacted] (2020a) 1,2-Benzisothiazol-3(2H)-one – Route and Rate of Degradation of [14C]-1,2-Benzisothiazol-3(2H)-one in Four Soils under Aerobic Conditions. [redacted], GLP, non-published, 20 December 2018, Amended 28 January 2020. |
| **Data protection** | Yes |
| • Data owner | Laboratorios Miret, S.A., LANXESS Deutschland GmbH, Lonza Ltd, Specialty Electronic Materials Switzerland GmbH (former The Dow Chemical Company), Thor GmbH, Troy Chemical Company BV |
| • Criteria for data protection | Data on existing a.s. submitted for first entry into the European list of approved biocidal active substance |
| | • **GUIDELINES AND QUALITY ASSURANCE** |
| **Guideline study** | Yes<br>(OECD Guideline 307 (Adopted 24th April 2002)<br>OPPTS 835.4100, US EPA, October 2008) |
| **GLP** | Yes |
| **Deviations** | No |
| | • **MATERIALS AND METHODS** |
| **Test material** | Test substance details are summarised below |
| • General information | 1,2-Benzisothiazol-3(2H)-one; CAS number: 2634-33-5; Molecular formula: $C_7H_5NOS$; Molecular weight: 151.19 g/mol |
| • Labelled test material (Lot/Batch number; purity) | 1,2-[ring-U-$^{14}$C]Benzisothiazol-3(2H)-one (thereafter referred to as [$^{14}$C]Benzisothiazolone); (CFQ43104; radiochemical purity: 96.6%) |
| • Unlabelled test material (Lot/Batch number; purity, description) | 1,2-Benzisothiazol-3(2H)-one; (MKBZ4459V; purity: 99.3%; white to yellow and faint beige to beige powder) |
| • Reference items | MET1 (R1): Hydroxy-1,2-benzisothiazolin-3-one<br>MET2 (R2): 1,2-Benzisothiazolin-3-one-1-oxide<br>MET3 (R3): Dihydroxy-1,2-benzisothiazolin-3-one<br>MET4 (R4): o-Sulphobenzamide (sodium salt)<br>MET7 (R7): N-(4-amino-4-hydroxy-buta-1,3-dienyl)-benzamide<br>Saccharin (R8): 1,2-Benzisothiazolin-3-one-1-dioxide<br>2-Sulphanylbenzamide (R9)<br>2-Sulphobenzoic acid hydrate (R11) |
| • Stability | Stability was determined before and after application. Test substance was stable |

**Section A7.2.2.3/01** **Aerobic degradation in soil, further studies:**
**Annex Point IIIA, XII.1.4** **Extent and nature of bound residues**

during the application procedure.

| | |
|---|---|
| **Test system** | Laboratory test |
| • Soil type | Four standard representative fresh field soils with a wide range of soil properties were used: Soil I: Speyer 2.4 (loam), Soil II: Speyer 5M (sandy loam), Soil III: RefeSol 02-A (silt loam) and Soil IV: RefeSol 04-A (loamy sand). |
| **Treatment and sampling** | Soil samples of 100 g (equivalent dry weight) were treated initial concentration of 0.5 mg per kg dry soil equivalent. Samples were incubated under aerobic conditions in the dark in an air-conditioned room at a temperature of 20.8 ± 0.2°C and 20.9 ± 0.2°C and a soil moisture content of pF 2. |
| **Extraction and analytics** | After extraction of soil samples with acetonitrile, acetonitrile/water (4:1, v:v), acetonitrile/water (1:1, v:v) and acetonitrile/0.1 hydrochloric acid (1:1, v:v), Soxhlet extraction using acetonitrile/water/32% hydrochloric acid (80:20:0.1, v:v:v) was performed. If non-extractable radioactivity is > 10% AR after Soxhlet extraction, additional harsh extraction with 0.1 M hydrochloric acid under reflux conditions followed by organic matter fractionation according to Stevenson (1982) was performed, to determine the amount of radioactivity in humin fractions and fulvic and humic acids. Extracts from harsh extractions were concentrated under reduced pressure in a rotary evaporator at about 30 °C. The concentrated extracts were measured by LSC for recovery and submitted for HPLC analysis. |

- **RESULTS**

| | |
|---|---|
| **Analytical results** | Non-extractable residues increased from 27.6, 10.3, 13.0 and 6.9% AR on 0.00 DAT to maximum levels of 52.0, 42.9, 44.6 and 45.6% AR on 56 DAT respectively for bioactive soils tested, and from 20.3, 5.8, 8.1 and 4.8% AR on 0.00 DAT to maximum levels of 40.7, 37.7, 36.4 and 47.0% AR on 13-28 DAT, respectively, for the four sterile soils tested. At the end of incubation, amounts were 48.6, 39.9, 43.2 and 41.9% AR respectively for the four bioactive soils and 36.2, 33.1, 31.3 and 41.8% AR, respectively, for the four sterile soils tested. Harsh extraction of bioactive soil samples from 56 DAT under reflux conditions further released 5.7, 3.7, 7.3 and 5.7 % AR from the soil matrix, proving that only small amounts might become bioavailable in addition. The HPLC analysis of the resulting extracts showed that they comprised of several discrete radio components, including parent and MET2. Benzisothiazolone was found at levels of ≤0.6% AR for all soils. The maximum level of any single degradate was ≤2.7% AR in all soils. Subsequent allocation of the non-extractable radioactivity to the organic matter fractions revealed that 8.0-12.7%, 2.1-14.8% and 4.7-32.3% AR were associated with the fulvic acid, humic acid and humin fractions, respectively. |

- **APPLICANT'S SUMMARY AND CONCLUSION**

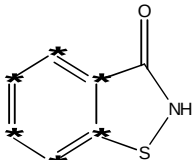
| | |
|---|---|
| **Materials and methods** | After incubation of treated soils samples, the soil samples were extracted four times at room temperature followed by Soxhlet extraction. If non-extractable radioactivity is > 10% AR after Soxhlet extraction, additional harsh extraction with 0.1 M hydrochloric acid under reflux conditions followed by organic matter fractionation according to Stevenson (1982) was performed. Extracts were |

**Section A7.2.2.3/01** **Aerobic degradation in soil, further studies:**
**Annex Point IIIA, XII.1.4** **Extent and nature of bound residues**

| | |
|---|---|
| | measured by LSC for recovery and submitted for HPLC analysis. |
| **Results and discussion** | Non-extractable residues remaining >10 % AR after Soxhlet extraction were further characterised. Harsh extraction under reflux conditions further released 5.7, 3.7, 7.3 and 5.7 % AR from the soil matrix. The HPLC analysis of the resulting extracts showed that they comprised of several discrete radio components, including parent (≤0.6% AR) and MET2. Subsequent allocation of the non-extractable radioactivity to the organic matter fractions revealed that 8.0-12.7%, 2.1-14.8% and 4.7-32.3% AR were associated with the fulvic acid, humic acid and humin fractions, respectively. |
| **Conclusion** | A fast degradation of [$^{14}$C]Benzisothiazolone in soil was observed. Bound residues were formed to maximum levels of 52.0, 42.9, 44.6 and 45.6% AR on 56 DAT respectively for bioactive soils tested, and to maximum levels of 40.7, 37.7, 36.4 and 47.0% AR on 13-28 DAT, respectively, for the four sterile soils tested. Harsh extraction further released 5.7, 3.7, 7.3 and 5.7 % AR from the soil matrix. Organic matter fractions revealed that 8.0-12.7%, 2.1-14.8% and 4.7-32.3% AR were associated with the fulvic acid, humic acid and humin fractions, respectively. |
| • Reliability | 1 |
| • Deficiencies | No |

| EVALUATION BY COMPETENT AUTHORITIES | |
|---|---|
| | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *18/2/20* |
| **Materials and Methods** | *Adopt applicant's version* |
| **Results and discussion** | *Adopt applicant's version* |
| **Conclusion** | *Adopt applicant's: A fast degradation of [14C]Benzisothiazolone in soil was observed. Bound residues were formed to maximum levels of 52.0, 42.9, 44.6 and 45.6% AR on 56 DAT respectively for bioactive soils tested, and to maximum levels of 40.7, 37.7, 36.4 and 47.0% AR on 13-28 DAT, respectively, for the four sterile soils tested. Harsh extraction further released 5.7, 3.7, 7.3 and 5.7 % AR from the soil matrix. Organic matter fractions revealed that 8.0 12.7%, 2.1-14.8% and 4.7-32.3% AR were associated with the fulvic acid, humic acid and humin fractions, respectively.* |
| **Reliability** | *1* |
| **Acceptability** | acceptable |
| **Remarks** | |
| | **COMMENTS FROM** |
| **Date** | *Give date of the comments submitted* |
| **Materials and Methods** | *Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion. Discuss if deviating from view of rapporteur member state* |
| **Results and discussion** | *Discuss if deviating from view of rapporteur member state* |
| **Conclusion** | *Discuss if deviating from view of rapporteur member state* |
| **Reliability** | *Discuss if deviating from view of rapporteur member state* |
| **Acceptability** | *Discuss if deviating from view of rapporteur member state* |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.2** | **Fate and Behaviour in Soil** |
| **Subsection A7.2.3** | **Adsorption and mobility in soil, further studies** |
| **Annex point IIIA XII, 1.2-1.3** | **Aged Column Leaching Study** |

| | | | Official use only |
|---|---|---|---|
| | | **1. REFERENCE** | |
| **1.1** | **Reference** | [redacted] (2008). *[$^{14}$C]-BIT: Aged Soil Leach*, [redacted] and Rohm and Haas Technical Report No. TR-08-067, 5 March 2009. Unpublished | |
| **1.2** | **Data Protection** | Yes | |
| 1.2.1 | Data Owner | Rohm and Haas Company, Philadelphia, PA USA | |
| 1.2.2 | Criteria for data protection | Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I/IA.<br>Data protection claimed in accordance with Article 12.1(c) (ii), as data generated after the entry into force of the Directive. | |
| | | **2. GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **Guideline Study** | Yes; OECD Guideline 312, Leaching in Soil Columns (April 2004) | |
| **2.2** | **GLP** | Yes | |
| **2.3** | **Deviations** | No | |
| | | **3. MATERIAL AND METHODS** | |
| **3.1** | **Test Material** | $^{14}$C-BIT (1,2-benzisothiazolin-3-one)<br>  <br><br>* position of the $^{14}$C-label | |
| 3.1.1 | Lot/Batch number | Lot 1069.00 (sublot 1069.0005) | |
| 3.1.2 | Specification | The test material used in this study is not as specified in section 2. As specified in the study guidelines, $^{14}$C -material was employed. Specifications for the $^{14}$C-material are listed in section 3.1. | |
| 3.1.3 | Purity | Radiopurity > 98% | |

| | | |
|---|---|---|
| 3.1.4 | Further relevant properties | • Soil adsorption $K_f$ = 55.6<br>• Water solubility (deionized water) > 0.7 g/L<br>• Half-life in aerobic soil simulation study is 5.6 hours at 20°C |
| **3.2** | **Reference substance** | No reference substances were employed to validate the study. The following compounds were used as chromatography standards.<br>2,3-dihydroxybenzoic acid, Lot 09026KB, Purity: 99.9%<br>Benzene sulfonamide, Lot 14024BB, Purity: 99.0%<br>Catechol, Lot 03812AD, Purity: 99.2%<br>2-sulfobenzoic acid, Lot 15101MB, Purity: 75.4%<br>Saccharin, Lot 11330EA-385, Purity: 99% |
| **3.3** | **Soil Type** | The soil used for both aging BIT and in the soil column was a sandy loam soil from Baylham, Ipswich, UK. The physical and chemical characteristics of the soils appear in Table A7.2.3.1-1. The soil was collected shortly before testing was initiated and divided into two batches;<br>1)passed through at 2 mm sieve with a minimum of air drying and<br>2) air dried and passed through a 1 mm sieve. Prior to use the soil was stored at 4 ± 2°C. |
| **3.4** | **Testing procedure** | |
| 3.4.1 | Test system/ conditions | <u>Method Development</u><br>To six incubation flasks, 100 g (dry weight) of sandy loam soil was added. Moistened air was drawn through the soil flasks and a series of volatiles traps. The trap closest to the soil flask was empty to capture any back flow, the second contained ethanediol (trap polar organics), the third contained 2% paraffin in xylene (trap nonpolar organics), and the final two contained 2M NaOH traps (trap $CO_2$). The flasks were maintained at 20 ± 2°C and a single flask was removed at 0, 2, 4, and 6 hours. The soil was extracted three times (15 minutes each time) with 100 ml each time of acetonitrile:1% ammonia (1:1) and centrifuged (10 minutes). The combined extracts were radioassayed and an aliquot removed for immediate chromatographic analysis. Aliquots of the volatile traps were radioassayed.<br>A second study using only two flasks was conducted as described above except the extraction and centrifugation periods were shorten to 10 and 5 minutes respectively. Immediately after dosing, the soil was extracted and one of the combined extracts was neutralized with |

formic acid. The extracts were radioassayed and aliquots removed for immediate chromatographic analaysis.

Definitive Soil Aging

For each soil column, 3 flasks were prepared as described in the method development experiments (6 flasks total). One flask was removed immediately after dosing (Time 0) and extracted with three times for 10 minutes with 100 ml of acetonitrile:1% ammonia. The extract was centrifuged, radioassayed, chromatographed, and the volatile traps radioassayed. The two remaining flasks were removed about 6 hours after dosing. One was treated as the Time 0 trap while the aged soil in the other flasks was transferred to the top of a prepared soil column. The soil was pressed in place and a filter paper placed on top and leaching initiated by the addition of 0.01M $CaCl_2$ solution.

Column Leach

Two soil columns were prepared containing sandy loam soil by fixing together 6 glass rings (5cm id x 6 cm high) and attaching them to a conical funnel which was plugged with glass wool and acid washed sand. Air dried soil was added and packing assisted by vibration to achieve a 30 cm leach column. The column was wrapped in aluminum foil to exclude light and stored at 20 ± 2°C. After adding the soil to the top of the column, approximately 393 ml of 0.01M $CaCl_2$ solution was added over 48 hours. The leachate was collected in a glass jar.

3.4.2 Preparation of test solution

A $^{14}$C-BIT stock solution was prepard by dissolving 18.9 mg in 9 mL of acetonitrile. Based on radioassay the concentration was 2.046mg/ml.

The application rate was 5µg/g soil (or 500 µg.100 g soil). The dosing solution was dispensed dropwise over the soil surface and the solvent allowed to evaporate prior to the test substance being mixed into the soil by hand rotating the flask. The application was as tabulated below.

| Test Procedure | No. of Flask | Volume of Stock Solution (µl) | Weight of Test Substance (µg) | Radioactivity (kBq) |
|---|---|---|---|---|
| Method Development Test 1 | 5 | 245 | 501 | 993.6 |
| Method Development Test 1 | 1 | 240 | 504 | 998.0 |
| Method Development Test 2 | 2 | 240 | 504 | 998.0 |
| Definitive Test 1 | 3 | 240 | 503 | 996.4 |
| Definitive Test 2 | 3 | 240 | 505 | 1001 |

3.4.3 Duration of test and sampling intervals

First Method Development Test: Samples were removed at 0, 2, 4, and 6 hours

Second Method Development Test: Duplicate samples were removed at Time 0.

Definitive Soil Aging: Sampling was at Time 0 and Hour 6.

Column Leach: It took approximately 48 hours for 393 ml of 0.01M $CaCl_2$ solution to be eluted.

3.4.4 Replicates

First Method Development Test: Single samples at each time interval.

Definitive Test: Duplicate soil samples at Hours 0 and 6 and duplicate soil columns.

| | |
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| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.2** | **Fate and Behaviour in Soil** |
| **Subsection A7.2.3** | **Adsorption and mobility in soil, further studies** |
| **Annex point IIIA XII, 1.2-1.3** | **Aged Column Leaching Study** |

| | | | |
|---|---|---|---|
| 3.4.5 | Sampling and extraction details | Method Development: Flasks were removed and immediately extracted for 15 minutes three times with 100 ml of acetonitrile:1% ammonia (1:1). The resulting mixture was centrifuged and the supernatant removed. The combined supernatant extracts were radioassayed. An aliquot was also removed for immediate chromatographic analysis. The remaining solid residue was air-dried, homogenized, and aliquots combusted in an oxidizer prior to radioassay. In a repeat test, the extraction time was shortened to 10 minutes and centrifugation time to 5 minutes. Aliquots were removed from traps at sample intervals and radioassayed.<br><br>Definitive test: Soil was extracted similar to that describe for the method development test using three 10 minute acetonitrile:1% ammonia extraction and a 5 minute centrifugation. Head space of extracts and post extraction solids were sparged with nitrogen to minimize oxidation.<br><br>The column leachate was collected and radioassayed. At completion, an aliquot was removed for immediate chromatographic analysis.<br><br>The combined extracts that contained ≥ 5% of the applied activity were analyzed by HPLC and compared to standards listed in section 3.2 above. BIT was confirmed by HPLC and TLC cochromatography with a $^{12}$C-standard. Selected extracts were analyzed by LC-MS to identify the metabolites in both the soil and the leachate. | |
| 3.4.6 | Bound residues—extent and nature | The two top segments from each column were further extracted for bound residues using 0.5M NaOH. After 24 hrs the sample was centrifuged and the solid humin fraction was washed with an additional 25 mL of NaOH. The combined supernatant fraction was acidified with HCl (~pH1). The resulting supernatant, fulvic acid fraction, was radioassayed. The precipitate resulting from acidification, humic acid fraction, was redissolved in 0.5M NaOH and radioassayed. The remaining insoluble matrix, humin, was radioassayed by combustion. | |

| | | | |
|---|---|---|---|
| 3.4.7 | Analytical Methods | Soil extracts were initially chromatographed by reversed phase HPLC using a Restek Ultra Aqueous C-18 column. The mobile phase consisted of a gradient of 0.5% Formic acid in water and 0.5% formic acid in methanol. A second system was employed in an attempt to separate the Polar Material using an amino column and a gradient of water and 50 nM ammonium acetate. Radioactivity was monitored with a flow through radioactivity monitor and UV at 254 nm.<br><br>TLC was initially performed on silica gel plates using ethyl acetate: methanol:acetonitrile:acetic acid (90:5:5:1, v/v/v/v) as the eluant. A second solvent system, ethyl acetate:acetic acid:methanol:water (60:15:15:10, v/v/v/v) was also employed. Radioactivity was imaged using a phosphorimager and non-radioactive standards using a 254 nm UV light.<br><br>Liquid scintillation spectrometry was performed using Packard liquid scintillation spectrometers. Radiocombustion was performed in a Harvey Biological Sample Oxidizer and subsequently quantitated by liquid scintillation spectrometry.<br><br>For metabolite identification, accurate masses were obtained using an LC-Fourier Transform MS. Two systems were employed:<br><br>1) Restek Ultra Aqueous C-18 with a gradient of 0.5% formic acid and methanol with 0.5% formic acid and<br><br>2) an Econosphere Amino column and a gradient consisting of methanol:water (1:1) and methanol: 50 nM ammonium acetate (1:1). The LC effluent was introduced into the MS via an API interface and both positive and negative ionization was employed. | |
| 3.4.8 | Degradation Products | The representative soil extracts and leachates listed below were analyzed by LC-MS:<br><br>• Column A5, Leachate (Polar Material/Unknown 3)<br>• Flask A3, Solvent Extract from the 6 hour aged soil (Polar Material and Unknown 2)<br>• Column A6, Segment 5, Soil extract (Polar Material)<br><br>An aliquot of the above was reduced to dryness on a rotary evaporator, reconstituted in acetonitrile:water (1:3), and centrifuged prior to LC-MS analysis. | |
| | | **4. RESULTS** | |
| **4.1** | **Radiochemical purity** | Prior to commencing the method development studies the radiopurity was determined by HPLC and TLC and found to be greater than 99% | |

| | | | |
|---|---|---|---|
| **4.2** | **Method Development** | The method development tests were performed to examine the extraction and chromatography methods and to estimate the half-life of BIT in sandy loam soil. It was found that a 10 minute extraction period was sufficient and that neutralization of the basic extract reduced degradation but since there was still degradation, samples were chromatographed immediately. The procedure yielded good extractability of Time 0 samples with recoveries being greater than 91%.<br><br>The distribution of radioactivity from the flasks dosed for 0, 2, 4, and 6 hours is presented in Table A7.2.3.1-2. Recovery of applied $^{14}$C-activity ranged from 93.1% to 102.7% (average; 97.9 ± 4.6%). Solvent extractability of $^{14}$C-activity decreased from 93.1% at 0 hours to 83.9% at 6 h (however, the 2 hr interval extractability was 74.7%). There was no detectable activity in the traps. Therefore traps were not necessary during the column leach period.<br><br>The results from the chromatographic quantitation are presented in Table A7.2.3.1-3. BIT decreased from 86.5% of the applied activity at 0 hours to 44.7% at 6 hours. After 6 hours, Polar Material was present at 9%, Unknown 1 at 21.2%, and Unknown 2 at 7.7%. Unknown 3 was present at less than 1% of the applied activity after 6 hours.<br><br>The half-life of $^{14}$C-BIT in the soil was 6.3 hours. | |
| **4.3** | **Definitive Test** | | |
| 4.3.1 | Distribution and recovery of radioactivity | The distribution of radioactivity in soil where BIT has been aged for 0 hrs and 6 hrs is presented in Table A7.2.3.1-4. The $^{14}$C-activity extracted from soil decreased during the 6 hours from 89% to 83% while the bound residue increased from 6.8% to 14.4%. There were no volatiles detected after 6 hours. Recovery of applied radioactivity was 97.0 ± 0.5%<br><br>The distribution and recovery of applied radioactivity in the soil columns is presented in Table A7.2.3.1-5. The total activity detected in the soil segments averaged 77.8% of the applied activity while 17.6% leached entirely through the column. The percent of applied activity decreased rapidly with increasing column depth with over 50% in the top two segments. Combining all the soil segments, about 50% of the total was extractable and 50% unextractable. However, with increasing depth (and thus decreasing $^{14}$C-activity) the percentage of unextractable became significantly less than extractable. The recovery of total applied radioactivity (soil segments and leachate) averaged 95.4%. | |

4.3.2 Characterization and quantitation of $^{14}C$-activity

The percent of BIT and metabolites detected in soil after aging for 6 hours is presented in Table A7.2.3.1-6. At Time 0, BIT accounted for 84% of the applied activity but decreased to 49% after 6 hours and there were 2 metabolites (Polar Material and Unknown 2) detected as tabulated below.

| Sample Interval (h) | Percent of Applied | | |
|---|---|---|---|
| | BIT | Unknown 2 | Polar Material |
| 0 | 84.0 | 5.5 | < 0.1 |
| 6 | 49.0 | 30.5 | 3.0 |

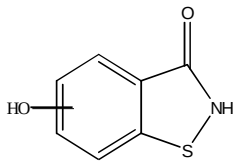
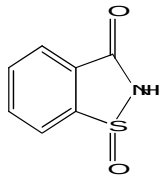
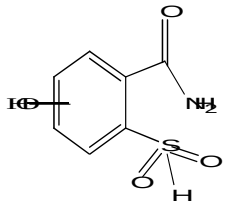
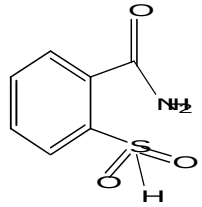
In the soil column segments and leachate (Table A7.2.3.1-6), BIT and 5 metabolites were detected. However, Unknown 1 and Unknown 4 were present at less than 2% of the applied activity. Subsequent analysis showed that Unknown 3 and Polar Material were the same compounds. The results are summarized below.

| Fraction | Percent of Applied | | |
|---|---|---|---|
| | BIT | Unknown 2 | Polar Material/ Unknown 3 |
| Segments | 13.8 | 7.4 | 9.4 |
| Leachate | < 0.1 | < 0.1 | 16.4 |
| Total | 13.8 | 7.4 | 25.7 |

BIT was only detected in the soil segments, not the leachate. This indicates that probably as a result of biodegradation BIT will not significantly leach in the environment and therefore is unlikely to persist in groundwater.

4.3.3 Identification of metabolites

Unknown 2 and the Polar Material (including Unknown 3) were the only metabolites greater than 5% of the applied activity. There structures were obtained by LC-MS.

| Designation | Structure/Name |
|---|---|
| Unknown 2 |  <br>Hydroxy-1,2-benzisothiazolin-3-one |
| | OR |
| |  <br>1,2-benzisothiazolin-3-one-1-oxide |
| Polar Material/ Unknown 3 ( 2 metabolites) |  <br>Hydroxy-2-sulfonylbenzamide |
| |  <br>2-sulfonylbenzamide |

| | | |
|---|---|---|
| 4.3.4 | Extent and nature of bound residue | The bound $^{14}$C-residue remaining in the top segment from each column after extensive extraction with acetonitrile:1% ammonia was subjected a 24 hour extraction with NaOH. The results are presented in Table A7.2.3.1-7. After extraction with NaOH, the acid soluble fraction, the fulvic acid fraction, comprised about 6% of the applied radioactivity while the acid insoluble fraction, the humic acid fraction, comprised about 9%. The base insoluble fraction (humin) comprised 10.6% of the applied activity. |
| 4.3.5 | Metabolic Pathway | Metabolic pathway is presented in Figure A7.2.3.1-1. |

**5. APPLICANTS SUMMARY AND CONCLUSION**

**5.1 Material and methods**

The test guidelines were OECD Guideline 312, Leaching in Soil Columns.

A preliminary study was performed to determine the half-life and time necessary to aged BIT in soil.

In the definitive study, 100 g soil (dry weight) was added to flasks and they were dosed at 5 ppm $^{14}$C-BIT. Volatiles were trapped and the system was maintained at 20°C. At time 0 and 6 h, flask were removed, extracted with acetonitrile:1% ammonia and the extract and remaining soil residue radioassayed. An aliquot of the extract was immediately chromatographed (HPLC). Volatile traps were radioassayed. After 6 h of aging BIT in soil, additional flask were removed and added to the top of soil columns.

Leaching columns were prepared by placing sieved sandy loam soil into duplicate glass segmented (5 cm id x 6 cm height) columns with a funnel attached at the bottom containing a glass fiber plug and sand. The final length of the soil column was about 30 cm. he soils were wetted with 0.01M $CaCl_2$. Sandy loam soil containing aged $^{14}$C-BIT was placed atop the columns and approximately 393 mL of 0.01M $CaCl_2$ was added over a 48 hour period and the leachate collected. At the conclusion, the soil column was separated into segments (5 column segments, 1 aged soil segment, and sand from the funnel). The leachate and soil segments were radioassayed and an aliquot of the leachate chromatographed (HPLC). Soil was extracted with acetonitrile:1% ammonia and radioassayed. A aliquot of the extract was immediately chromatographed (HPLC). Selected extracts were further analyzed by LC-MS to identify metabolites. Bound residue was extracted with NaOH and fractionated into fulvic acid, humic acid, and humin.

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.2** | **Fate and Behaviour in Soil** |
| **Subsection A7.2.3** | **Adsorption and mobility in soil, further studies** |
| **Annex point IIIA XII, 1.2-1.3** | **Aged Column Leaching Study** |

| | | | |
|---|---|---|---|
| **5.2** | **Results** | The measured half-life in soil was about 6 hours.<br><br>Most of the applied activity did not leach through the column; about 18% of the applied activity in the leachate and 78% in the column soil. The top two segments (aged soil and first segment) contained about 60% of the applied activity demonstrating that activity decreased with increasing column depth and there is limited leaching of the applied radioactivity.<br><br>At the end of the leach period the top segment (applied aged soil segment) contained 11.1% of the applied activity detected as BIT and the remaining 5 segments a total of 2.7%, whereas there was no BIT in the leachate. This indicates that BIT will not leach appreciably and should not be persistent in the environment.<br><br>Two metabolites were detected at greater than 5% of the applied activity. Unknown 2 (identified by LC-MS either hydroxy-1,2-benzisothiazolin-3-one or 1,2-benzisothiazolin-3-one-1-oxide comprised) 7.4% of the applied activity in the total soil segments but was not detected in the leachate. Polar Material/Unknown 3 (identified by LC-MS as two compounds; hydroxy-2-sulfonyl-benzamide and 2 sulfonyl-benzamide) comprised 9.4% in the soil segments and 16.8% in the leachate for a total of 25.7% of the applied activity. The results suggest that Unknown 2 and BIT degraded to Polar Metabolites/Unknown 3. | |
| **5.3** | **Conclusion** | This study confirms the quick biodegradation of BIT in soil and the resulting fast half-life (ca. 6 h). Probably due to biodegradation, BIT shows limited mobility with within soil and should not be persistent in ground water. Two major metabolites were detected. The major metabolic reactions were oxidation of either the benzene ring or the sulfur and cleavage of the N-S isothizolone bond. | |
| **5.4** | **Reliability** | 1 | |
| **5.5** | **Deficiencies** | None | |
| | | | |

| | | |
|---|---|---|
| | **Evaluation by Competent Authorities** | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | *May 2013* | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.2** | **Fate and Behaviour in Soil** |
| **Subsection A7.2.3** | **Adsorption and mobility in soil, further studies** |
| **Annex point IIIA XII, 1.2-1.3** | **Aged Column Leaching Study** |

| | | |
|---|---|---|
| **Materials and Methods** | *Applicant's version is adopted, although the following deficiencies were detected:*<br>▪ *Only one type of soil was used, while the OECD 312 guideline recommends four soil types.*<br>▪ *No reference substances were employed to validate the study* | |
| **Results and discussion** | *Applicant's version is adopted with the following information.*<br>*For ionisable test substances the selected soils should cover a wide range of pH, in order to evaluate the mobility of the substance in its ionized and unionized forms.* | |
| **Conclusion** | *The transformation products are important and studies with more soil type is necessary. However, this study can be used as additional information.* | |
| **Reliability** | *3* | |
| **Acceptability** | *Acceptable as additional information.* | |
| **Remarks** | | |
| **Remarks** | | |

**Table A7.2.1-1: Physical and Chemical Properties of Sandy Loam Soil**

| Characteristic | Sandy Loam |
|---|---|
| Percent Sand | 70 |
| Percent Silt | 16 |
| Percent Clay | 14 |
| Percent Organic Matter | 2.8 |
| Cation Exchange Capacity (meq/100 g) | 15.4 |
| pH | 4.9 |
| Percent Moisture (1/3 bar) | 11.4 |
| Bulk Density (g/cc) | 1.1 |

**Table 7.2.3.1-2: Method Development Test: Distribution of Applied Radioactivity**

| Sample Interval | Percent of Applied | | | | |
|---|---|---|---|---|---|
| | Soil Extract | Unextracted from Soil | Total in Soil | Total in Traps | Recovery |
| 0 | 93.1 | NA[1] | 93.1 | NA | 93.1 |
| 2 | 74.7 | 28.0 | 102.7 | ND[2] | 102.7 |
| 4 | 87.3 | 7.5 | 94.8 | ND | 94.8 |
| 6 | 83.9 | 16.9 | 100.8 | ND | 100.8 |

[1] NA = Not Applicable [2] ND = Not Deteccted (<0.1%)

**Table 7.2.3.1-3: Method Development Test: Quantitation of Parent and Metabolites in Soil Extract**

| Sample Interval | Percent of Applied | | | | |
|---|---|---|---|---|---|
| | BIT | Polar Material | Unknown 1 | Unknown 2 | Unknown 3 |
| 0 | 86.5 | ND[1] | 4.6 | 1.3 | ND |
| 2 | 54.2 | 2.1 | 13.1 | 5.0 | ND |
| 4 | 55.2 | 3.2 | 20.4 | 7.7 | ND |
| 6 | 44.7 | 9.0 | 21.2 | 7.7 | 0.5 |

1 ND = Not Dected (< 0.1%)

**Table A7.2.3.1-4: Definitive Study: Distribution of Radioactivity in Aged Soil.**

| Sample Interval | Percent of Applied Radioactivity[1] | | | | |
|---|---|---|---|---|---|
| | Soil Extract | Unextracted from Soil | Total in Soil | Total Trapped Volatiles | Recovery |
| 0 | 89.9 | 6.8 | 96.6 | NA[2] | 96.6 |
| 6 | 83.0 | 14.4 | 97.3 | ND[3] | 97.3 |

[1] Average of duplicate flasks

[2] NA = Not Applicable

[3] ND = Not Detected (< 0.1%)

**Table A7.2.3.1-5: Definitive Study: Distribution of Radioactivity in Soil Columns**

| Segment | Soil Extract | | | Unextracted from Soil | | | Total | | |
|---|---|---|---|---|---|---|---|---|---|
| | Col 1 | Col 2 | Average | Col 1 | Col 2 | Average | Col 1 | Col 2 | Average |
| Leachate | 18.1 | 17.0 | 17.6 | NA[1] | NA | | 18.1 | 17.0 | 17.6 |
| Segment 1 | 17.6 | 16.9 | 17.3 | 29.2 | 27.7 | 28.5 | 46.8 | 44.6 | 45.7 |
| Segment 2 | 5.5 | 6.2 | 5.9 | 5.3 | 8.1 | 6.7 | 10.8 | 14.3 | 12.6 |
| Segment 3 | 4.7 | 4.9 | 4.8 | 1.7 | 2.3 | 2.0 | 6.4 | 7.2 | 6.8 |
| Segment 4 | 4.3 | 5.1 | 4.7 | 0.7 | 0.8 | 0.8 | 5.0 | 5.9 | 5.5 |
| Segment 5 | 3.0 | 3.1 | 3.1 | 0.3 | 0.3 | 0.3 | 3.3 | 3.4 | 3.4 |
| Segment 6 | 2.6 | 1.9 | 2.3 | 0.2 | 0.1 | 0.2 | 2.8 | 2.0 | 2.4 |
| Sand Segment | 1.6 | 1.4 | 1.5 | ND[2] | 0.1 | 0.1 | 1.6 | 1.5 | 1.6 |
| Total Activity in Column Segments | | | | | | | 76.7 | 78.9 | 77.8±1.6 |
| Total Activity in Leachate | | | | | | | 18.1 | 17.0 | 17.6±0.8 |
| Total Activity Column Segments and Leachate | | | | | | | 94.8 | 95.9 | 95.4±0.8 |

[1] NA = Not Applicable [2] ND = Not Detected (< 0.1%)

**Table A7.2.3.1-6: Definitive Study: Quantitation of Parent and Metabolites in Soil Extracts and Leachate**

| Segment | Percent of Applied[1] | | | | | |
|---|---|---|---|---|---|---|
| | BIT | Polar Material | Unknown 1 | Unknown 2 | Unknown 3 | Unknown 4 |
| Soil Aged 0 hrs | 84.0 | ND | ND | 5.5 | ND | ND |
| Soil Aged 6 hrs | 49.0 | 3.0 | ND | 30.5 | ND | ND |
| Segment 1 | 11.1 | 1.1 | ND | 1.8 | ND | ND |
| Segment 2 | 1.3 | 1.6 | ND | 1.6 | ND | ND |
| Segment 3 | 0.6 | 1.5 | 0.3 | 2.1 | ND | ND |
| Segment 4 | 0.5 | 1.8 | 0.6 | 0.9 | ND | ND |
| Segment 5 | 0.1 | 2.4 | 0.2 | 0.4 | ND | ND |
| Segment 6 | 0.2 | 1.1 | 0.1 | 0.6 | ND | ND |
| Total, Segments | 13.8 | 9.4 | 1.1 | 7.4 | ND | ND |
| Leachate | ND[2] | 1.8 | ND | ND | 14.6 | 1.0 |
| Total, Column | 13.8 | 11.1 | 1.1 | 7.4 | 14.6 | 1.0 |

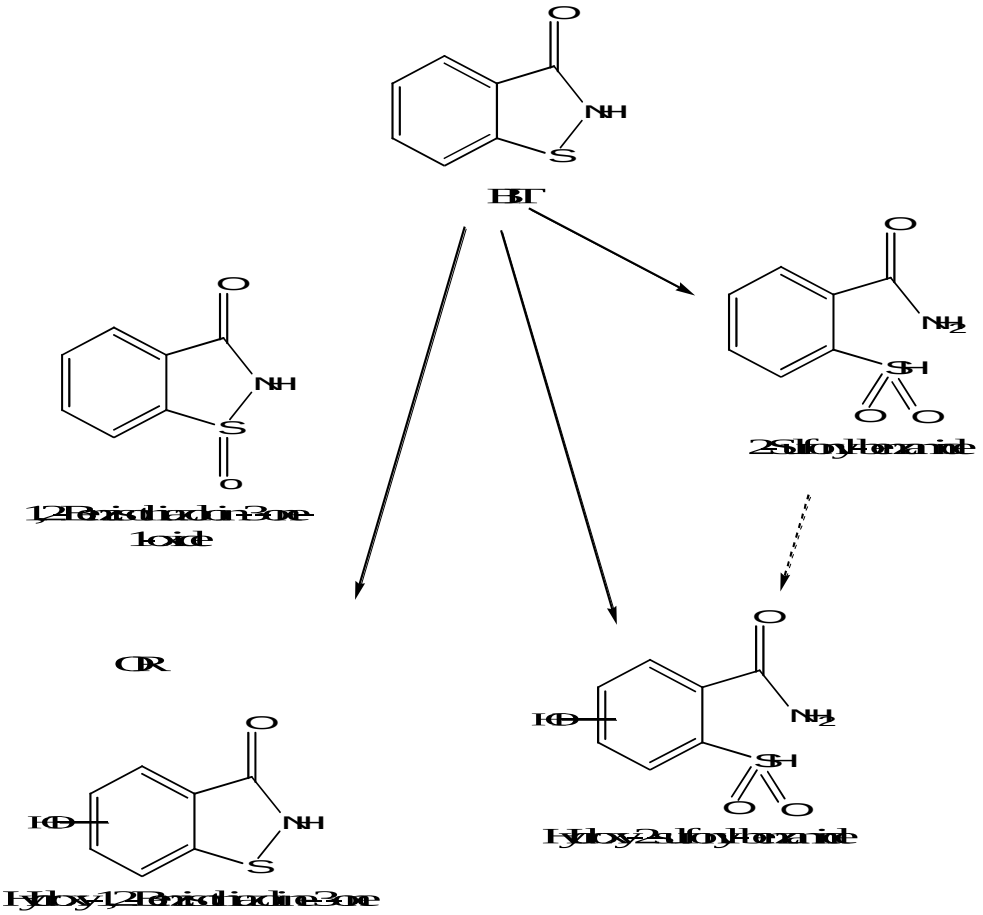
[1] Average of duplicate columns

[2] ND = Not Detected (<0.1%)

**Table A7.2.3.1-7: Definitive Study: Extend and Nature of Bound Residues**

| Column | Segment | Percent of Applied Activity | | | | | |
|---|---|---|---|---|---|---|---|
| | | Initial Residue | Bound Residue Extract | Fulvic Acid | Humic Acid | Humin | Total |
| 1 | 1 | 29.2 | 18.2 | 5.9 | 9.2 | 10.9 | 26.1 |
| 2 | 1 | 27.7 | 17.7 | 5.7 | 8.2 | 10.3 | 24.2 |
| Average | | 28.5 | 18.0 | 5.8 | 8.8 | 10.6 | 25.2 |

**Figure A7.2.1-2: Metabolic Pathway for BIT in Aged Sandy Loam Soil**



**Section A7** **Ecotoxicological Profile Including Environmental Fate and Behaviour**

**Subsection A7.3** **Fate and Behaviour in Air**

**Subsection A7.3.1** **Phototransformation in air**

**Annex Point IIIA, VII.5**

| | | Official use only |
|---|---|---|
| | **1 REFERENCE** | |
| **1.1 Reference** | **A7.3.1/01** [redacted] (2007) Calculation of Tropospheric Phototransformation of 1,2-Benzisothiazolin-3-one, Rohm and Haas Company, Rohm and Haas Technical Report N° TR-07-003 (April 19, 2007), Unpublished. | |
| **1.2 Data protection** | Yes | |
| 1.2.1 Data owner | Rohm and Haas Company | |
| 1.2.2 Companies with letter of access | | |
| 1.2.3 Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into Annex I/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1 Guideline study** | Yes-Technical Guidance Document, Chapter 3, Section 7.3.1 | |
| **2.2 GLP** | Not Applicable (This is a calculation method and not a laboratory experiment) | |
| **2.3 Deviations** | None | |
| | **3 MATERIALS AND METHODS** | |
| **3.1 Test material** | BIT (1,2-Benzisothiazolin-3-one) | |
| 3.1.1 Lot/Batch number | Not applicable | |
| 3.1.2 Specification | Not applicable | |
| 3.1.3 Purity | Not applicable | |
| 3.1.4 Radiolabeling | Not applicable | |
| 3.1.5 UV/VIS absorption spectra and value | Not applicable | |
| 3.1.6 Further relevant properties | Vapor Pressure at 25°C: 2.3 x $10^{-4}$ Pa<br>Octanol:Water Partition Coefficient: 15.4 (pH = 7)<br>Solubility in Water: 1.15 g/L at pH 7 and 20°C<br>Aqueous Photolytic half-life: < 9 hours | |

| | | 1 REFERENCE | Official use only |
|---|---|---|---|
| **3.2** | **Reference** | Environment Monograph. Application of Structure-Activity Relationships to the Estimation of Properties Important in Exposure Assessment. No 67. Environment Directorate, Paris, 1993. | |
| **3.3** | **Test solution** | Not applicable | |
| **3.4** | **Testing procedure** | As described in the Technical Guidance Document, Chapter 3, section 7.3.1, a first approach to the phototransformation of a biocide in air is to determine the first order degradation rate constant by Structure-Activity Relationship (SAR) methods.<br><br>SAR recognizes that organic compounds emitted into the troposphere are mainly removed by reactions with OH radicals during the daylight hours and $NO_3$ radicals during night.<br><br>SAR utilizes the fact that a number of separate OH radical reactions occur and that they can be dealt with individually in terms of the rate constant, $k_{OH}$, including: a) hydrogen atom abstraction from C-H bonds in alkanes, carbonyls, and other saturated organics; b) addition to >C=C< and -C≡C- unsaturated bonds; c) addition to aromatic rings; and d) interaction with –$NH_2$, >NH, >N-, -SH, and –S- groups) *i.e.*:<br><br>$k_{OH}$ = k(hydrogen atom abstraction from C-H bonds)<br>+ k(radical addition to >C=C< and -C≡C- bonds)<br>+ k(radical addition to aromatic rings)<br>+ k(radical interaction with –$NH_2$, >NH, >N-, -SH, -S-)<br><br>Since little is known about the reaction mechanism of $NO_3$ radicals with organic compounds and no database for $NO_3$ radical reactions is available, the rate constant $k_{NO3}$ is estimated by correlations between $k_{NO3}$ and $k_{OH}$, *i.e.*:<br><br>$-\log k_{NO3} = -18.86 + 3.05 \times (-\log k_{OH})$<br><br>SAR calculates phototransformation half-life of a specific organic compound ($t_{1/2}$) based on its phototransformation rate constant k and the concentration of OH and $NO_3$ radicals in the troposphere, i.e.:<br><br>$t_{½} = \ln 2/(k [C])$<br><br>Where k is the phototransformation rate constant and [C] is the concentration of the radicals in the troposphere such as OH and $NO_3$. | |
| 3.4.1 | Test system | Not applicable | |
| 3.4.2 | Properties of light source | Not applicable | |
| 3.4.3 | Determination of | Not applicable | |

| | 1 REFERENCE | Official use only |
|---|---|---|
| irradiance | | |
| 3.4.4 Temperature | Not applicable | |
| 3.4.5 pH | Not applicable | |
| 3.4.6 Duration of test | Not applicable | |
| 3.4.7 Number of replicate | Not applicable | |
| 3.4.8 Sampling | Not applicable | |
| 3.4.9 Analytical method | Not applicable | |
| **3.5 Transformation products** | Potential phototransformation products are hypothesized based on previously conducted environmental fate studies, *i.e.* aqueous photolysis, hydrolysis, and water/soil metabolism:<br>$H_2NC(O)PhSH$<br>$(H_2NC(O)PhS)_2$<br>HSPhCOOH<br>$H_2NC(O)PhSO_3H$<br>$H_2NSO_2PhCOOH$<br>$HSO_3PhCOOH$<br>$HSO_3Ph(OH)OH$<br>HOPh(OH)COOH<br>HOPhOH<br>$HOOCCH_2CHCHC(O)COOH$<br>where Ph = phenyl ring | |
| 3.5.1 Method of analysis for transformation procedure | Same as that of the parent (see section 3.4). | |
| | **4 RESULTS** | |
| **4.1 CMIT** | | |
| 4.1.1 $K_{OH}$ | The first order degradation rate constant ($k_{OH}$) from $OH^-$ radicals is calculated as the sum of bond $k_{OH}$'s. This is presented in Table A7.3.1-1. The $k_{OH}$ for BIT is $287.47 \times 10^{-13}$ $cm^3$. $molecule^{-1}$. $sec^{-1}$. | |

| | | 1 REFERENCE | Official use only |
|---|---|---|---|
| 4.1.2 | Half-life ($OH^•$) | The half-life due to the hydroxyl radical is determined as follows:<br>$t_{½} = ln2/(k_{OH}) \times [OH]$<br>$= 0.693/(287.47 \times 10^{-13}$ $cm^3$-molec.$^{-1}$-sec$^{-1}$ $\times 6.5 \times 10^5$ molecule.$cm^{-3}$)<br>$= 3.71 \times 10^4$ sec<br>= 10.3 hours | |
| 4.1.3 | $K_{NO3}$ | The first order degradation rate constant ($k_{NO3}$) from $NO_3^•$ radicals is determined as follows:<br>$-\log k_{NO3} = -18.86 + 3.05 \times (-\log k_{OH})$<br>$= -18.86 + 3.05 \times (-\log 287.47 \times 10^{-13})$<br>$= -18,86 + 3.05 \times (10.541)$<br>$= -13.291$<br>$k_{NO3}$ = antilog (-13.291)<br>$= 0.512 \times 10^{-13}$ $cm^3$.molecule$^{-1}$.sec$^{-1}$ | |
| 4.1.4 | Half-life ($NO_3^•$) | The half-life due to the nitrate radical is calculated similarly to the hydroxyl (described above) and is 15.7 hours. | |
| **4.2 Transformations products** | | | |
| 4.2.1 | $K_{OH}$ | The first order degradation rate constant ($k_{OH}$) from $OH^•$ radicals for the potential transformation products is presented in Table A7.3.1-2 | |
| 4.2.2 | Half-life ($OH^•$) | The half-life of the potential transformation products due to the hydroxyl radical is presented in Table A7.3.1-2. The half-lives range from 5.2-237.1 hours. | |
| | | **5 APPLICANT'S SUMMARY AND CONCLUSION** | |
| **5.1 Materials and methods** | | The guideline followed is that described in the Technical Guidance Document, Chapter 3, Section 7.3.1<br>The phototransformation rate constant of BIT is calculated using SAR method.<br>Global average OH and $NO_3$ radical concentrations in daylight and night hours are used.<br>Potential phototransformation products of BIT are hypothesized based on available information.<br>The estimation is demonstrated to be accurate by comparing the rate constant of BIT with that of six compounds which have similar bond | |

| | 1 REFERENCE | Official use only |
|---|---|---|
| | types. | |
| **5.2 Results and discussion** | Due to relative low vapor pressure and high water solubility, the concentration of BIT in the troposphere is expected to be low. This ensures that the photodegradation of the radicals with BIT follows a pseudo first-order kinetics required by SAR calculation method.<br>Due to the presence of nitrogen and sulfur bonds, BIT has a large phototransformation rate constant. The parent compound quickly photodegrades during the daylight with half-life of 12.6 hours.<br>All potential photodegradation products are expected to be very reactive to photodegradation with half-lives ranging from 5.4-237.1 hours. | |
| **5.3 Conclusion** | Daylight photolysis is the dominant phototransformation procedure for BIT and its potential metabolites.<br>BIT photodegrades quickly with half-life of 10.3 hours and the half-lives of its metabolites range from 5.4 – 237.1 hours.<br>Due to very low production and usage volume, the effect from BIT and its potential photodegradation products towards global warming is minimal. Therefore, BIT and its photodegradation metabolites impose no effect to global warming. | |
| 5.3.1 Reliability | 1-valid without restrictions | |
| 5.3.2 Deficiencies | No | |
| | | |

| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *November 2010* |
| **Materials and Methods** | *Applicant's version is accepted. Test method considers the photodegradation of BIT due to reactions with OH radicals and with $NO_3$ radicals.* |
| **Results and discussion** | *Applicant's version is accepted, but with the following comments:*<br>*3.5. Transformation products. It is recommended to carry out further studies focused on the environmental behavior of the compound identified as metabolite 10. However, due to the low vapor pressure of BIT, its concentration on troposphere is expected to be low.*<br>*4.1 CMIT should read BIT* |

**Section A7** **Ecotoxicological Profile Including Environmental Fate and Behaviour**

**Subsection A7.3** **Fate and Behaviour in Air**

**Subsection A7.3.1** **Phototransformation in air**

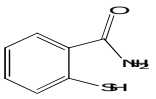
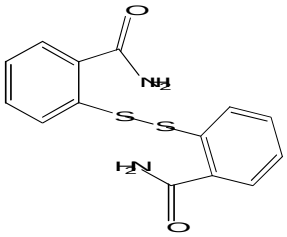
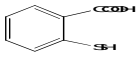
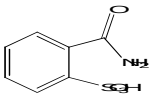
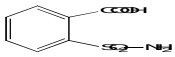
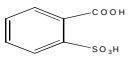
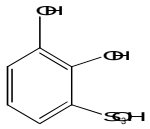
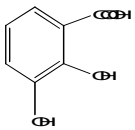
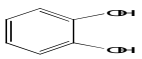
**Annex Point IIIA, VII.5**

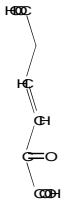
| | **1 REFERENCE** | **Official use only** |
|---|---|---|
| **Conclusion** | *Daylight photolysis is the dominant phototransformation procedure for BIT and its potential metabolites.*<br>*BIT photodegrades quickly with half-life of 10.3 hours and the half-lives of its metabolites range from 5.4 – 237.1 hours.*<br>*Due to very low production and usage volume, the effect from BIT and its potential photodegradation products towards global warming is minimal. Therefore, BIT and its photodegradation metabolites impose no effect to global warming.* | |
| **Reliability** | *1* | |
| **Acceptability** | *Acceptable* | |
| **Remarks** | | |

**Table A7.3.1-1: Hydroxyl Rate Constants of Different Types of Reactions for BIT**

| **Bond Type** | **$k_{OH}$ ($10^{-13}$ $cm^3$.$molecule^{-1}$.$sec^{-1}$)** | **Number of Bonds** | **Total ($10^{-13}$ $cm^3$.$molecule^{-1}$.$sec^{-1}$)** |
|---|---|---|---|
| C-H | 0.14 | 4 | 0.56 |
| >C=C< | 11.0 | 6 | 66.0 |
| >C=O | 0.31 | 1 | 0.31 |
| >N- | 60.2 | 3 | 180.6 |
| -S- | 20.0 | 2 | 40.0 |
| | | | 287.47 |

**Table A7.3.1-2: Reaction Rate Constant $k_{OH}$ and Half-Life of Transformation Products**

| Compound | $k_{OH}$ ($10^{-13}$ $cm^3.molecule^{-1}.sec^{-1}$) | $t_{½}$ (hours) |
|---|---|---|
|  | 287.47 | 10.3 |
|  | 574.94 | 5.2 |
|  | 106.87 | 27.7 |
|  | 287.47 | 10.3 |
|  | 287.47 | 10.3 |
|  | 106.87 | 27.7 |
|  | 106.87 | 27.8 |
|  | 66.56 | 44.4 |
|  | 66.56 | 44.5 |

| Compound | $k_{OH}$ ($10^{-13}$ $cm^3$.$molecule^{-1}$.$sec^{-1}$) | $t_{½}$ (hours) |
|---|---|---|
|  | 12.49 | 237.1 |

| | | |
|---|---|---|
| **Section A7**<br><br>**Subsection A7.3**<br>**Section A7.3.2**<br>**Annex point IIIA, XII.3** | **Ecotoxicological Profile Including Environmental Fate and Behaviour**<br>**Fate and Behaviour in Air**<br>**Fate and behaviour in air, further studies** | |
| | **JUSTIFICATION FOR NON-SUBMISSION OF DATA** | **Official use only** |
| **Other existing data [X]** | **Technically not feasible [ ] Scientifically unjustified [ ]** | |
| **Limited exposure [X]** | **Other justification [ ]** | |
| **Detailed justification:** | Due to the rapid decline of parent and metabolites calculated in Section 7.3.1, BIT does not trigger the need for additional fate and behaviour in air studies. | |
| **Undertaking of intended data submission [ ]** | No studies are planned. | |
| | | |
| | | |
| | **Evaluation by Competent Authorities** | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | *January 2011* | |
| **Evaluation of applicant's justification** | *Applicant's justification is accepted.* | |
| **Conclusion** | *Applicant's justification is accepted.* | |
| **Remarks** | | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Section A7.4.1.1a/01**<br>**Annex Point IIA VII.7.1** | **Acute toxicity of BIT to fish-Fresh water, Rainbow trout** |

| | | | Official use only |
|---|---|---|---|
| | | **1 REFERENCE** | |
| **1.1** | **Reference** | **A7.4.1.1.a/01** [redacted]. (2006a) 1,2-Benzisothiazolin-3-one: A 96-hour flow-through acute toxicity test with the rainbow trout (*Oncorhynchus mykiss*); [redacted] Rohm and Haas Report N° 06RC-082 (October 6, 2006), Unpublished. | |
| **1.2** | **Data protection** | Yes | |
| 1.2.1. | Data owner | Rohm and Haas Company | |
| 1.2.2. | Companies with letter of access | | |
| 1.2.3. | Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into Annex I/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **Guideline study** | Yes, OECD 203, US EPA OPPTS 850.1075 | |
| **2.2** | **GLP** | Yes | |
| **2.3** | **Deviations** | No | |
| | | **3 MATERIALS AND METHODS** | |
| **3.1** | **Test material** | 1,2-Benzisothiazolin-3-one | |
| 3.1.1 | Lot/Batch number | 2005-051 | |
| 3.1.2 | Specification | As given in section 2 | |
| 3.1.3 | Purity | 89.8% | |
| 3.1.4 | Composition of Product | not applicable | |
| 3.1.5 | Further relevant properties | not applicable | |
| 3.1.6 | Method of analysis | High performance liquid chromatography (HPLC) with UV detector | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Section A7.4.1.1a/01**<br>**Annex Point IIA VII.7.1** | **Acute toxicity of BIT to fish-Fresh water, Rainbow trout** |

| | | |
|---|---|---|
| **3.2 Preparation of TS solution for poorly soluble or volatile test substances** | | see Table A7.4.1.1.a/01-1 |
| **3.3 Reference substance** | | No |
| 3.3.1 | Method of analysis for reference substance | not tested |
| **3.4 Testing procedure** | | |
| 3.4.1 | Dilution water | see Table A7.4.1.1.a/01-2 |
| 3.4.2 | Test organisms | see Table A7.4.1.1.a/01-3 |
| 3.4.3 | Test system | see Table A7.4.1.1.a/01-4 |
| 3.4.4 | Test conditions | see Table A7.4.1.1.a/01-5 |
| 3.4.5 | Duration of the test | 96 h |
| 3.4.6 | Test parameter | mortality: see table A7.4.1.1.a/01-6 |
| 3.4.7 | Sampling | Water samples were collected from one test chamber of each treatment and control group three days prior to the start of the test after conditioning the diluter for three days. The samples were collected from mid-depth in each test chamber, placed in glass vials and processed immediately for analysis. |
| 3.4.8 | Monitoring of TS concentration | Yes, 0, 48 and 96 hours of the study |
| 3.4.9 | Statistics | Mortality data were canalyzed using the omputer program of C.E. Stephan (Methods for calculating an $LC_{50}$, *Aquatic Toxicology and Hazard Evaluations*. American Society for Testing and Materials. Publication Number STP 634, pages 65-84). Binomial probability was used to calculate the 24 and 48-hour $LC_{50}$ values and the probit method was used to calculate the 72 and 96-hour $LC_{50}$ values. The no-mortality and the NOEC were determined by visual interpretation of the mortality and observation data. |
| | | **4 RESULTS** |
| **4.1 Limit Test** | | Not performed |

**4.2 Results test substance**

4.2.1 Initial concentrations of test substance

Nominal (mg BIT/L)

0.31, 0.63, 1.3, 2.5, 5.0

4.2.2 Actual concentrations of test substance

Measured concentrations (mg BIT/L) in test samples

| Nominal | 0 hr | 48 hr | 96 hr | Mean |
|---|---|---|---|---|
| 0.31 | 0.281 | 0.270 | 0.268 | 0.27 |
| 0.63 | 0.594 | 0.581 | 0.580 | 0.59 |
| 1.3 | 1.24 | 1.20 | 1.22 | 1.2 |
| 2.5 | 2.37 | 2.37 | 2.29 | 2.3 |
| 5.0 | 5.13 | 5.14 | 100% mortal-ity, no sample | 5.1 |

4.2.3 Effect data (Mortality)

see Table A7.4.1.1.a/01-6; see table A7.4.1.1.a/01-7

4.2.4 Concentration / response curve

See Figure A7.41.1.a/01-1.

4.2.5 Other effects

One lethargic fish in the 1.2 mg BIT/L group and one fish lying on the bottom of the tank in the 2.3 mg BIT/L group. All other surviving fish appeared normal at test termination. All test solutions appeared clear and colorless in the diluter mixing chambers and in the test chambers at test intiation and termination.

**4.3 Results of controls**

4.3.1 Number/ percentage of animals showing adverse effects

no adverse effects

4.3.2 Nature of adverse effects

not applicable

**4.4 Test with reference substance**

Not performed

**5 APPLICANT'S SUMMARY AND CONCLUSION**

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Section A7.4.1.1a/01**<br>**Annex Point IIA VII.7.1** | **Acute toxicity of BIT to fish-Fresh water, Rainbow trout** |

| | | | |
|---|---|---|---|
| **5.1 Materials and methods** | | OECD 203, US EPA OPPTS 850.1075, Acute flow-through 96 h fish study with analytical confirmation of test solution concentrations. | |
| **5.2 Results and discussion** | | 96 h NOEC = 0.27 mg BIT/L | |
| 5.2.1 | $LC_0$ | 96 h = 0.27 mg BIT/L | |
| 5.2.2 | $LC_{50}$ | 96 h = 1.9 mg BIT/L | |
| 5.2.3 | $LC_{100}$ | 96 h = 5.1 mg BIT/L | |
| **5.3 Conclusion** | | see validity criteria summarized in table A7.4.1.1.a/01-8 | |
| 5.3.1 | Other Conclusions | None | |
| 5.3.2 | Reliability | (1), valid without restriction | |
| 5.3.3 | Deficiencies | No | |

| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *March 2013* |
| **Materials and Methods** | *The applicants version is accepted* |
| **Results and discussion** | *Applicant's version is adopted* |
| **Conclusion** | *Applicant's version is adopted* |
| **Reliability** | *1* |
| **Acceptability** | *Acceptable* |
| **Remarks** | |

**Table A7.4.1.1.a/01-1: Preparation of TS solution for poorly soluble or volatile test substances**

| Criteria | Details |
|---|---|
| Dispersion | Yes, sonic bath and mixed by inversion |
| Vehicle | Yes, Dimethyl formamide (DMF) |
| Concentration of vehicle | The concentration of DMF in the solvent control and all treatment groups was 0.1 ml/L |
| Vehicle control performed | Yes, DMF |
| Other procedures | not applicable |

**Table A7.4.1.1.a/01-2: Dilution water**

| Criteria | Details |
|---|---|
| Source | Filtered, UV-sterilized, well water, 40 meters deep located at the Wildlife International site |
| Alkalinity | 182 mg/L (as $CaCO_3$) |
| Hardness | 136 mg/L (as $CaCO_3$) |
| pH | 8.2 |
| Oxygen content | ≥ 8.2 mg/L (76% of saturation) |
| Conductance | 313 µMhos/cm |
| Holding water different from dilution water | No |

**Table A7.4.1.1.a/01-3: Test organisms**

| Criteria | Details |
|---|---|
| Species/strain | rainbow trout, *Oncorhynchus mykiss* |
| Source | Thomas Fish Company, Anderson, California, USA |
| Wild caught | No |
| Age/size | Juveniles, the length of the longest fish measured was no more than twice the length of the shortest fish. |
| Kind of food | Holding period: trout were fed a commercially prepared diet supplied by Ziegler Brothers, Inc., Gardners, Pennsylvania, USA |
| Amount of food | *ad libitum* |

| | |
|---|---|
| Feeding frequency | Daily during 2-week holding period |
| Pretreatment | The fish were not fed for at least two days prior to testing. |
| Feeding of animals during test | No |

**Table A7.4.1.1.a/01-4: Test system**

| Criteria | Details |
|---|---|
| Test type | Flow-through |
| Renewal of test solution | A continuous flow diluter was used to deliver each concentration of TS, solvent control (DMF) and a negative control (dilution water). A calibrated syringe pump was used to deliver the TS and controls into mixing chambers. The diluter was adjusted so that each test chamber received approximately 10 volume additions of test water every 24 hours. The five stock solutions were injected into the diluting mixing chambers (at a rate of 20 µL/minute) where they were mixed with well water (at a rate of 200 mL/minute) to achieve the desired test concentrations. |
| Volume of test vessels | 25 L stainless steel chambers filled with approximately 15 L of test water to achieve a depth of 18.7 cm. |
| Volume/animal | 1.5 L |
| Number of animals/vessel | 10 |
| Number of vessels/ concentration | 2 |
| Test performed in closed vessels due to significant volatility of TS | No |

**Table A7.4.1.1.a/01-5: Test conditions**

| Criteria | Details |
|---|---|
| Test temperature (degree C) | 11.3 – 12.6 ºC |
| Dissolved oxygen (mg/L) | ≥ 8.2 mg/L (76% of saturation) |
| pH | 8.0 – 8.1 |
| Adjustment of pH | Not described |
| Aeration of dilution water | Yes, flow-through |
| Intensity of irradiation | Fluorescent light bulbs that emit wavelengths similar to natural sunlight |

| | |
|---|---|
| Photoperiod | 16 h daylight, 8 h darkness |

**Table A7.4.1.1.a/01-6: Mortality data**

| Test-Substance Concentration (mean measured) [mg BIT/L] | Mortality | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Number | | | | | Percentage | | | | |
| | 2.5 h | 24 h | 48 h | 72 h | 96 h | 2.5 h | 24 h | 48 h | 72 h | 96 h |
| Negative control | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 | 0 | 0 | 0 | 0 | 0 |
| DMF solvent control | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 | 0 | 0 | 0 | 0 | 0 |
| 0.27 | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 | 0 | 0 | 0 | 0 | 0 |
| 0.59 | 0/20 | 0/20 | 0/20 | 0/20 | 1/20 | 0 | 0 | 0 | 0 | 5 |
| 1.2 | 0/20 | 2/20 | 3/20 | 3/20 | 3/20 | 0 | 10 | 15 | 15 | 15 |
| 2.3 | 0/20 | 1/20 | 4/20 | 6/20 | 12/20 | 0 | 5 | 20 | 30 | 60 |
| 5.1 | 0/20 | 19/20 | 20/20 | 20/20 | 20/20 | 0 | 95 | 100 | 100 | 100 |
| **Temperature [°C]** | 11.8-12.6 | -- | -- | -- | 11.3-12.1 | | | | | |
| **pH** | 8.0-8.1 | 8.0-8.1 | 8.0 | 8.0-8.1 | 8.0 | | | | | |
| **Oxygen [mg/l]** | 8.7-9.0 | 8.2-8.6 | 8.2-8.5 | 8.2-8.4 | 8.4-8.7 | | | | | |

**Table A7.4.1.1.a/01-7: Effect data**

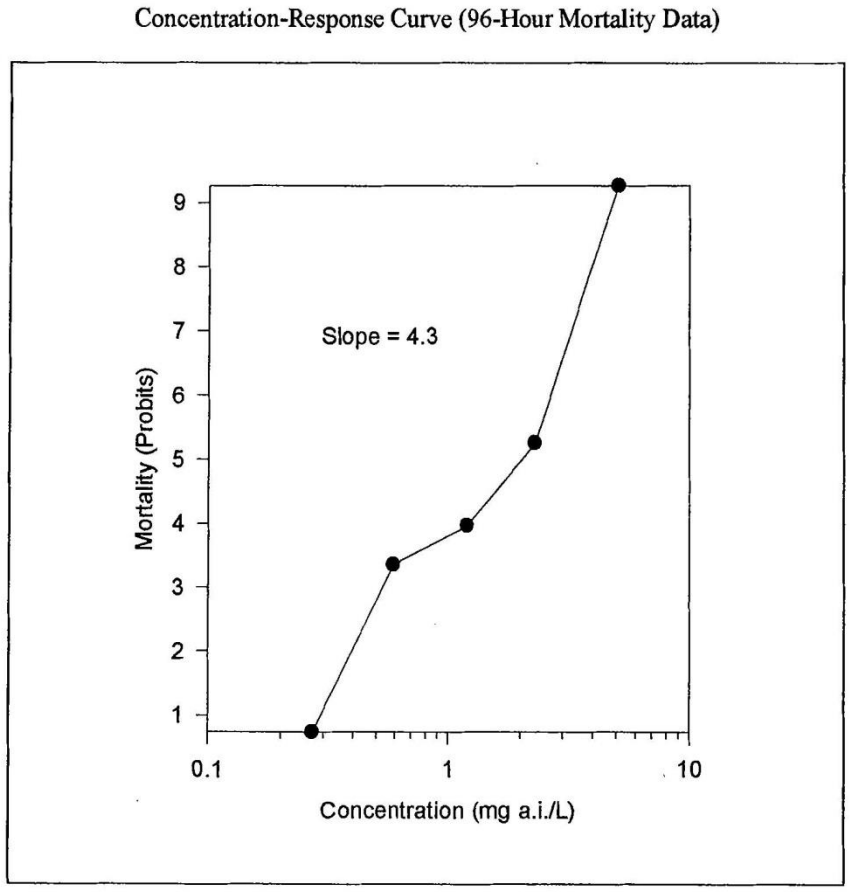
| | 24 h [ mg BIT/L][1] | 95 % C.I. | 48 h [mg BIT/L][1] | 95 % C.I. | 72 h [mg BIT/L][1] | 95 % C.I. | 96 h [mg BIT/L][1] | 95 % C.I. |
|---|---|---|---|---|---|---|---|---|
| $LC_{50}$ | 3.4 | 2.3 – 5.1 | 2.9 (m) | 2.3 – 5.1 | 2.4 | 2.0 – 3.0 | 1.9 (m) | 1.5 – 2.4 |

[1] effect data are based on measured (m) concentrations

**Table A7.4.1.1.a/01-8: Validity criteria for acute fish test according to OECD Guideline 203**

| | fulfilled | Not fullfilled |
|---|---|---|
| Mortality of control animals < 10% | yes | |
| Concentration of dissolved oxygen in all test vessels > 60% saturation | yes | |
| Concentration of test substance ≥ 80% of initial concentration during test | yes | |

**Figure A7.4.1.1.a/01-1: 96-hour dose-response line for Rainbow trout (*Oncorhynchus mykiss*) exposed to BIT**



| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.1b/01**<br>**Annex Point IIA VII.7.1** | **Acute toxicity of BIT to fish-Marine water, Sheepshead Minnow** |

| | | | Official use only |
|---|---|---|---|
| | | **1 REFERENCE** | |
| **1.1** | **Reference** | **A7.4.1.1.b/01** [redacted] (2006c) 1,2-Benzisothiazolin-3-one: A 96-hour flow-through acute toxicity test with the sheepshead minnow (*Cyprinodon variegatus*), [redacted], Rohm and Haas Report N° 06RC-083 (December 20, 2006), Unpublished. | |
| **1.2** | **Data protection** | Yes | |
| 1.2.1. | Data owner | Rohm and Haas Company | |
| 1.2.2. | Companies with letter of access | | |
| 1.2.3. | Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into Annex I/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **Guideline study** | Yes, US-EPA OPPTS 850.1075 | |
| **2.2** | **GLP** | Yes | |
| **2.3** | **Deviations** | No | |
| | | **3 MATERIALS AND METHODS** | |
| **3.1** | **Test material** | 1,2-Benzisothiazolin-3-one (BIT) | |
| 3.1.1 | Lot/Batch number | 2005-051 | |
| 3.1.2 | Specification | As given in section 2 | |
| 3.1.3 | Purity | 89.8% BIT | |
| 3.1.4 | Composition of Product | not applicable | |
| 3.1.5 | Further relevant properties | not applicable | |
| 3.1.6 | Method of analysis | High performance liquid chromatography with UV detection | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.1b/01**<br>**Annex Point IIA VII.7.1** | **Acute toxicity of BITto fish-Marine water, Sheepshead Minnow** |

| | | |
|---|---|---|
| **3.2 Preparation of TS solution for poorly soluble or volatile test substances** | | see table A7.4.1.1.b/01-1 |
| **3.3 Reference substance** | | No |
| 3.3.1 | Method of analysis for reference substance | not tested |
| **3.4 Testing procedure** | | |
| 3.4.1 | Dilution water | see Table A7.4.1.1.b/01-2 |
| 3.4.2 | Test organisms | see Table A7.4.1.1.b/01-3 |
| 3.4.3 | Test system | see Table A7.4.1.1.b/01-4 |
| 3.4.4 | Test conditions | see Table A7.4.1.1.b/01-5 |
| 3.4.5 | Duration of the test | 96 h |
| 3.4.6 | Test parameter | mortality: see Table A7.4.1.1.b/01-6 |
| 3.4.7 | Sampling | The water samples were collected from mid-depth in the test chambers, placed in gless vials and processed immediately for analysis of BIT. |
| 3.4.8 | Monitoring of TS concentration | Yes, 0, 48 hours and 96 hours |
| 3.4.9 | Statistics | The mortality data were analysed using the computer program of C.E. Stephan [(U.S. Environmental Protection Agency. 1985. Standard Evaluation Procedure, *Acute Toxicity Test for Freshwater Estuarine and Marine Organisms (Estuarine Fish 96-Hour Acute Toxicity Test)*. Hazard Evaluation Division. Office of Pesticide Programs. EPA-540/9-85-006. Washington D.C.]. The program was designed to calculate the $LC_{50}$ value and the 95% confidence interval by probit analysis, the moving average method, and binomial probability with nonlinear interpolation (Finney, D.J. 1971. *Statistical Methods in Biological Assay*. Second edition. Griffin press, London) ( Thompson, W.R. 1947. Bacteriological Reviews. Vol. II, No. 2, pages 115-145) (C.E. Stephan 1977. Methods for calculating an $LC_{50}$, *Aquatic Toxicology and Hazard Evaluations*. American Society for Testing and Materials. Publication Number STP 634, pages 65-84). Binomial probability was used to calculate the 48, 72 and 96-hour $LC_{50}$ values. The no-mortality and the NOEC were determined by visual interpretation of the mortality and |

observation data.

**4 RESULTS**

**4.1 Limit Test** Not performed

**4.2 Results test substance**

4.2.1 Initial concentrations of test substance

Nominal (mg BIT/L)

1.9, 3.8, 7.5, 15 and 30

4.2.2 Actual concentrations of test substance

Measured concentrations of BIT in test samples (mg BIT/L)

| Nominal | 0 h | 48 h | 96 h | Mean measured |
|---|---|---|---|---|
| 0, Negative control | < LOQ | < LOQ | < LOQ | -- |
| 0, solvent control | < LOQ | < LOQ | < LOQ | -- |
| 1.9 | 1.80 | 1.73 | 1.90 | 1.8 |
| 3.8 | 3.49 | 3.45 | 3.60 | 3.5 |
| 7.5 | 7.07 | 6.81 | 7.18 | 7.0 |
| 15 | 13.1 | 13.7 | 14.1 | 14 |
| 30 | 22.4 | 21.9 | 28.6 | 24 |

4.2.3 Effect data (Mortality) see Table A7.4.1.1.b/01-6; see Table A7.4.1.1.b/01-7

4.2.4 Concentration / response curve The slope of the 96-hour mortality concentration-response line was 12.5. See Figure A7.4.1.1.b/01-1.

4.2.5 Other effects Lethargy, surfacing, lying on the bottom of the aquarium, loss of equilibrium and erratic swimming

**4.3 Results of controls**

| | | |
|---|---|---|
| 4.3.1 | Number/ percentage of animals showing adverse effects | no adverse effects |
| 4.3.2 | Nature of adverse effects | not applicable |
| **4.4 Test with reference substance** | | Not performed |
| | | **5 APPLICANT'S SUMMARY AND CONCLUSION** |
| **5.1 Materials and methods** | | US EPA Guideline OPPTS 850.1075, Acute flow-through 96h fish study with analytical confirmation of test solution concentrations. |
| **5.2 Results and discussion** | | The test solutions appeared clear and colorless in all test chambers at test initiation and test termination. A white precipitate was observed in the diluter mixing chamber for the 30 mg BIT/L solution which indicated that the test was conducted to the limit of water solubility. All water quality parameters were within acceptable limits during the test. All fish in the negative and solvent control groups and in the 1.8 and 3.5 mg BIT/L treatment groups appeared normal throughout the test. No mortality was observed in the 7.0 mg BIT/L group though the fish were lethargic at 48 hours. Percent mortality was 5 and 90% for the 14 and 24 mg BIT/L groups, respectively. Signs of toxicity observed in fish in the the 14 and 24 mg BIT/L groups during the test included lethargy, loss of equilibrium, erratic swimming, surfacing and.or lying on the bottom of the aquarium. 96 h NOEC = 3.5 mg BIT/L based on survival. |
| 5.2.1 | $LC_0$ | 96 h = 7.0 mg BIT/L |
| 5.2.2 | $LC_{50}$ | 96 h = 19 mg BIT/L |
| 5.2.3 | $LC_{100}$ | Not applicable |
| **5.3 Conclusion** | | See validity criteria summarized in table A7.4.1.1.b/01-8 |
| 5.3.1 | Other Conclusions | None |
| 5.3.2 | Reliability | (1) reliable without restriction |
| 5.3.3 | Deficiencies | No |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.1b/01**<br>**Annex Point IIA VII.7.1** | **Acute toxicity of BITto fish-Marine water, Sheepshead Minnow** |

| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *December 2010* |
| **Materials and Methods** | *Applicant's version is acceptable* |
| **Results anddiscussion** | *Applicant's version is adopted* |
| **Conclusion** | *Applicant's version is adopted* |
| **Reliability** | *2* |
| **Acceptability** | *Acceptable* |
| **Remarks** | |

**Table A7.4.1.1.b/01-1: Preparation of TS solution for poorly soluble or volatile test substances**

| Criteria | Details |
|---|---|
| Dispersion | Yes, sonication and inversion |
| Vehicle | Yes, dimethyl formamide (DMF) |
| Concentration of vehicle | 0.1 ml/L in solvent control and in all BIT treatment groups |
| Vehicle control performed | Yes |
| Other procedures | not applicable |

**Table A7.4.1.1.b/01-2: Dilution water**

| Criteria | Details |
|---|---|
| Source | filtered natural seawater from Indian River Inlet, Delaware, USA |
| Alkalinity | not described |
| Hardness | not described |
| pH | 7.7 to 8.1 |
| Oxygen content | 7.3 to 7.7 mg/L |
| Conductance | not described |
| Holding water different from dilution water | not described |

**Table A7.4.1.1.b/01-3: Test organisms**

| Criteria | Details |
|---|---|
| Species/strain | sheepshead minnow, *Cyrinodon variegatus* |
| Source | Aquatic BioSystems, Inc., Fort Collins, Colorado, USA |
| Wild caught | No |
| Age/size | Juveniles. Average total length of 10 negative control fish measured at the end of the test was 1.6 cm with a range of 1.3 to 1.8 cm. The average wet weight (blotted dry) of 10 negative control fish measured at the end of the test was 0.06 grams with a range of 0.03 to 0.09 grams. |
| Kind of food | Commercially prepared diet supplemented with brine shrimp nauplii (*Artemia* species) |
| Amount of food | *Ad libitum* |
| Feeding frequency | Daily |
| Pretreatment | Fish were held for at least 14 days prior to the test in water from the same source and the same temperature as used during the test. The fish were not fed for two days prior to the test initiation. |
| Feeding of animals during test | No |

**Table A7.4.1.1.b/01-4: Test system**

| Criteria | Details |
|---|---|
| Test type | Flow-through |
| Renewal of test solution | During the test the continuous flow diluter was adjusted so that each test chamber received approximately 10 volume additions per 24 hours. |
| Volume of test vessels | 25 L Teflon-lined stainless steel aquaria filled with 15 L test water |
| Volume/animal | 1.5 L |
| Number of animals/vessel | 10 |
| Number of vessels/ concentration | 2 |
| Test performed in closed vessels due to significant volatility of TS | No |

**Table A7.4.1.1.b/01-5: Test conditions**

| Criteria | Details |
|---|---|
| Test temperature | 21.6 to 22.0 ° C |
| Dissolved oxygen | 7.3 to 7.7 mg/L (at or above 93% saturation) |
| pH | 7.7 to 8.1 |
| Adjustment of pH | not described |
| Salinity | 20 parts per thousand |
| Aeration of dilution water | Yes |
| Intensity of irradiation | fluorescent lights, 145 lux |
| Photoperiod | 16 h daylight, 8 h dark, 30 minute transition period of low light intensity |

**Table A7.4.1.1.b/01-6: Mortality data**

| Test-Substance Concentration (measured)[1] [mg BIT/L] | Mortality | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Number | | | | Percentage | | | |
| | 24 h | 48 h | 72 h | 96 h | 24 h | 48 h | 72 h | 96 h |
| Negative control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| DMF solvent control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1.8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 14 | 0 | 1 | 1 | 1 | 0 | 5 | 5 | 5 |
| 24 | 10 | 18 | 18 | 18 | 50 | 90 | 90 | 90 |
| **Temperature [°C]** | 21.7-22.0 | 21.7-21.8 | 21.6-21.8 | 21.6-21.9 | | | | |
| **pH** | 7.8-8.1 | 7.9-8.1 | 7.7-8.0 | 7.8-8.1 | | | | |
| **Oxygen [mg/l]** | 7.3-7.4 | 7.5-7.6 | 7.6-7.7 | 7.6-7.7 | | | | |

[1] TS concentrations were measured

**Table A7.4.1.1.b/01-7: Effect data**

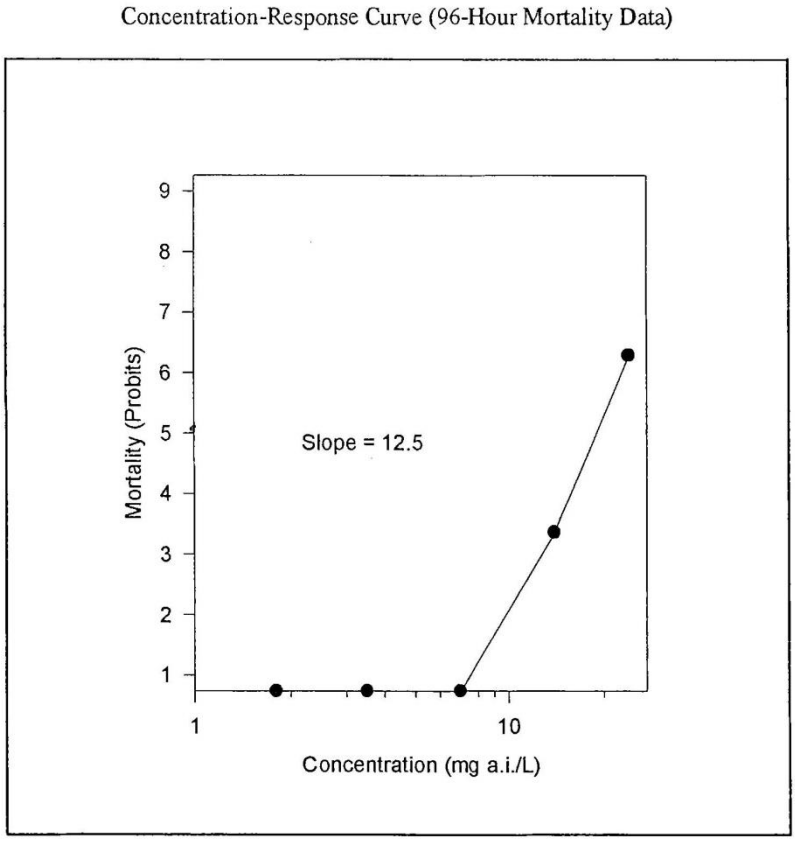
| | 48 h [mg BIT/L][1] | 95 % C.I. | 96 h [mg BIT/L][1] | 95 % C.I. |
|---|---|---|---|---|
| **$LC_0$** | 7.0 (m) | Not applicable | 7.0 (m) | Not applicable |
| **$LC_{50}$** | 19 (m) | 17-21 | 19 (m) | 17-21 |

[1] effect data are based on measured (m) concentrations

**Table A7.4.1.1.b/01-8: Validity criteria for acute fish test according to OECD Guideline 203**

| | fulfilled | Not fullfilled |
|---|---|---|
| Mortality of control animals < 10% | **yes** | |
| Concentration of dissolved oxygen in all test vessels > 60% saturation | **yes** | |
| Concentration of test substance ≥ 80% of initial concentration during test | **yes** | |

**Figure A7.4.1.1.b/01-1: Survival of organisms exposed to BIT for 96 hours**



| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.2.a/01** | **Acute toxicity of BIT to invertebrates-Fresh water, *Daphnia magna*** |
| **Annex Point IIA VII.7.2** | |

| | | | |
|---|---|---|---|
| | | **1 REFERENCE** | **Official use only** |
| **1.1** | **Reference** | **A7.4.1.2.a/01** [redacted] (2006b) 1,2-Benzisothiazolin-3-one: A 48-hour flow-through acute toxicity test with the cladoceran (*Daphnia magna*), [redacted], Rohm and Haas Report N° 06RC-084 (September 28, 2006), GLP, Unpublished. | |
| **1.2** | **Data protection** | Yes | |
| 1.2.1. | Data owner | Rohm and Haas Company | |
| 1.2.2. | Companies with letter of access | | |
| 1.2.3. | Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into Annex I/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **Guideline study** | Yes, OECD 202, US EPA 850.1010 | |
| **2.2** | **GLP** | Yes | |
| **2.3** | **Deviations** | No | |
| | | **3 MATERIALS AND METHODS** | |
| **3.1** | **Test material** | 1,2-Benzisothiazolin-3-one | |
| 3.1.1 | Lot/Batch number | 2005-051 | |
| 3.1.2 | Specification | As given in section 2 | |
| 3.1.3 | Purity | 89.8% BIT | |
| 3.1.4 | Composition of Product | not applicable | |
| 3.1.5 | Further relevant properties | not applicable | |

| | | | |
|---|---|---|---|
| 3.1.6 | Method of analysis | Reverse phase high performance liquid chromatography (HPLC) | |
| **3.2 Preparation of TS solution for poorly soluble or volatile test substances** | | see Table A7.4.1.2.a/01-1 | |
| **3.3 Reference substance** | | No | |
| 3.3.1 | Method of analysis for reference substance | Not tested | |
| **3.4 Testing procedure** | | | |
| 3.4.1 | Dilution water | see Table A7.4.1.2.a/01-2 | |
| 3.4.2 | Test organisms | see Table A7.4.1.2.a/01-3 | |
| 3.4.3 | Test system | see Table A7.4.1.2.a/01-4 | **X** |
| 3.4.4 | Test conditions | see Table A7.4.1.2.a/01-5 | |
| 3.4.5 | Duration of the test | 48 h | |
| 3.4.6 | Test parameter | immobilization : see table A7.4.1.2.a/01-6 | |
| 3.4.7 | Sampling | The samples were collected from mid-depth in each test chamber, placed in glass vials and processed immediately for BIT concentration. | |
| 3.4.8 | Monitoring of TS concentration | Yes, 0 and 48 hours of the study | |
| 3.4.9 | Statistics | The 24 and 48 hour mortality and immobility data were analyzed using the computer program of C.E. Stephan (C.E. Stephan, 1978, US EPA, Environmental Research Laboratory, Duluth, Minnesota, USA, personal communication). The binomial probability was used to calculate the 24 hour $EC_{50}$ value and the probit method was used to calculate the 48 hour $EC_{50}$ value.. | |
| | | **4 RESULTS** | |
| **4.1 Limit Test** | | Not performed | |

**4.2 Results test substance**

4.2.1 Initial concentrations of test substance

Nominal (mg BIT/L)

0, 1.3, 2.5, 5.0, 10, and 20

4.2.2 Actual concentrations of test substance

measured concentrations (mg BIT/L)

| Nominal | 0 h | 48 h | mean |
|---|---|---|---|
| 1.3 | 1.08 | 1.19 | 1.1 |
| 2.5 | 2.88 | 2.92 | 2.9 |
| 5.0 | 5.13 | 4.98 | 5.1 |
| 10 | 10.3 | 9.62 | 10 |
| 20 | 21.5 | 20.6 | 21 |

4.2.3 Effect data (Immobilisation)

see table A7.4.1.2.a/01-6; see table A7.4.1.2.a/01-7

4.2.4 Concentration / response curve

48 hour mortality/immobility data, see Figure A7.4.1.2.a/01-1.

4.2.5 Other effects

Mortality, lethargy

**4.3 Results of controls**

normal in appearance and behavior

**4.4 Test with reference substance**

Not performed

**5 APPLICANT'S SUMMARY AND CONCLUSION**

**5.1 Materials and methods**

US EPA Guideline 72-2, Acute flow-through 48h *Daphnia magna* study with analytical confirmation of test solution concentrations. There were no guideline deviations.

**5.2 Results and discussion**

*Daphnia magna* were exposed to five concentrations of BIT, a dilution water control (negative control) and a solvent control (dimethyl formamide) under flow-through conditions for 48 hours. The test solutions appeared clear and colorless in all test chambers at test initiation and termination. Analytical recoveries ranged from 83 to 115% of nominal concentrations on Day 0 and from 92 to 117% of nominal concentrations on Day 2. At test termination, all daphnids in the negative control and the solvent control appeared normal with no

| | | | |
|---|---|---|---|
| | | mortalities or immobile daphnids noted. Percent mortality/immobility at test termination in the 1.1, 2.9, 5.1, 10 and 21 mg BIT/L treatment groups was 5, 35, 60, 100 and 100%, respectively. | |
| 5.2.1 | $EC_0$ | Not applicable | |
| 5.2.2 | $EC_{50}$ | 3.7 mg BIT/L (95% confidence interval of 2.9 to 6.4 mg BIT/L) | |
| 5.2.3 | $EC_{100}$ | 10 mg BIT/L | |
| **5.3 Conclusion** | | see table A7.4.1.2.a/01-8 | |
| 5.3.1 | Reliability | (1), reliable without restriction | |
| 5.3.2 | Deficiencies | No | |

| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *December 2010* |
| **Materials and Methods** | ***3.4.3**: The OECD Guideline 202 recommends four groups of 5 animals each instead of two groups of 10 animals.* |
| **Results and discussion** | *Applicant's version adopted* |
| **Conclusion** | *Applicant's version adopted* |
| **Reliability** | *2* |
| **Acceptability** | *Acceptable* |
| **Remarks** | |

**Table A7.4.1.2.a/01-1: Preparation of TS solution for poorly soluble or volatile test substances**

| Criteria | Details |
|---|---|
| Dispersion | Mixed by inversion |

| | |
|---|---|
| Vehicle | DMF (dimethyl formamide) |
| Concentration of vehicle | 0.1 mL/L in solvent control and in all BIT treatment groups |
| Vehicle control performed | yes |
| Other procedures | Not applicable |

**Table A7.4.1.2.a/01-2: Dilution water**

| Criteria | Details |
|---|---|
| Source | Well water, approximately 40 meters deep, located at Wildlife International, Easton, Maryland, USA |
| Alkalinity | 180 to 182 mg/L as $CaCO_3$ |
| Hardness | Moderately hard, 132 to 136 mg/L as $CaCO_3$ |
| pH | 8.0 to 8.2 |
| Ca / Mg ratio | Not described |
| Na / K ratio | Not described |
| Oxygen content | ≥ 7.9 mg/L (88% of saturation) |
| Conductance | 305 to 320 µmhos/cm |
| Holding water different from dilution water | Well water was sand filtered, pumped into a storage tank and aerated. Prior to use, the water was filtered to 0.45 µm and passed through an ultraviolet sterilizer. |

**Table A7.4.1.2.a/01-3: Test organisms**

| Criteria | Details |
|---|---|
| Strain | *Daphnia magna* |
| Source | In-house daphnid culture |
| Age | first instar daphnids (<24 h old) |
| Breeding method | not described |
| Kind of food | Mixture of yeast, cereal grass media and trout chow and a suspension of freshwater green alga, *Selenastrum capricornutum* |
| Amount of food | *ad libitum* |
| Feeding frequency | Daily prior to test initiation |
| Pretreatment | None |
| Feeding of animals during test | No |

**Table A7.4.1.2.a/01-4: Test system**

| Criteria | Details |
|---|---|
| Renewal of test solution | Flow-through using a calibrated syringe pump to deliver the desired test concentration. Diluter was adjusted so that each test chamber received approximately 5 volume additions of test water every 24 hours. |
| Volume of test vessels | 25 liter stainless steel aquarium containing 22 liters of test water |
| Volume/animal | 2.2 liters |
| Number of animals/vessel | 10 |
| Number of vessels/ concentration | 2 |
| Test performed in closed vessels due to significant volatility of TS | No |

**Table A7.4.1.2.a/01-5: Test conditions**

| Criteria | Details |
|---|---|
| Test temperature | 19.9 to 20.1 °C |
| Dissolved oxygen | ≥ 7.9 mg/L (88% of saturation) |
| pH | 8.0 to 8.1 |
| Adjustment of pH | not described |
| Aeration of dilution water | Yes |
| Quality/Intensity of irradiation | 183 lux |
| Photoperiod | 16 hr daylight, 8 hours darkness |

**Table A7.4.1.2.a/01-6: Immobilisation data**

| Test-Substance Concentration (mean measured) [1] [mg BIT/L] | Mortality/Immobility *Daphnia* | | | | | | |
|---|---|---|---|---|---|---|---|
| | Number | | Percentage (%) | | Oxygen [mg/L] | pH | Tempera-ture [°C] |
| | 24 h | 48 h | 24 h | 48 h | 48 h | 48 h | 48 h |
| Negative control | 0/10 | 0/10 | 0 | 0 | 8.4 | 8.0 | 20.1 |
| DMF solvent control | 0/10 | 0/10 | 0 | 0 | 8.4 | 8.0 | 20.1 |
| 1.1 | 0/10 | 1/10 | 0 | 5 | 8.5 | 8.1 | 20.0 |
| 2.9 | 0/10 | 3.5/10 | 0 | 35 | 8.2 | 8.1 | 20.0 |
| 5.1 | 0/10 | 6/10 | 0 | 60 | 8.3 | 8.0 | 20.0 |
| 10 | 0/10 | 10/10 | 0 | 100 | 8.2 | 8.0 | 19.9 |
| 21 | 10/10 | 10/10 | 100 | 100 | 8.3 | 8.0 | 19.9 |

[1] TS concentrations were measured

**Table A7.4.1.2.a/01-7: Effect data**

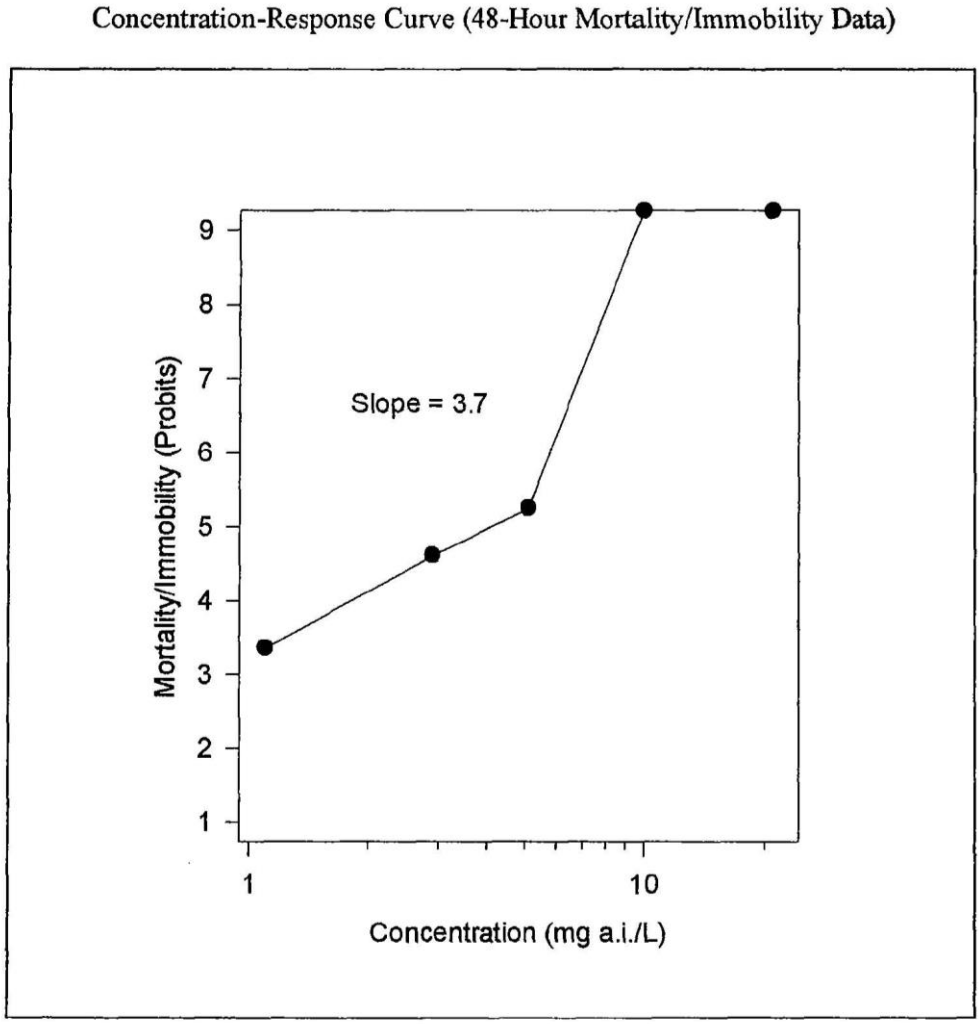
| | $EC_{50}$ [1] | 95 % C.I | $EC_0$ [1] | $EC_{100}$ [1] |
|---|---|---|---|---|
| **24 h [mg BT/L]** | | | Not applicable | |
| **48 h [mg BIT/L]** | 3.7 | 2.9 to 4.6 | Not applicable | |

[1] effect data are based on measured (m) concentrations

**Table A7.4.1.2.a/01-8: Validity criteria for acute daphnia immobilistaion test according to OECD Guideline 202**

| | fulfilled | Not fullfilled |
|---|---|---|
| Immobilisation of control animals < 10% | **yes** | |
| Control animals not staying at the surface | **yes** | |
| Concentration of dissolved oxygen in all test vessels > 3 mg/L | **yes** | |
| Concentration of test substance ≥ 80% of initial concentration during test | **yes** | |

**Figure A7.4.1.2.a/01-1: 48-hour dose-concentration response curve for *Daphnia magna* exposed to BIT**



| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.2.b/01** | **Acute toxicity of BIT to invertebrates-Marine water, Mysid** |
| **Annex Point IIA VII.7.2** | |

| | | | Official use only |
|---|---|---|---|
| | | **1 REFERENCE** | |
| **1.1** | **Reference** | **A7.4.1.2.b/01** [redacted] (2007a) 1,2-Benzisothiazolin-3-one: A 96-hour flow-through acute toxicity test with the saltwater mysid (*Americamysis bahia*), [redacted], Rohm and Haas Report N° 06RC-085 (January 15, 2007), Unpublished | |
| **1.2** | **Data protection** | Yes | |
| 1.2.1. | Data owner | Rohm and Haas Company | |
| 1.2.2. | Companies with letter of access | | |
| 1.2.3. | Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into Annex I/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **Guideline study** | Yes, US EPA OPPTS 850.1035 | |
| **2.2** | **GLP** | Yes | |
| **2.3** | **Deviations** | No | |
| | | **3 MATERIALS AND METHODS** | |
| **3.1** | **Test material** | 1,2-Benzisothiazolin-3-one | |
| 3.1.1 | Lot/Batch number | 2005-051 | |
| 3.1.2 | Specification | As given in section 2 | |
| 3.1.3 | Purity | 89.8% BIT | |
| 3.1.4 | Composition of Product | Not applicable | |
| 3.1.5 | Further relevant properties | Not applicable | |

| | | | |
|---|---|---|---|
| 3.1.6 | Method of analysis | Reverse phase high performance liquid chromatography (HPLC) | |
| **3.2 Preparation of TS solution for poorly soluble or volatile test substances** | | see Table A7.4.1.2.b/01-1 | |
| **3.3 Reference substance** | | No | |
| **3.4 Testing procedure** | | | **X** |
| 3.4.1 | Dilution water | see Table A7.4.1.2.b/01-2 | |
| 3.4.2 | Test organisms | see Table A7.4.1.2.b/01-3 | |
| 3.4.3 | Test system | see Table A7.4.1.2.b/01-4 | |
| 3.4.4 | Test conditions | see Table A7.4.1.2.b/01-5 | **X** |
| 3.4.5 | Duration of the test | 96 h | |
| 3.4.6 | Test parameter | mortality see table A7.4.1.2.b/01-6 | |
| 3.4.7 | Sampling | Samples were collected from mid-depth in the test chambers, placed in glass vials and processed immediately for analysis. | |
| 3.4.8 | Monitoring of TS concentration | Yes, 0, 48 and 96 hours of the study. A problem occurred with the analytical method during the analysis of the 48 h samples. Additional samples were collected at 72 hours from the 1.3, 2.5 and 5.0 mg BIT/L samples to confirm that nominal concentrations were achieved. | |
| 3.4.9 | Statistics | The mortality data were analysed using the computer program of C.E. Stephan [(U.S. Environmental Protection Agency. 1985. Standard Evaluation Procedure, *Acute Toxicity Test for Freshwater Estuarine and Marine Organisms (Estuarine Fish 96-Hour Acute Toxicity Test)*. Hazard Evaluation Division. Office of Pesticide Programs. EPA-540/9-85-006. Washington D.C.]. The program was designed to calculate the $LC_{50}$ value and the 95% confidence interval by probit analysis, the moving average method, and binomial probability with nonlinear interpolation (Finney, D.J. 1971. *Statistical Methods in Biological Assay*. Second edition. Griffin press, London) (Thompson, W.R. 1947. Bacteriological Reviews. Vol. II, No. 2, pages 115-145) (C.E. Stephan 1977. Methods for calculating an $LC_{50}$, *Aquatic Toxicology and Hazard Evaluations*. American Society for Testing and Materials. Publication Number STP 634, pages 65-84). Binomial probability was used to calculate the 48, 72 and 96-hour $LC_{50}$ values. The no-mortality and the NOEC were detesrmined by visual interpretation of the mortality and observation data. | |

**4 RESULTS**

**4.1 Limit Test** Not performed

**4.2 Results test substance**

4.2.1 Initial concentrations of test substance

Nominal (mg BIT/L)

0.31, 0.63, 1.3, 2.5, and 5.0

4.2.2 Actual concentrations of test substance

measured concentration (mg BIT/L)

| Nominal | 0 h | 48 h | 72 h | 96 h | Mean measured |
|---|---|---|---|---|---|
| 0.31 | 0.296 | 0.298 | -- | 0.232 | 0.28 |
| 0.63 | 0.624 | 0.639 | -- | 0.573 | 0.61 |
| 1.3 | 1.29 | -- | 1.21 | 1.21 | 1.2 |
| 2.5 | 2.55 | -- | 2.39 | 2.36 | 2.4 |
| 5.0 | 5.09 | -- | 4.87 | -- | 5.0 |

4.2.3 Effect data (Mortality) see Table A7.4.1.2.b/01-6; see Table A7.4.1.2.b/01-7

4.2.4 Concentration / response curve See Figure A7.4.1.2.b/01-1.

4.2.5 Other effects Mortality, lethargy and erratic swimming

**4.3 Results of controls** No adverse effects

**4.4 Test with reference substance** Not performed

**5 APPLICANT'S SUMMARY AND CONCLUSION**

**5.1 Materials and methods** Yes, US EPA OPPTS 850.1035, Acute 96h mysid flow-through study with analytical confirmation of test solution concentrations.

**5.2 Results and discussion** The test solutions appeared clear and colorless in all test chambers at test initiation and test termination. All water quality parameters were within acceptable limits during the test. Percent mortality in the 2.4 and 5.0 mg BIT/L treatment groups was 80 and 100%, respectively. Surviving mysids in the 2.4 mg BIT/L group exhibited lethargy and

| | | | |
|---|---|---|---|
| | | erratic swimming behavior at test termination. The single mortalities in the 0.28 and 1.2 mg BIT/L groups were not considered to be treatment related. | |
| 5.2.1 | $LC_0$ | 96 h = 1.2 mg BIT/L | |
| 5.2.2 | $LC_{50}$ | 96 h = 1.9 mg BIT/L | |
| 5.2.3 | $LC_{100}$ | 96 h = 5.0 mg BIT/L | |
| **5.3 Conclusion** | | see validity criteria summarized in Table A7.4.1.2.b/01-8 | |
| 5.3.1 | Reliability | (1) reliable without restriction | |
| 5.3.2 | Deficiencies | No | |

| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *December 2010.* |
| **Materials and Methods** | ***3.4**: TA range-finding test (following EPA Guideline 850.1035) should be conducted with both newly hatched (< 24 h) and young adult (5-6 d old) to assess which age-class must be used in the definitive test.*<br>***3.4.4**: The photoperiod used in this study is different that recommended by the EPA Guideline* |
| **Results and discussion** | *Applicant's version is adopted* |
| **Conclusion** | *Applicant's version is adopted* |
| **Reliability** | *2* |
| **Acceptability** | *Acceptable* |
| **Remarks** | |

**Table A7.4.1.2.b/01-1: Preparation of TS solution for poorly soluble or volatile test substances**

| Criteria | Details |
|---|---|
| Dispersion | Yes, sonicated and mixed by inversion |
| Vehicle | Yes, dimethyl formamide (DMF) |
| Concentration of vehicle | 0.1 ml/L in solvent control and in all BIT treatment groups |
| Vehicle control performed | Yes |
| Other procedures | Not applicable |

**Table A7.4.1.2.b/01-2: Dilution water**

| Criteria | Details |
|---|---|
| Source | Filtered seawater from the Indian River Inlet, Delaware, USA |
| Alkalinity | Not described |
| Hardness | Not described |
| PH | 8.0 to 8.3 |
| Aeration | Yes |
| Salinity | 18 to 20 ppt (part per thousand) |
| Ca / Mg ratio | Not described |
| Na / K ratio | Not described |
| Oxygen content | 6.6 to 7.7 mg/L (≥ 90% of saturation) |
| Conductance | Not described |
| Holding water different from dilution water | No |

**Table A7.4.1.2.b/01-3: Test organisms**

| Criteria | Details |
|---|---|
| Strain | Mysid (*Americamysis bahia*) |
| Source | Aquatic BioSystems, Inc., Fort Collins, Colorado, USA |
| Age | Juvenile, < 24 h old |
| Breeding method | Adult mysids were held in the laboratory for 12 days prior to collection of the juveniles |
| Kind of food | Live brine shrimp *Artemia* nauplii |
| Amount of food | *ad libitum* |
| Feeding frequency | Daily |
| Pretreatment | Adult mysids were held for 12 days in water from the same source and temperature as used during the test |
| Feeding of animals during test | Yes, live brine shrimp *Artemia* nauplii daily |

**Table A7.4.1.2.b/01-4: Test system**

| Criteria | Details |
|---|---|
| Renewal of test solution | Test substance was supplied by a continuous flow diluter for 10 volume additions of test water every 24 hourss |
| Volume of test vessels | 25 liter stainless steel aquaria that contained 15-liters of test solution |
| Volume/animal | 1.5 L |
| Number of animals/vessel | 10 |
| Number of vessels/ concentration | 2 |
| Test performed in closed vessels due to significant volatility of TS | No |

**Table A7.4.1.2.b/01-5: Test conditions**

| Criteria | Details |
|---|---|
| Test temperature | 24.2 – 24.7 °C at test initiation |
| Dissolved oxygen | 7.0-7.3 mg/L at test initiation |
| pH | 8.0-8.1 at test initiation |
| Adjustment of pH | not described |
| Salinity | 20 ppt |
| Aeration of dilution water | Yes |
| Quality/Intensity of irradiation | Fluorescent light bulbs, 159 lux |
| Photoperiod | 16 h light and 8 h dark with 30 minute transition period of low light intensity |

**Table A7.4.1.2.b/01-6: Mortality data**

| Test-Substance<br>Concentration (mean measured)<br>[mg BIT/L] | Mortality | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Number | | | | Percentage (%) | | | |
| | 24 h | 48 h | 72 h | 96 h | 24 h | 48 h | 72 h | 96 h |
| Negative control | 0 | 0 | 0 | 0 | -- | -- | -- | -- |
| DMF solvent control | 0 | 0 | 0 | 0 | -- | -- | -- | -- |
| 0.28 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0.5 |
| 0.61 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1.2 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0.5 |
| 2.4 | 0 | 11 | 11 | 16 | 0 | 55 | 55 | 80 |
| 5.0 | 9 | 20 | 20 | 20 | 45 | 100 | 100 | 100 |
| **Temperature [°C]** | -- | -- | -- | 24.5-24.7 | | | | |
| **pH** | 8.0 | 8.1 | 7.9-8.0 | 8.0-8.1 | | | | |
| **Oxygen [mg/l]** | 7.4-7.5 | 7.0-7.3 | 6.0-7.3 | 6.1-7.1 | | | | |
| **Salinity [ppt]** | 20 ppt | 20 ppt | 20 ppt | 20 ppt | | | | |

[1] TS concentrations were measured

**Table A7.4.1.2.b/01-7: Effect data**

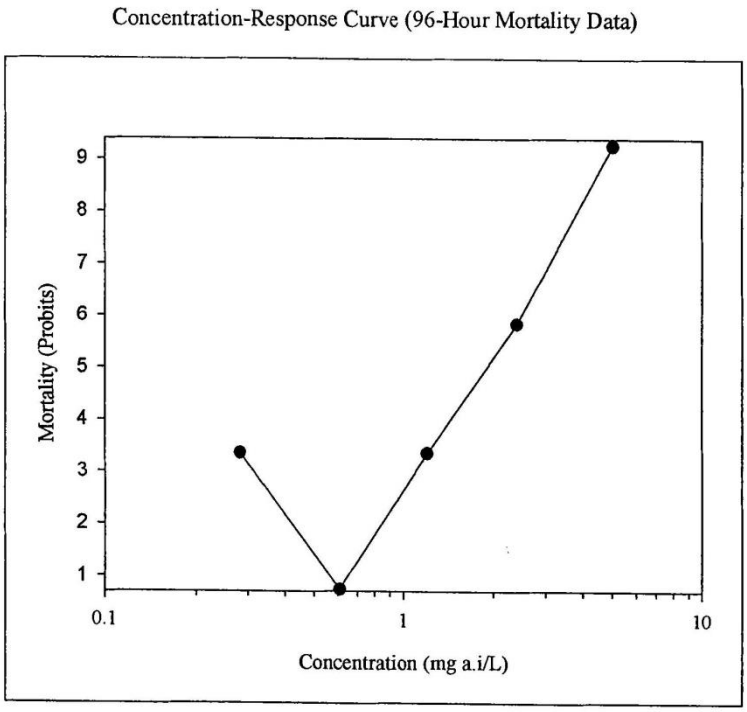
| | $LC_{50}$[1] | 95 % C.I. | $LC_0$[1] | $LC_{100}$[1] |
|---|---|---|---|---|
| **24 h [mg BIT/L]** | > 5.0 (m) | -- | 2.4 (m) | -- |
| **48 h [mg BIT/L]** | 2.3 (m) | 1.2 – 5.0 | 1.2 (m) | 5.0 (m) |
| **72 h [mg BIT/L]** | 2.3 (m) | 1.2 – 5.0 | 1.2 (m) | 5.0 (m) |
| **96 h [mg BIT/L]** | 1.9 (m) | 1.2 – 2.4 | 1.2 (m) | 5.0 (m) |

[1] effect data are based on measured (m) concentrations

**Table A7.4.1.2.b/01-8: Validity criteria**

| | fulfilled | Not fullfilled |
|---|---|---|
| Mortality of control animals < 10% | **yes** | |
| Concentration of dissolved oxygen in all test vessels > 3 mg/L | **yes** | |
| Concentration of test substance ≥ 80% of initial concentration during test | **yes** | |

**Figure A7.4.1.2.b/01-1: Survival of organisms exposed to the test substance, BIT, for 96 hours**



| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.3a/01** | **Growth inhibition test of BIT on algae-Fresh water, *Pseudokirchneriella subcapitata*** |
| **Annex Point IIA VII.7.3** | |

| | | | Official use only |
|---|---|---|---|
| | | **1 REFERENCE** | |
| **1.1** | **Reference** | **A7.4.1.3.a/01** [redacted] (2006b) 1,2--Benzisothiazolin-3-one: A 96-hour toxicity test with the freshwater alga *(Pseudokirchneriella subcapitata)*, [redacted] Rohm and Haas Report N° 06RC-086 (September 20, 2006), Unpublished. | |
| **1.2** | **Data protection** | Yes | |
| 1.2.1. | Data owner | Rohm and Haas Company | |
| 1.2.2. | Companies with letter of access | | |
| 1.2.3. | Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into Annex I/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **Guideline study** | Yes, OECD Guideline 201, EEC Method C.3, US EPA OPPTS 850.5400 | |
| **2.2** | **GLP** | Yes | |
| **2.3** | **Deviations** | No | |
| | | **3 MATERIALS AND METHODS** | |
| **3.1** | **Test material** | 1,2--Benzisothiazolin-3-one (BIT) | |
| 3.1.1 | Lot/Batch number | 2005-051 | |
| 3.1.2 | Specification | As given in section 2 | |
| 3.1.3 | Purity | 89.8% BIT | |
| 3.1.4 | Composition of Product | Not applicable | |
| 3.1.5 | Further relevant properties | Due to the decline in BIT concentrations over the duration of the study, the biological endpoints were based on Day 0 measured concentrations. | |

| | | | |
|---|---|---|---|
| 3.1.6 | Method of analysis | High performance liquid chromatography (HPLC) with UV detector | |
| **3.2 Preparation of TS solution for poorly soluble or volatile test substances** | | see Table A7.4.1.3.a/01-1 | |
| **3.3 Reference substance** | | No | |
| **3.4 Testing procedure** | | | |
| 3.4.1 | Culture medium | Stock nutrient solutions were prepared by adding reagent-grade chemicals to purified well water. The pH of the medium was adjusted to pH 8.0 with 10% HCl and the medium was sterilized by filtration (0.22 µm) prior to use. | |
| 3.4.2 | Test organisms | see Table A7.4.1.3.a/01-2 | |
| 3.4.3 | Test system | see Table A7.4.1.3.a/01-3 | |
| 3.4.4 | Test conditions | see Table A7.4.1.3.a/01-4 | **X** |
| 3.4.5 | Duration of the test | 96 h | |
| 3.4.6 | Test parameter | cell multiplication inhibition | |
| 3.4.7 | Sampling | 0 h: aliquots were collected from the individual batches of test solution prepared for each treatment and control group prior to addition of the algae. At 96 h: samples were from pooled replicates from each treatment and control group. All samples were collected in glass vials and were processed on the day of collection and analyzed as soon as possible. | |
| 3.4.8 | Monitoring of TS concentration | Yes, 0 and 96 h | |
| 3.4.9 | Statistics | The calculation of cell densities, area under the growth curve, growth rates and percent inhibition values, as well as all statistical analyses, were conducted using "SAS System for Windows", Version 8.02 (SAS Institute, Inc., 1999, Cary, North Carolina, USA). The data were evaluated for normality and homogeneity of variance (p = 0.05) using the Shapiro-Wilk's and Levene's tests, respectively. | |
| | | **4 RESULTS** | |
| **4.1 Limit Test** | | Not performed | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.3a/01** | **Growth inhibition test of BIT on algae-Fresh water, *Pseudokirchneriella subcapitata*** |
| **Annex Point IIA VII.7.3** | |

| | | | |
|---|---|---|---|
| **4.2 Results test substance** | | | |
| 4.2.1 | Initial concentrations of test substance | Nominal: 0 (negative control), 0.018, 0.041, 0.091, 0.20, 0.45 and 1.0 mg BIT/L | |
| 4.2.2 | Actual concentrations of test substance | Day 0 Measured: negative control < LOQ (limit of quantitation), 0.019, 0.043, 0.095, 0.21, 0.47 and 1.1 mg BIT/L<br>Day 4 (96 hours) all BIT concentrations were <LOQ. | |
| 4.2.3 | Growth curves | see attached Figure A7.4.1.3.a/01-1 for growth of *Pseudokirchneriella subcapitata* in the negative control | |
| 4.2.4 | Concentration / response curve | see attached Figure A7.4.1.3.a/01-2 | |
| 4.2.5 | Cell concentration data | Not described in report | **X** |
| 4.2.6 | Effect data (cell multiplication inhibition) | 72 h $EC_{50}$ = 0.32 mg BIT/L<br>72 h $E_rC_{50}$ = 0.80 mg BIT/L<br>72 h $E_bC_{50}$ = 0.32 mg BIT/L | **X** |
| 4.2.7 | Other observed effects | Not applicable | |
| **4.3 Results of controls** | | control results performed as expected | |
| **4.4 Test with reference substance** | | Not performed | |
| | | **5 APPLICANT'S SUMMARY AND CONCLUSION** | |
| **5.1 Materials and methods** | | US EPA OPPTS 850.5400, OECD Guideline 201, EEC Method C.3, Acute static 96 h algal study with analytical confirmation of test solution concentrations. | |
| **5.2 Results and discussion** | | The 96 hour $EC_{50}$ is equal to 0.38 mg BIT/L. The freshwater alga was exposed to a geometric series of six test concentrations and a negative control under static conditions for 96 hours. All stock solutions and test solutions appeared clear and colourless at preparation and no precipitates were observed in the test solutions during the test. Samples of test medium collected and analyzed for BIT concentrations resulted in recoveries that ranged from 105 to 106% of nominal concentrations on Day 0 and all < LOQ on Day 4. | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.3a/01** | **Growth inhibition test of BIT on algae-Fresh water, *Pseudokirchneriella subcapitata*** |
| **Annex Point IIA VII.7.3** | |

| | | | |
|---|---|---|---|
| 5.2.1 | $NOE_rC$ | 96 h = 0.47 mg BIT/L | |
| 5.2.2 | $E_rC_{50}$ | 96 h = 0.98 mg BIT/L | |
| 5.2.3 | $E_bC_{50}$ | 96 h = 0.36 mg BIT/L | |
| **5.3 Conclusion** | | see validity criteria in Table A7.4.1.3.a/01-6 | |
| 5.3.1 | Reliability | (1), reliable without restriction | |
| 5.3.2 | Deficiencies | No | |

| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *March 2015* |
| **Materials and Methods** | |
| **Results and discussion** | *The test fulfills the Validity criteria in OECD 201:*<br>• *It fulfills exponential growth criteria.*<br>• *Mean coefficient of variation section by section at 96h = 0.169 and at 72h = 0.2. Meets the criteria and does not exceeds 35%.*<br>• *Coef. of variation of average specific growth rates for 72h = 0.0056 and for 96h = 0.022 meets the criteria and does not exceeds 7%.*<br>• *Initial cell density is 10000 cells/ml fulfilling criteria.* |
| **Conclusion** | *The endpoints were recalculated.*<br>*Initial measured concentrations were used for endpoints calculation since 24h* |

| | |
|---|---|
| | *represents the most sensitive endpoint. An $E_rC50$ = 0.33 mg BIT/l and a ErC10 = 0.032 mg BIT/l was calculated.* |
| **Reliability** | *2* |
| **Acceptability** | *Acceptable* |
| **Remarks** | Cell Density By Replicate Over the 96-Hour Exposure Period |

| Day 0 Measured Test Concentration (mg a.i./L) | Replicate | Cell Density (cells/mL) 24 Hours [1] | 48 Hours | 72 Hours | 96 Hours |
|---|---|---|---|---|---|
| Negative Control | A | 34,334 | 190,493 | 1,233,029 | 6,572,691 |
| | B | 36,993 | 183,054 | 1,185,600 | 6,117,897 |
| | C | 32,655 | 174,583 | 1,170,767 | 5,008,024 |
| 0.019 | A | 31,684 | 189,538 | 1,212,067 | 5,962,431 |
| | B | 33,299 | 184,889 | 1,129,678 | 5,645,357 |
| | C | 30,520 | 178,450 | 1,211,324 | 5,095,540 |
| 0.043 | A | 27,793 | 170,002 | 1,261,301 | 5,864,374 |
| | B | 27,843 | 168,182 | 1,103,894 | 5,435,972 |
| | C | 29,883 | 184,396 | 1,303,178 | 5,673,872 |
| 0.095 | A | 24,085 | 155,191 | 1,042,784 | 5,162,959 |
| | B | 27,532 | 161,607 | 876,990 | 4,108,751 |
| | C | 27,885 | 175,917 | 1,133,576 | 5,539,338 |
| 0.21 | A | 23,365 | 153,781 | 827,483 | 4,029,426 |
| | B | 20,297 | 111,828 | 815,122 | 3,496,899 |
| | C | 23,118 | 136,224 | 593,587 | 3,930,307 |
| 0.47 | A | 18,014 | 100,331 | 680,436 | 3,548,696 |
| | B | 14,693 | 58,432 | 272,661 | 1,486,388 |
| | C | 13,987 | 66,990 | 449,224 | 2,661,253 |
| 1.1 | A | 11,257 | 42,862 | 121,509 | 661,433 |
| | B | 14,545 | 22,565 | 30,012 | 99,220 |
| | C | 10,829 | 21,495 | 21,802 | 46,757 |

[1] The initial cell density of the stock culture was determined and an inoculum volume was administered to each test chamber to yield a cell density of approximately 10,000 cells/mL at test initiation (0 hours).

***Calculation of endpoints:***

*The endpoints evaluated were the 50% effect concentration for growth rate (ErC50), 10% effect concentration (ErC10) and NOEC. They have been estimated fitting the curve taking into account negative growths and later calculating the 50% of the upper asymptote of the curve. NOEC values are estimated using Dunnett's test.*

**Section A7** **Ecotoxicological Profile Including Environmental Fate and Behaviour**

**Subsection A7.4** **Fate and Behaviour in the Environment**

**Subsection A7.4.1** **Aquatic toxicity initial (acute) tests**

**Subsection A7.4.1.3a/01** **Growth inhibition test of BIT on algae-Fresh water, *Pseudokirchneriella subcapitata***

**Annex Point IIA VII.7.3**

| Period | eCA | | |
|---|---|---|---|
| | **ErC50** | **ErC10** | **NOEC** |
| **0-24** | 0.33 (0.26-0.4) | 0.032 (0.01 -0.05) | 0.04 |
| **0-48** | 0.8(0.59-1.02) | 0.19 (0.14 -0.25) | 0.21 |
| **0-72** | 0.99 (0.74 - 1.24) | 0.24 (0.16 - 0.32) | 0.47 |
| **0-96** | 1.31 (0.88 - 1.74) | 0.34 (0.25 – 0.45) | 0.47 |

**Table A7.4.1.3.a/01-1: Preparation of TS solution for poorly soluble or volatile test substances**

| Criteria | Details |
|---|---|
| Dispersion | Yes |
| Vehicle | Yes, purified well water |
| Concentration of vehicle | Not applicable |
| Vehicle control performed | Yes dilution water control |
| Other procedures | Not applicable |

**Table A7.4.1.3.a/01-2: Test organisms**

| Criteria | Details |
|---|---|
| Species | *Pseudokirchneriella subcapitata* |
| Strain | Not applicable |
| Source | University of Toronto Culture Collection 37 |
| Laboratory culture | Yes |
| Method of cultivation | sterile algal medium identical to medium used in the toxicity test |
| Pretreatment | Actively growing in culture medium for at least two weeks prior to test initiation |
| Initial cell concentration | 1.0 x $10^6$ cells/ml; each test vessel was inoculated with 1.0 ml to yield 10,000 cells/mL at test initiation |

**Table A7.4.1.3.a/01-3: Test system**

| Criteria | Details |
|---|---|
| Volume of culture flasks | 250 mL containing 100 mL test solution |
| Culturing apparatus | haemocytometer and a microscope |
| Light quality | cool-white fluorescent lights |
| Procedure for suspending algae | rotary shaker adjusted to 100 rpm |
| Number of vessels/ concentration | 3 |
| Test performed in closed vessels due to significant volatility of TS | Erlenmeyer flasks were plugged with foam stoppers |

**Table A7.4.1.3.a/01-4: Test conditions**

| Criteria | Details |
|---|---|
| Test temperature | 24 ± 2 °C |
| pH | 7.9 to 8.0 on Day 0 and 8.1 to 8.4 on Day 4 |
| Aeration of dilution water | Not described |
| Light intensity | 4300 ± 10% lux |
| Photoperiod | 24 h photoperiod daily |

**Table A7.4.1.3.a/01-5: Cell concentration data**

| Test-Substance Concentration (measured)[1] [mg BIT/L] | Cell density (mean values) [cells x $10^3$/ml] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean cell density | | | | Percent inhibition | | | |
| | 24 h | 48 h | 72 h | 96 h | 24 h | 48 h | 72 h | 96 h |
| 0 (control) | 35 | 183 | 1196 | 5899 | -- | -- | -- | -- |
| 0.019 | 32 | 184 | 1184 | 5568 | 8.2 | -0.87 | 1.0 | 5.6 |
| 0.0.43 | 29 | 174 | 1223 | 5658 | 18 | 4.6 | -2.2 | 4.1 |
| 0.095 | 27 | 164 | 1018 | 4937 | 24 | 10 | 15 | 16 |
| 0.21 | 22 | 134 | 745 | 3819 | 36 | 27 | 38 | 35 |
| 0.47 | 16 | 75 | 467 | 2565 | 55 | 59 | 61 | 57 |
| 1.1 | 12 | 29 | 58 | 269 | 65 | 84 | 95 | 95 |
| **Temperature [°C]** | 24.5 | 24.6 | 24.5 | 24.0 | | | | |
| **pH** | 7.9 to 8.0 on Day 0; 8.1 to 8.4 on Day 4 | | | | | | | |

[1] TS concentrations were Day 0 measured concentrations

**Table A7.4.1.3.a/01-6: Validity criteria for algal growth inhibition test**

| | fulfilled | Not fulfilled |
|---|---|---|
| Cell concentration in control cultures increased at least by a factor of 16 within 3 days | **yes** | |
| Concentration of test substance ≥ 80% of initial concentration during test | | **yes** |

**Figure A7.4.1.3.a/01-1: Growth of the freshwater alga, *Pseudokirchneriella subcapitata*, in the negative control during the toxicity test with BIT**

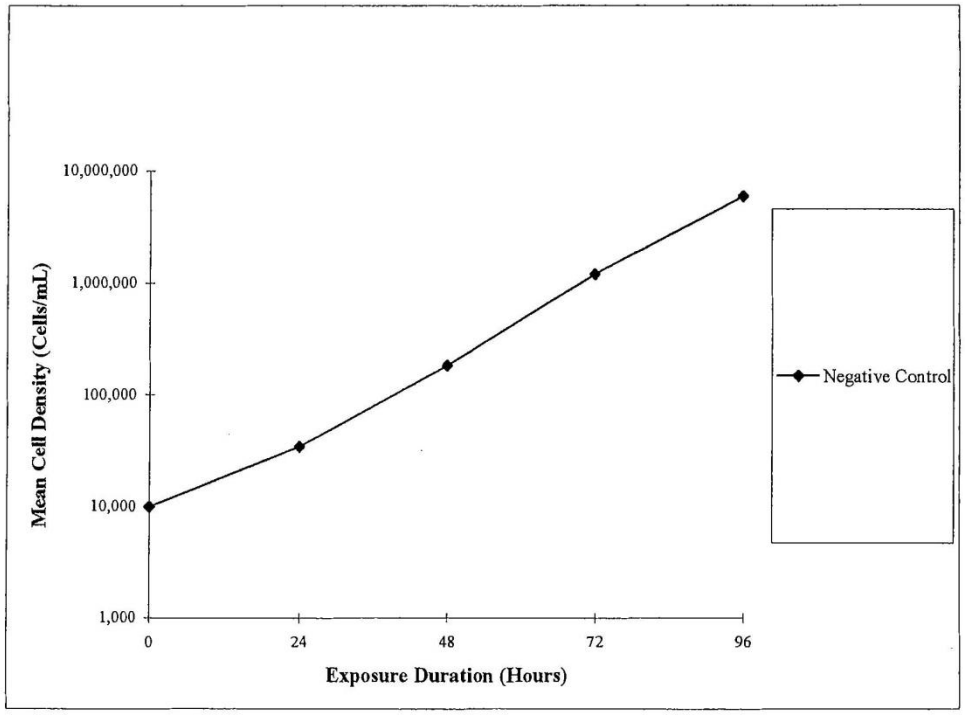
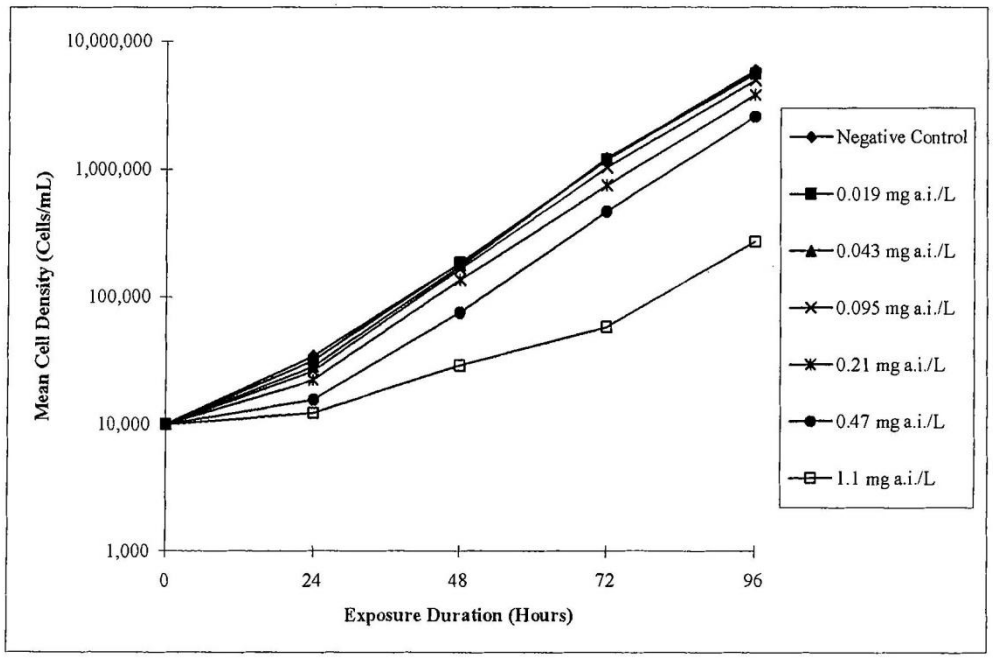



**Figure A7.4.1.3.a/01-2: Concentration response curve for *Pseudokirchneriella subcapitata*, exposed to BIT for 96 hours, expressed as cell density**



| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.3.b/01** | **Growth inhibition test of BIT on algae-Marine water, *Skeletonema costatum*** |
| **Annex Point IIA VII.7.3** | |

| | | | |
|---|---|---|---|
| | | **1 REFERENCE** | **Official use only** |
| **1.1** | **Reference** | **A7.4.1.3.b/01** [redacted] (2006a) 1,2-Benzisothiazolin-3-one: A 96-hour toxicity test with the marine diatom (*Skeletonema costatum*), [redacted], Rohm and Haas Report N° 06RC-087 (September 18, 2006), GLP, Unpublished. | |
| **1.2** | **Data protection** | Yes | |
| 1.2.1. | Data owner | Rohm and Haas Company | |
| 1.2.2. | Companies with letter of access | | |
| 1.2.3. | Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into Annex I/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **Guideline study** | Yes, OECD 201, EU Directive 92/69/EEC Method C.3, US EPA OPPTS 850.5400 | |
| **2.2** | **GLP** | Yes | |
| **2.3** | **Deviations** | No | |
| | | **3 MATERIALS AND METHODS** | |
| **3.1** | **Test material** | | |
| 3.1.1 | Lot/Batch number | 1,2-Benzisothiazolin-3-one | |
| 3.1.2 | Specification | 2005-051 | |
| 3.1.3 | Purity | As given in section 2 | |
| 3.1.4 | Composition of Product | 89.8% | |
| 3.1.5 | Further relevant properties | not applicable | |
| 3.1.6 | Method of | not applicable | |

| | | |
|---|---|---|
| | analysis | |
| **3.2 Preparation of TS solution for poorly soluble or volatile test substances** | | see Table A7.4.1.3.b/01-1 |
| **3.3 Reference substance** | | No |
| **3.4 Testing procedure** | | |
| 3.4.1 | Culture medium | Saltwater algal medium at a salinity of 30 parts per thousand was adjusted to pH 8.0 ± 0.1 with 10% HCl and was sterilized by filtration (0.22 µm) prior to use. |
| 3.4.2 | Test organisms | see Table A7.4.1.3.b/01-2 |
| 3.4.3 | Test system | see Table A7.4.1.3.b/01-3 |
| 3.4.4 | Test conditions | see Table A7.4.1.3.b/01-4 |
| 3.4.5 | Duration of the test | 96 h |
| 3.4.6 | Test parameter | algal growth inhibition |
| 3.4.7 | Sampling | 0 h samples were collected from test solutions prior to the addition of algae. 96 h samples were collected from pooled replicates from test solutions. All samples were collected in glass vials and were processed on the day of collection and analyzed as soon as possible. |
| 3.4.8 | Monitoring of TS concentration | Yes, 0 and 96 h |
| 3.4.9 | Statistics | The calculation of cell densities, areas under the growth curve, growth rates and percent inhibition values and all statistical analyses were conducted using "The SAS System for Windows", Version 8.02 (The SAS System for Windows, 1999, version 8.02. SAS Institute, Cary, North Carolina, USA). Non-linear regression was used to calculate $EC_{50}$ values and their corresponding 95% confidence intervals for each 24 hour exposure period. (Bruce, R.D. and Versteeg, D.J., 1992. A Statistical Procedure for Modeling Continuous Toxicity Data. Environmental Toxicology and Chemistry 11: 1485-1494) The data were evaluated for normality and homogeneity of variance (p=0.05) using the Shapiro-Wilk's and Levene's tests, respectively. The results of the statistical analyses and an evaluation of the concentration-response pattern were used to determine the NOEC at 72 and 96 hours. |

| | | |
|---|---|---|
| | | **4 RESULTS** |
| **4.1 Limit Test** | | Not performed |
| **4.2 Results test substance** | | |
| 4.2.1 | Initial concentrations of test substance | Nominal (mg BIT/L)<br>0, 0.019, 0.038, 0.075, 0.15, 0.30 and 0.60 |
| 4.2.2 | Actual concentrations of test substance | measured (mg BIT/L)<br>LOQ = limit of quantitation |
| 4.2.3 | Growth curves | see Figure A7.4.1.3.b/01-1 |
| 4.2.4 | Concentration / response curve | see Figure A7.4.1.3.b/01-2 |
| 4.2.5 | Cell concentration data | see table A7.4.1.3.b/01-5 |
| 4.2.6 | Effect data (cell multiplication inhibition) | 72 h (mg BIT/L)<br>$EC_{50}$, cell density: 0.26; NOAEC, cell density: 0.074<br>$E_bC_{50}$, area under the growth curve: 0.19; NOAEC: 0.074<br>$E_rC_{50}$, growth rate: 0.35; NOAEC: 0.15<br><br>96 h (mg BIT/L) |

| 0 h | 96 h |
|---|---|
| 0.017 | < LOQ |
| 0.039 | < LOQ |
| 0.074 | < LOQ |
| 0.15 | < LOQ |
| 0.31 | < LOQ |
| 0.60 | 0.018 |

| | | | |
|---|---|---|---|
| | | $EC_{50}$, cell density: 0.40; NOAEC, cell density: 0.15<br>$E_bC_{50}$, area under the growth curve: 0.23; NOAEC: 0.074<br>$E_rC_{50}$, growth rate: 0.42; NOAEC: 0.15 | |
| 4.2.7 | Other observed effects | Not applicable | |
| **4.3 Results of controls** | | control results performed as expected | **X** |
| **4.4 Test with reference substance** | | Not performed | |
| | | **5 APPLICANT'S SUMMARY AND CONCLUSION** | |
| **5.1 Materials and methods** | | Yes, OECD 201, EU Directive 92/69/EEC Method C.3, US EPA OPPTS 850.5400, static 96 h marine algal study with analytical confirmation of test solution concentrations | |
| **5.2 Results and discussion** | | All stock solutions and test solutions appeared clear and colorless at preparation and no precipitates were observed. Five of the six measured concentrations in test media samples collected at 96 h were < the limit of quantitation (< 0.010 mg BIT/L). | |
| 5.2.1 | $NOE_rC$ | 0.15 mg BIT/L (95% confidence interval: | |
| 5.2.2 | $E_rC_{50}$ | 0.42 mg BIT/L (95% confidence interval: 0.39 - 0.47 mg BIT/L)) | |
| 5.2.3 | $E_bC_{50}$ | 0.23 mg BIT/L (95% confidence interval: 0.21 - 0.26 mg BIT/L) | |
| **5.3 Conclusion** | | see validity criteria in table, below | |
| 5.3.1 | Reliability | (1), reliable without restriction | |
| 5.3.2 | Deficiencies | No | |

| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *March 2015* |
| **Materials and Methods** | *A 96-hour toxicity test with the marine diatom (Skeletonema costatum) of BIT (purity 89.8%) was conducted following OECD TG 201, EC Method C.3, and US EPA* |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.3.b/01** | **Growth inhibition test of BIT on algae-Marine water, *Skeletonema costatum*** |

**Annex Point IIA VII.7.3**

| | |
|---|---|
| | *OPPTS 850.5400.*<br>*The initial cell density was ca. 77000 cells/ml for each test flask.* |
| **Results and discussion** | *Nominal concentrations of BIT were: 0, 0.019, 0.038, 0.075, 0.15, 0.30 and 0.60 mg BIT/L. Analytical confirmation of test solution concentrations was performed at 0 h and at 96 h. It was shown that at 96 h the concentrations decreased to below the LOQ.*<br>*The endpoints were estimated on the basis of nominal concentrations because it was considered that measured concentrations do not represent well the exposure during the test.*<br>*In the control there was not an exponential growth during the test, at least the cell density did not increase by a factor of 16 within the 72-hour test period. Thus the validity criteria were not met.*<br>*At 72 h the following endpoints were calculated:*<br>*$EC_{50}$, cell density: 0.26; NOAEC, cell density: 0.074*<br>*$E_bC_{50}$, area under the growth curve: 0.19; NOAEC: 0.074*<br>*$E_rC_{50}$, growth rate: 0.35; NOAEC: 0.15* |
| **Conclusion** | *The test did not pass the validity criteria. However the test was well conducted and there is sufficient information to recalculate the endpoints in a different way (see details below), based on measured concentrations and corrected by the purity of BIT.*<br>*The relevant endpoints are: 24h-$E_rC_{50}$ = 0.030 mg BIT/L and 24h-$NOE_rC$ = 0.019 mg BIT/L.*<br>*These values can be considered as additional information.* |
| **Reliability** | *3 (supporting information)* |
| **Acceptability** | *Non-acceptable* |
| **Remarks** | ***4.3**: The increase in biomass in the controls was lower than the factor (16) recommended by the OECD TG201.*<br>*Calculation of endpoints:*<br>*The Applicant calculated the endpoints based on nominal concentrations because measured concentrations at 96h were below the limit of quantification (LOQ). The OECD Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures recommends in these cases to take as final concentrations the half of the LOQ. Thus to estimate the relevant exposure concentrations the RMS has taken the geometric means of the initial measured concentrations (at 0 h) and the ½ LOQ. The LOQ of the analytical method was 0.01 mg BIT/L.* |

| *Nominal [mg/L BIT]* | *Actual [mg/L BIT]* | | *Geometric Mean [mg/L BIT]* |
|---|---|---|---|
| | *0 h* | *96 h* | |
| *0* | *0.005* | *0.005* | *0.0050* |
| *0.019* | *0.017* | *0.005* | *0.0092* |
| *0.038* | *0.039* | *0.005* | *0.0140* |
| *0.075* | *0.074* | *0.005* | *0.0192* |
| *0.15* | *0.15* | *0.005* | *0.0274* |
| *0.3* | *0.31* | *0.005* | *0.0394* |
| *0.6* | *0.6* | *0.005* | *0.0548* |

*Additionally, isothiazolones are known to have a very rapid toxicity action towards algae. To take this into account the algal growth inhibition should be taken at the time when the algae show the highest growth inhibition. A comparison of the $E_rC_{50}$ values calculated by the RMS is presented below.*

| | |
|---|---|
| *$E_rC_{50}$ (24 h)* | ***0.030 mg/L*** |
| *$E_rC_{50}$ (48 h)* | *0.034 mg/L* |
| *$E_rC_{50}$ (72 h)* | *0.048 mg/L* |
| *$E_rC_{50}$ (96 h)* | *0.043 mg/L* |

*A comparison of the $E_rC_{50}$ values shows that the most sensitive endpoint is the one estimated at 24 hours. This confirms the mode of action of BIT to algae. Therefore this last value can be considered the best estimation for BIT under the test conditions.*

*The $NOE_rC$ at 24 h is 0.019 mg BIT/L.*

**Table A7.4.1.3.b/01-1: Preparation of TS solution for poorly soluble or volatile test substances**

| Criteria | Details |
|---|---|
| Dispersion | Yes, sonic bath and mixed by inversion |
| Vehicle | Yes, Dimethyl formamide (DMF) |
| Concentration of vehicle | The concentration of DMF in the solvent control and all treatment groups was 0.1 mL/L |
| Vehicle control performed | Yes, dimethylformamide |
| Other procedures | Not applicable |

**Table A7.4.1.3.b/01-2: Test organisms**

| Criteria | Details |
|---|---|
| Species | *Skeletonema costatum* |
| Strain | CCMP 1332 |
| Source | CCMP-Provasoli-Guillard National Center for the Culture of Marine Phytoplankton |
| Laboratory culture | Yes |
| Method of cultivation | Not described |
| Pretreatment | The algal culture used for this toxicity test had been actively growing in culture medium for at least two weeks prior to test initiation. The culture was last transferred to fresh medium three days prior to test initiation. |
| Initial cell concentration | approximately 77000 cells/ml for each test flask |

**Table A7.4.1.3.b/01-3: Test system**

| Criteria | Details |
|---|---|
| Volume of culture flasks | 250 mL Erlenmeyer flasks containing 100 mL test solution plugged with foam stoppers |
| Culturing apparatus | The flasks were shaken continuously at 100 rpm; cell counts were determined using a hemacytometer and a microscope |
| Light quality | cool white fluorescent lights |
| Procedure for suspending algae | Not described |
| Number of vessels/ concentration | 3 |
| Test performed in closed vessels due to significant volatility of TS | No |

**Table A7.4.1.3.b/01-4: Test conditions**

| Criteria | Details |
|---|---|
| Test temperature | 20 ± 2 °C |
| pH | 7.9 to 8.6 |
| Aeration of dilution water | Not described |
| Light intensity | 4310 ± 650 lux |
| Photoperiod | 16 h light, 8 h darkness |

**Table A7.4.1.3.b/01-5: Cell concentration data**

| Test-Substance Concentration (measured)[1] [mg BIT/L] | Cell concentrations (mean values) [cells x $10^3$/mL] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | measured | | | | Percent Inhibition | | | |
| | 24 h | 48 h | 72 h | 96 h | 24 h | 48 h | 72 h | 96 h |
| 0 (control) | 220 | 856 | 991 | 901 | -- | -- | -- | -- |
| 0.0174 | 200 | 778 | 976 | 844 | 9.2 | 9.1 | 1.5 | 6.3 |
| 0.0386 | 184 | 786 | 954 | 772 | 16 | 8.1 | 3.8 | 14 |
| 0.0740 | 178 | 805 | 965 | 857 | 19 | 6.0 | 2.7 | 4.9 |
| 0.153 | 142 | 539 | 758 | 864 | 35 | 37 | 24 | 4.0 |
| 0.312 | 82 | 170 | 379 | 647 | 63 | 80 | 62 | 28 |
| 0.602 | 67 | 100 | 113 | 94 | 69 | 88 | 89 | 90 |
| **Temperature [°C]** | 20 ± 2 °C | | | | | | | |
| **pH** | 7.9 on Day 0 and 8.2 to 8.6 on Day 4 | | | | | | | |

[1] TS concentrations were Day 0 measured concentrations

**Table A7.4.1.3.b/01-6: Validity criteria for algal growth inhibition test**

| | fulfilled | Not fullfilled |
|---|---|---|
| Cell concentration in control cultures increased at least by a factor of 16 within 3 days | | **yes** |
| Concentration of test substance ≥ 80% of initial concentration during test | | **yes** |

**Figure A7.4.1.3.b/01-1: Growth of the marine alga, *Skeletonema costatum*, during the toxicity test with BIT**

**Figure 1.** Growth of *Skeletonema costatum* in the negative control over the 96-hour exposure period, expressed as cell density.

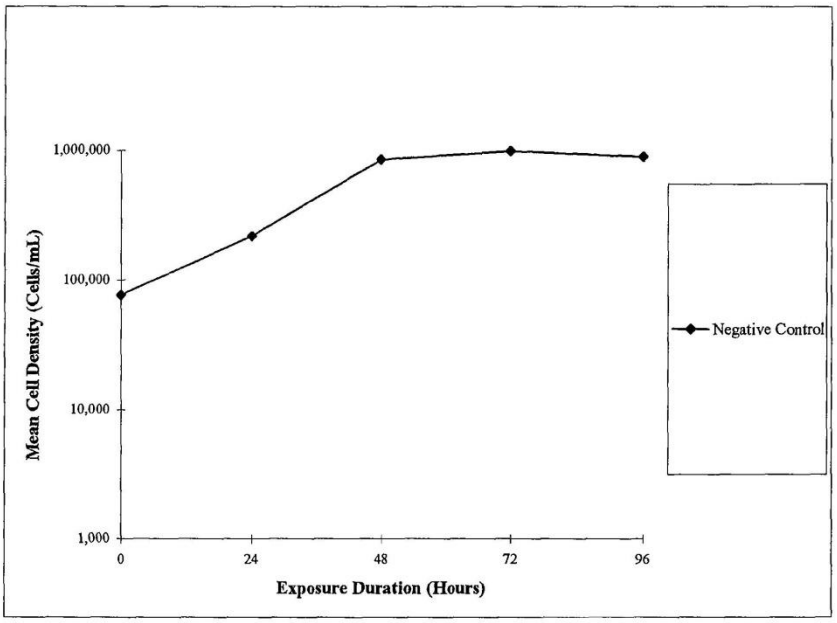
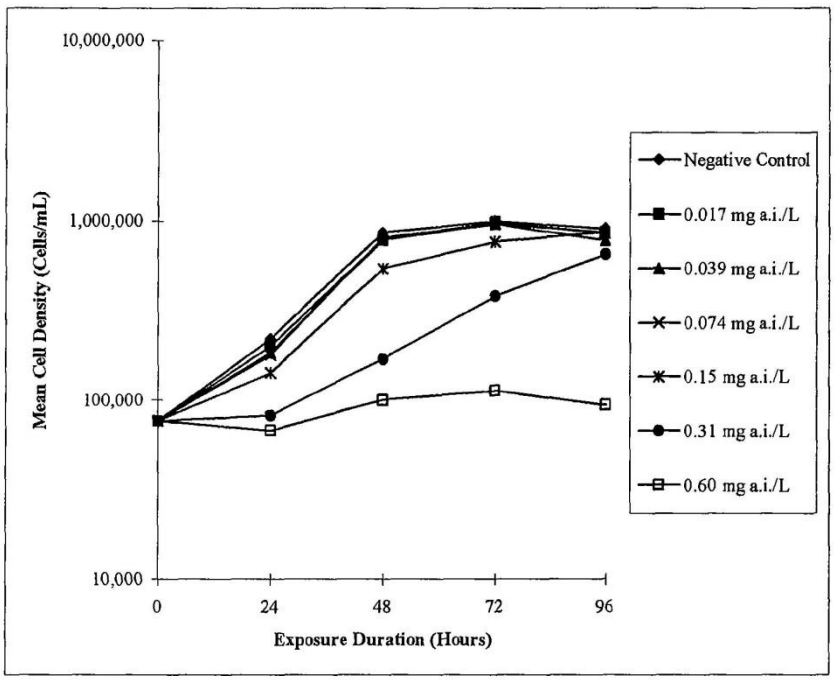



**Figure A7.4.1.3.b/01-2: Concentration/response curve of the marine alga, *Skeletonema costatum*, during the toxicity test with BIT**

**Figure 2.** Concentration-response curve for *Skeletonema costatum* exposed to 1,2-Benzisothiazolin-3-one for 96 hours, expressed as cell density.



| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.4**<br>**Annex Point IIA VII.7.4 and IIIA VII.3** | **Inhibition to microbial activity (aquatic, activated sludge)** |

**Official use only**

**1 REFERENCE**

| | | |
|---|---|---|
| **1.1 Reference** | | **A7.4.1.4/01** [redacted] (2006) 1,2-Benzisothiazolin-3-one: An activated sludge, respiration inhibition test; [redacted], Rohm and Haas Report N° 06RC-088 (August 14, 2006), Unpublished. |
| **1.2 Data protection** | | Yes |
| 1.2.1. | Data owner | Rohm and Haas Company |
| 1.2.2. | Companies with letter of access | |
| 1.2.3. | Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into AnnexI/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. |

**2 GUIDELINES AND QUALITY ASSURANCE**

| | |
|---|---|
| **2.1 Guideline study** | Yes, OECD 209 |
| **2.2 GLP** | Yes |
| **2.3 Deviations** | No |

**3 MATERIALS AND METHODS**

| | | |
|---|---|---|
| **3.1 Test material** | | 1,2-benzisothiazolin-3-one |
| 3.1.1 | Lot/Batch number | 2005-051 |
| 3.1.2 | Specification | As given in section 2. |
| 3.1.3 | Purity | 89.9% BIT |
| 3.1.4 | Composition of Product | Not applicable |
| 3.1.5 | Further relevant properties | Not applicable |
| 3.1.6 | Method of analysis | High performance liquid chromatography (HPLC) |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.4** | **Inhibition to microbial activity (aquatic, activated sludge)** |
| **Annex Point IIA VII.7.4 and IIIA VII.3** | |

**Official use only**

**1 REFERENCE**

| | |
|---|---|
| **1.1 Reference** | **A7.4.1.4/01** [redacted] (2006) 1,2-Benzisothiazolin-3-one: An activated sludge, respiration inhibition test; [redacted], Rohm and Haas Report N° 06RC-088 (August 14, 2006), Unpublished. |
| **1.2 Data protection** | Yes |
| 1.2.1. Data owner | Rohm and Haas Company |
| 1.2.2. Companies with letter of access | |
| 1.2.3. Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into AnnexI/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. |

**2 GUIDELINES AND QUALITY ASSURANCE**

| | |
|---|---|
| **2.1 Guideline study** | Yes, OECD 209 |
| **2.2 GLP** | Yes |
| **2.3 Deviations** | No |

**3 MATERIALS AND METHODS**

| | |
|---|---|
| **3.1 Test material** | 1,2-benzisothiazolin-3-one |
| 3.1.1 Lot/Batch number | 2005-051 |
| 3.1.2 Specification | As given in section 2. |
| 3.1.3 Purity | 89.9% BIT |
| **3.2 Preparation of TS solution for poorly soluble or volatile test substances** | see Table A7.4.1.4/01-1 |
| **3.3 Reference substance** | 3,5-dichlorophenol |
| 3.3.1 Method of analysis for reference substance | Not measured in this assay. |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.4** | **Inhibition to microbial activity (aquatic, activated sludge)** |
| **Annex Point IIA VII.7.4 and IIIA VII.3** | |

**Official use only**

**1 REFERENCE**

**1.1 Reference**

**A7.4.1.4/01** [redacted] (2006) 1,2-Benzisothiazolin-3-one: An activated sludge, respiration inhibition test; [redacted], Rohm and Haas Report N° 06RC-088 (August 14, 2006), Unpublished.

**1.2 Data protection** Yes

1.2.1. Data owner — Rohm and Haas Company

1.2.2. Companies with letter of access

1.2.3. Criteria for data protection — Data on existing a.s. submitted for the first time in support of the first inclusion into AnnexI/IA.

Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive.

**2 GUIDELINES AND QUALITY ASSURANCE**

**2.1 Guideline study** Yes, OECD 209

**2.2 GLP** Yes

**2.3 Deviations** No

**3 MATERIALS AND METHODS**

**3.1 Test material** 1,2-benzisothiazolin-3-one

3.1.1 Lot/Batch number — 2005-051

3.1.2 Specification — As given in section 2.

3.1.3 Purity — 89.9% BIT

**3.4 Testing procedure**

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.4**<br>**Annex Point IIA VII.7.4 and IIIA VII.3** | **Inhibition to microbial activity (aquatic, activated sludge)** |

**Official use only**

**1 REFERENCE**

**1.1 Reference**

**A7.4.1.4/01** [redacted]. (2006) 1,2-Benzisothiazolin-3-one: An activated sludge, respiration inhibition test; [redacted], Rohm and Haas Report N° 06RC-088 (August 14, 2006), Unpublished.

**1.2 Data protection**

Yes

1.2.1. Data owner

Rohm and Haas Company

1.2.2. Companies with letter of access

1.2.3. Criteria for data protection

Data on existing a.s. submitted for the first time in support of the first inclusion into AnnexI/IA.

Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive.

**2 GUIDELINES AND QUALITY ASSURANCE**

**2.1 Guideline study**

Yes, OECD 209

**2.2 GLP**

Yes

**2.3 Deviations**

No

**3 MATERIALS AND METHODS**

**3.1 Test material**

1,2-benzisothiazolin-3-one

3.1.1 Lot/Batch number

2005-051

3.1.2 Specification

As given in section 2.

3.1.3 Purity

89.9% BIT

3.4.1 Culture medium

Activated sludge collected from the Denton Wastewater Treatment Plant, Denton, Maryland on June 5, 2006 was utilized as the inoculum for the test. The Denton facility receives wastes from predominately domestic sources. The sludge was sieved using a 2 mm screen and allowed to settle for approximately 30 minutes. The supernatant above the settled solids was removed and the total suspended solids (TSS) concentration of the settled sludge was determined. Total suspended solids in the settled sludge were adjusted to a nominal concentration of approximately 4000 mg/L by dilution with municipal water. 50 mL of synthetic sludge was added to each liter of adjusted sludge. The sludge was maintained at a temperature of 20 ± 2°C and continuously aerated overnight. Before use, the pH and total suspended solids concentration of the activated sludge were determined.

| | | | |
|---|---|---|---|
| **Section A7** | | **Ecotoxicological Profile Including Environmental Fate and Behaviour** | |
| **Subsection A7.4** | | **Fate and Behaviour in the Environment** | |
| **Subsection A7.4.1** | | **Aquatic toxicity initial (acute) tests** | |
| **Subsection A7.4.1.4**<br>**Annex Point IIA VII.7.4 and IIIA VII.3** | | **Inhibition to microbial activity (aquatic, activated sludge)** | |
| | | **1 REFERENCE** | **Official use only** |
| **1.1 Reference** | | **A7.4.1.4/01** [redacted] (2006) 1,2-Benzisothiazolin-3-one: An activated sludge, respiration inhibition test; [redacted], Rohm and Haas Report N° 06RC-088 (August 14, 2006), Unpublished. | |
| **1.2 Data protection** | | Yes | |
| 1.2.1. | Data owner | Rohm and Haas Company | |
| 1.2.2. | Companies with letter of access | | |
| 1.2.3. | Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into AnnexI/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1 Guideline study** | | Yes, OECD 209 | |
| **2.2 GLP** | | Yes | |
| **2.3 Deviations** | | No | |
| | | **3 MATERIALS AND METHODS** | |
| **3.1 Test material** | | 1,2-benzisothiazolin-3-one | |
| 3.1.1 | Lot/Batch number | 2005-051 | |
| 3.1.2 | Specification | As given in section 2. | |
| 3.1.3 | Purity | 89.9% BIT | |
| 3.4.2 | Inoculum/ test organism | see Table A7.4.1.4/01-2 | |
| 3.4.3 | Test system | see Table A7.4.1.4/01-3 | |
| 3.4.4 | Test conditions | see Table A7.4.1.4/01-4 | **X** |
| 3.4.5 | Duration of the test | 3 h contact time for each concentration of the reference substance or the TS with the activated sludge | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.4** | **Inhibition to microbial activity (aquatic, activated sludge)** |
| **Annex Point IIA VII.7.4 and IIIA VII.3** | |

| | | | Official use only |
|---|---|---|---|
| | | **1 REFERENCE** | |
| **1.1 Reference** | | **A7.4.1.4/01** [redacted] (2006) 1,2-Benzisothiazolin-3-one: An activated sludge, respiration inhibition test; [redacted] Rohm and Haas Report N° 06RC-088 (August 14, 2006), Unpublished. | |
| **1.2 Data protection** | | Yes | |
| 1.2.1. | Data owner | Rohm and Haas Company | |
| 1.2.2. | Companies with letter of access | | |
| 1.2.3. | Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into AnnexI/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1 Guideline study** | | Yes, OECD 209 | |
| **2.2 GLP** | | Yes | |
| **2.3 Deviations** | | No | |
| | | **3 MATERIALS AND METHODS** | |
| **3.1 Test material** | | 1,2-benzisothiazolin-3-one | |
| 3.1.1 | Lot/Batch number | 2005-051 | |
| 3.1.2 | Specification | As given in section 2. | |
| 3.1.3 | Purity | 89.9% BIT | |
| 3.4.6 | Test parameter | respiration inhibition | |
| 3.4.7 | Analytical parameter | dissolved oxygen concentrations | |
| 3.4.8 | Sampling | respiration rate was measured at 10 second intervals over a 10 minute period or until dissolved oxygen concentrations fell below 1.0 mg/L using a dissolved oxygen meter. | |
| 3.4.9 | Monitoring of TS concentration | No | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.4** | **Inhibition to microbial activity (aquatic, activated sludge)** |
| **Annex Point IIA VII.7.4 and IIIA VII.3** | |

**Official use only**

**1 REFERENCE**

| | |
|---|---|
| **1.1 Reference** | **A7.4.1.4/01** [redacted] (2006) 1,2-Benzisothiazolin-3-one: An activated sludge, respiration inhibition test; [redacted], Rohm and Haas Report N° 06RC-088 (August 14, 2006), Unpublished. |
| **1.2 Data protection** | Yes |
| 1.2.1. Data owner | Rohm and Haas Company |
| 1.2.2. Companies with letter of access | |
| 1.2.3. Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into AnnexI/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. |

**2 GUIDELINES AND QUALITY ASSURANCE**

| | |
|---|---|
| **2.1 Guideline study** | Yes, OECD 209 |
| **2.2 GLP** | Yes |
| **2.3 Deviations** | No |

**3 MATERIALS AND METHODS**

| | |
|---|---|
| **3.1 Test material** | 1,2-benzisothiazolin-3-one |
| 3.1.1 Lot/Batch number | 2005-051 |
| 3.1.2 Specification | As given in section 2. |
| 3.1.3 Purity | 89.9% BIT |
| 3.4.10 Controls | The control contained 9.6 mL of synthetic sewage, 120 mL of inoculum and municipal water to bring up the total volume to 300 ml. |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.4**<br>**Annex Point IIA VII.7.4 and IIIA VII.3** | **Inhibition to microbial activity (aquatic, activated sludge)** |

**Official use only**

## 1 REFERENCE

| | | |
|---|---|---|
| **1.1 Reference** | | **A7.4.1.4/01** [redacted]. (2006) 1,2-Benzisothiazolin-3-one: An activated sludge, respiration inhibition test; [redacted], Rohm and Haas Report N° 06RC-088 (August 14, 2006), Unpublished. |
| **1.2 Data protection** | | Yes |
| 1.2.1. | Data owner | Rohm and Haas Company |
| 1.2.2. | Companies with letter of access | |
| 1.2.3. | Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into AnnexI/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. |

## 2 GUIDELINES AND QUALITY ASSURANCE

| | |
|---|---|
| **2.1 Guideline study** | Yes, OECD 209 |
| **2.2 GLP** | Yes |
| **2.3 Deviations** | No |

## 3 MATERIALS AND METHODS

| | | |
|---|---|---|
| **3.1 Test material** | | 1,2-benzisothiazolin-3-one |
| 3.1.1 | Lot/Batch number | 2005-051 |
| 3.1.2 | Specification | As given in section 2. |
| 3.1.3 | Purity | 89.9% BIT |
| 3.4.11 | Statistics | A respiration rate was calculated for each test mixture and expressed in mg $O_2$/L/hour. The rate was calculated using dissolved oxygen (DO) values between 6.5 mg $O_2$/L and 2.5 mg $O_2$/L, or over a 10 minute period if the dissolved oxygen did not reach approximately 2.5 mg $O_2$/L. The respiration rate was calculated using the following calculation:<br>Respiration rate=[(initial DO-final DO)/(final time-initial time)]x3600 seconds/hour<br>Percent inhibition was calculated using the following calculation:<br>Percent Inhibition=[1-(2Rs/RC1 + RC2)] x 100<br>Rs = oxygen consumption rate at a given concentration of the TS<br>RC1 = oxygen consumption rate, control 1<br>RC2 = oxygen consumption rate, control 2 |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.4** | **Inhibition to microbial activity (aquatic, activated sludge)** |
| **Annex Point IIA VII.7.4 and IIIA VII.3** | |

**Official use only**

**1 REFERENCE**

**1.1 Reference**

**A7.4.1.4/01** [redacted] (2006) 1,2-Benzisothiazolin-3-one: An activated sludge, respiration inhibition test; [redacted] Rohm and Haas Report N° 06RC-088 (August 14, 2006), Unpublished.

**1.2 Data protection**

Yes

1.2.1. Data owner

Rohm and Haas Company

1.2.2. Companies with letter of access

1.2.3. Criteria for data protection

Data on existing a.s. submitted for the first time in support of the first inclusion into AnnexI/IA.

Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive.

**2 GUIDELINES AND QUALITY ASSURANCE**

**2.1 Guideline study**

Yes, OECD 209

**2.2 GLP**

Yes

**2.3 Deviations**

No

**3 MATERIALS AND METHODS**

**3.1 Test material**

1,2-benzisothiazolin-3-one

3.1.1 Lot/Batch number

2005-051

3.1.2 Specification

As given in section 2.

3.1.3 Purity

89.9% BIT

**4 RESULTS**

**4.1 Preliminary test**

Not Performed

4.1.1 Concentration

Not applicable

4.1.2 Effect data

Not applicable

**4.2 Results test substance**

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.4**<br>**Annex Point IIA VII.7.4 and IIIA VII.3** | **Inhibition to microbial activity (aquatic, activated sludge)** |

**Official use only**

**1 REFERENCE**

| | |
|---|---|
| **1.1 Reference** | **A7.4.1.4/01** [redacted] (2006) 1,2-Benzisothiazolin-3-one: An activated sludge, respiration inhibition test; [redacted], Rohm and Haas Report N° 06RC-088 (August 14, 2006), Unpublished. |
| **1.2 Data protection** | Yes |
| 1.2.1. Data owner | Rohm and Haas Company |
| 1.2.2. Companies with letter of access | |
| 1.2.3. Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into AnnexI/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. |

**2 GUIDELINES AND QUALITY ASSURANCE**

| | |
|---|---|
| **2.1 Guideline study** | Yes, OECD 209 |
| **2.2 GLP** | Yes |
| **2.3 Deviations** | No |

**3 MATERIALS AND METHODS**

| | |
|---|---|
| **3.1 Test material** | 1,2-benzisothiazolin-3-one |
| 3.1.1 Lot/Batch number | 2005-051 |
| 3.1.2 Specification | As given in section 2. |
| 3.1.3 Purity | 89.9% BIT |
| 4.2.1 Initial concentrations of test substance | Nominal: 1, 3, 10, 30, 100, 300 and 1000 mg a.i./L |
| 4.2.2 Actual concentrations of test substance | Not applicable |
| 4.2.3 Growth curves | Not applicable |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.4** | **Inhibition to microbial activity (aquatic, activated sludge)** |
| **Annex Point IIA VII.7.4 and IIIA VII.3** | |

**Official use only**

**1 REFERENCE**

| | |
|---|---|
| **1.1 Reference** | **A7.4.1.4/01** [redacted] (2006) 1,2-Benzisothiazolin-3-one: An activated sludge, respiration inhibition test; [redacted] Rohm and Haas Report N° 06RC-088 (August 14, 2006), Unpublished. |
| **1.2 Data protection** | Yes |
| 1.2.1. Data owner | Rohm and Haas Company |
| 1.2.2. Companies with letter of access | |
| 1.2.3. Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into AnnexI/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. |

**2 GUIDELINES AND QUALITY ASSURANCE**

| | |
|---|---|
| **2.1 Guideline study** | Yes, OECD 209 |
| **2.2 GLP** | Yes |
| **2.3 Deviations** | No |

**3 MATERIALS AND METHODS**

| | |
|---|---|
| **3.1 Test material** | 1,2-benzisothiazolin-3-one |
| 3.1.1 Lot/Batch number | 2005-051 |
| 3.1.2 Specification | As given in section 2. |
| 3.1.3 Purity | 89.9% BIT |
| 4.2.4 Cell concentration data | Not applicable |
| 4.2.5 Concentration/ response curve | See Figure A7.4.1.4/01-1 |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.4** | **Inhibition to microbial activity (aquatic, activated sludge)** |
| **Annex Point IIA VII.7.4 and IIIA VII.3** | |

**Official use only**

## 1 REFERENCE

**1.1 Reference**

**A7.4.1.4/01** [redacted] (2006) 1,2-Benzisothiazolin-3-one: An activated sludge, respiration inhibition test; [redacted], Rohm and Haas Report N° 06RC-088 (August 14, 2006), Unpublished.

**1.2 Data protection** Yes

1.2.1. Data owner — Rohm and Haas Company

1.2.2. Companies with letter of access

1.2.3. Criteria for data protection — Data on existing a.s. submitted for the first time in support of the first inclusion into AnnexI/IA.

Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive.

## 2 GUIDELINES AND QUALITY ASSURANCE

**2.1 Guideline study** Yes, OECD 209

**2.2 GLP** Yes

**2.3 Deviations** No

## 3 MATERIALS AND METHODS

**3.1 Test material** 1,2-benzisothiazolin-3-one

3.1.1 Lot/Batch number — 2005-051

3.1.2 Specification — As given in section 2.

3.1.3 Purity — 89.9% BIT

4.2.6 Effect data — Nominal concentration mg BIT per liter

| Concentration | Respiration Rate (mg $O_2$/L/hour) | Percent Inhibition |
|---|---|---|
| 1 | 52.4 | -36.3 |
| 3 | 33.5 | 12.9 |
| 10 | 33.5 | 12.9 |
| 30 | 18.3 | 52.4 |
| 100 | 6.1 | 84.1 |
| 300 | 1.8 | 95.3 |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.4** | **Inhibition to microbial activity (aquatic, activated sludge)** |
| **Annex Point IIA VII.7.4 and IIIA VII.3** | |

**Official use only**

**1 REFERENCE**

**1.1 Reference**

**A7.4.1.4/01** [redacted] (2006) 1,2-Benzisothiazolin-3-one: An activated sludge, respiration inhibition test; [redacted] Rohm and Haas Report N° 06RC-088 (August 14, 2006), Unpublished.

**1.2 Data protection** Yes

1.2.1. Data owner — Rohm and Haas Company

1.2.2. Companies with letter of access

1.2.3. Criteria for data protection — Data on existing a.s. submitted for the first time in support of the first inclusion into AnnexI/IA.

Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive.

**2 GUIDELINES AND QUALITY ASSURANCE**

**2.1 Guideline study** Yes, OECD 209

**2.2 GLP** Yes

**2.3 Deviations** No

**3 MATERIALS AND METHODS**

**3.1 Test material** 1,2-benzisothiazolin-3-one

3.1.1 Lot/Batch number — 2005-051

3.1.2 Specification — As given in section 2.

3.1.3 Purity — 89.9% BIT

4.2.7 Other observed effects

**4.3 Results of controls** respiration rate = 36.9 and 40.0 mg $O_2$/L/h

**4.4 Test with reference substance** Performed

4.4.1 Concentrations — 3,5-dichlorophenol: 3.0, 15, 50 mg/L

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.4** | **Inhibition to microbial activity (aquatic, activated sludge)** |
| **Annex Point IIA VII.7.4 and IIIA VII.3** | |

**Official use only**

**1 REFERENCE**

| | |
|---|---|
| **1.1 Reference** | **A7.4.1.4/01** [redacted] (2006) 1,2-Benzisothiazolin-3-one: An activated sludge, respiration inhibition test; [redacted] Rohm and Haas Report N° 06RC-088 (August 14, 2006), Unpublished. |
| **1.2 Data protection** | Yes |
| 1.2.1. Data owner | Rohm and Haas Company |
| 1.2.2. Companies with letter of access | |
| 1.2.3. Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into AnnexI/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. |

**2 GUIDELINES AND QUALITY ASSURANCE**

| | |
|---|---|
| **2.1 Guideline study** | Yes, OECD 209 |
| **2.2 GLP** | Yes |
| **2.3 Deviations** | No |

**3 MATERIALS AND METHODS**

| | |
|---|---|
| **3.1 Test material** | 1,2-benzisothiazolin-3-one |
| 3.1.1 Lot/Batch number | 2005-051 |
| 3.1.2 Specification | As given in section 2. |
| 3.1.3 Purity | 89.9% BIT |
| 4.4.2 Results | $EC_{50}$ = 15.86 mg/L (95% confidence limits: 3 and 50 mg/L) |

**5 APPLICANT'S SUMMARY AND CONCLUSION**

| | |
|---|---|
| **5.1 Materials and methods** | OECD 209, Activated sludge, respiration inhibition test |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.4**<br>**Annex Point IIA VII.7.4 and IIIA VII.3** | **Inhibition to microbial activity (aquatic, activated sludge)** |

**Official use only**

**1 REFERENCE**

| | |
|---|---|
| **1.1 Reference** | **A7.4.1.4/01** [redacted] (2006) 1,2-Benzisothiazolin-3-one: An activated sludge, respiration inhibition test; [redacted] Rohm and Haas Report N° 06RC-088 (August 14, 2006), Unpublished. |
| **1.2 Data protection** | Yes |
| 1.2.1. Data owner | Rohm and Haas Company |
| 1.2.2. Companies with letter of access | |
| 1.2.3. Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into AnnexI/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. |

**2 GUIDELINES AND QUALITY ASSURANCE**

| | |
|---|---|
| **2.1 Guideline study** | Yes, OECD 209 |
| **2.2 GLP** | Yes |
| **2.3 Deviations** | No |

**3 MATERIALS AND METHODS**

| | |
|---|---|
| **3.1 Test material** | 1,2-benzisothiazolin-3-one |
| 3.1.1 Lot/Batch number | 2005-051 |
| 3.1.2 Specification | As given in section 2. |
| 3.1.3 Purity | 89.9% BIT |
| **5.2 Results and discussion** | Nominal concentrations of BIT were used for all calculations. The respiration rates observed in the two controls were 36.9 and 40.0 mg $O_2$/L/h with a difference of approximately 7.8%. The $EC_{50}$ value for the reference substance was 15.86 mg/L with 95% confidence limits of 3 and 50 and was within the 5 to 30% mg/L range considered acceptable for the test. The $EC_{50}$ value for 1,2-Benzisothiazolin-3-one was 28.52 mg/L with 95% confidence limits of 10 and 100. The $EC_{50}$ and 95% confidence limits were calculated using binomial probability with nonlinear interpolation. Inhibitory effects upon respiration by 1,2-Benzisothiazolin-3-one at the concentrations evaluated in this study exhibited a concentration dependent dose response pattern. |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.4**<br>**Annex Point IIA VII.7.4 and IIIA VII.3** | **Inhibition to microbial activity (aquatic, activated sludge)** |

**Official use only**

**1 REFERENCE**

| | |
|---|---|
| **1.1 Reference** | **A7.4.1.4/01** [redacted]. (2006) 1,2-Benzisothiazolin-3-one: An activated sludge, respiration inhibition test; [redacted] Rohm and Haas Report N° 06RC-088 (August 14, 2006), Unpublished. |
| **1.2 Data protection** | Yes |
| 1.2.1. Data owner | Rohm and Haas Company |
| 1.2.2. Companies with letter of access | |
| 1.2.3. Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into AnnexI/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. |

**2 GUIDELINES AND QUALITY ASSURANCE**

| | |
|---|---|
| **2.1 Guideline study** | Yes, OECD 209 |
| **2.2 GLP** | Yes |
| **2.3 Deviations** | No |

**3 MATERIALS AND METHODS**

| | |
|---|---|
| **3.1 Test material** | 1,2-benzisothiazolin-3-one |
| 3.1.1 Lot/Batch number | 2005-051 |
| 3.1.2 Specification | As given in section 2. |
| 3.1.3 Purity | 89.9% BIT |
| 5.2.1 NOEC | 3 h = between 1 and 3 mg BIT/L |
| 5.2.2 EC50 | 3 h = 28.52 mg BIT/L (95% C.I. 10 and 100 mg/L) |
| 5.2.3 EC80 | Not calculated. 84.1% inhibition was observed at 100 mg BIT/L. |
| **5.3 Conclusion** | |
| 5.3.1 Reliability | (1), reliable without restriction |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.4**<br>**Annex Point IIA VII.7.4 and IIIA VII.3** | **Inhibition to microbial activity (aquatic, activated sludge)** |

| | | | Official use only |
|---|---|---|---|
| | | **1 REFERENCE** | |
| **1.1 Reference** | | **A7.4.1.4/01** [redacted] (2006) 1,2-Benzisothiazolin-3-one: An activated sludge, respiration inhibition test; [redacted] Rohm and Haas Report N° 06RC-088 (August 14, 2006), Unpublished. | |
| **1.2 Data protection** | | Yes | |
| 1.2.1. | Data owner | Rohm and Haas Company | |
| 1.2.2. | Companies with letter of access | | |
| 1.2.3. | Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into AnnexI/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1 Guideline study** | | Yes, OECD 209 | |
| **2.2 GLP** | | Yes | |
| **2.3 Deviations** | | No | |
| | | **3 MATERIALS AND METHODS** | |
| **3.1 Test material** | | 1,2-benzisothiazolin-3-one | |
| 3.1.1 | Lot/Batch number | 2005-051 | |
| 3.1.2 | Specification | As given in section 2. | |
| 3.1.3 | Purity | 89.9% BIT | |
| 5.3.2 | Deficiencies | No | |

| **Evaluation by Competent Authorities** | |
|---|---|
| **EVALUATION BY RAPPORTEUR MEMBER STATE** | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.4**<br>**Annex Point IIA VII.7.4 and IIIA VII.3** | **Inhibition to microbial activity (aquatic, activated sludge)** |

| | | | Official use only |
|---|---|---|---|
| | | **1 REFERENCE** | |
| **1.1 Reference** | | **A7.4.1.4/01** [redacted] (2006) 1,2-Benzisothiazolin-3-one: An activated sludge, respiration inhibition test; [redacted] Rohm and Haas Report N° 06RC-088 (August 14, 2006), Unpublished. | |
| **1.2 Data protection** | | Yes | |
| 1.2.1. | Data owner | Rohm and Haas Company | |
| 1.2.2. | Companies with letter of access | | |
| 1.2.3. | Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into AnnexI/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1 Guideline study** | | Yes, OECD 209 | |
| **2.2 GLP** | | Yes | |
| **2.3 Deviations** | | No | |
| | | **3 MATERIALS AND METHODS** | |
| **3.1 Test material** | | 1,2-benzisothiazolin-3-one | |
| 3.1.1 | Lot/Batch number | 2005-051 | |
| 3.1.2 | Specification | As given in section 2. | |
| 3.1.3 | Purity | 89.9% BIT | |

| | |
|---|---|
| **Date** | *December 2010* |
| **Materials and Methods** | *Applicant's version is adopted with the following remark:*<br>***3.4.4**: It is necessary to describe aeration of the dilution water and the air flow.* |
| **Results and discussion** | *Applicant's version is adopted.* |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.1** | **Aquatic toxicity initial (acute) tests** |
| **Subsection A7.4.1.4**<br>**Annex Point IIA VII.7.4 and IIIA VII.3** | **Inhibition to microbial activity (aquatic, activated sludge)** |

| | | | Official use only |
|---|---|---|---|
| | | **1 REFERENCE** | |
| **1.1 Reference** | | **A7.4.1.4/01** [redacted] (2006) 1,2-Benzisothiazolin-3-one: An activated sludge, respiration inhibition test; [redacted] Rohm and Haas Report N° 06RC-088 (August 14, 2006), Unpublished. | |
| **1.2 Data protection** | | Yes | |
| 1.2.1. | Data owner | Rohm and Haas Company | |
| 1.2.2. | Companies with letter of access | | |
| 1.2.3. | Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into AnnexI/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1 Guideline study** | | Yes, OECD 209 | |
| **2.2 GLP** | | Yes | |
| **2.3 Deviations** | | No | |
| | | **3 MATERIALS AND METHODS** | |
| **3.1 Test material** | | 1,2-benzisothiazolin-3-one | |
| 3.1.1 | Lot/Batch number | 2005-051 | |
| 3.1.2 | Specification | As given in section 2. | |
| 3.1.3 | Purity | 89.9% BIT | |

| | |
|---|---|
| **Conclusion** | *Applicant's version is adopted*<br>*Additionally eCA calculated EC10 using linear regression and the Michaelis Menten model which results in a EC10 = 4.12 mg a.s./l BIT.* |
| **Reliability** | *2* |
| **Acceptability** | *Acceptable* |
| **Remarks** | |

**Table A7.4.1.4/01-1: Preparation of TS solution for poorly soluble or volatile test substances**

| Criteria | Details |
|---|---|
| Dispersion | Yes |
| Vehicle | No |
| Concentration of vehicle | Not applicable |
| Vehicle control performed | Not applicable |
| Other procedures | Not applicable |

**Table A7.4.1.4/01-2: Inoculum / Test organism**

| Criteria | Details |
|---|---|
| Nature | activated sludge |
| Species | Not applicable |
| Strain | Not applicable |
| Source | municipal wastewater treatment plant in Denton, Maryland, USA which treats predominantly domestic waste |
| Sampling site | Not described |
| Laboratory culture | No |
| Method of cultivation | Not applicable |
| Preparation of inoculum for exposure | The sludge was sieved using a 2 mm screen and allowed to settle for approximately 30 minutes. The supernatant above the settled solids was removed and the total suspended solids (TSS) concentration of the settled sludge was determined. he sludge was maintained at a temperature of 20 ± 2 °C and continuously aerated overnight. Before use, the pH and total suspended solids concentration of the activated sludge were determined. |
| Pretreatment | Not described |
| Initial cell concentration | Total suspended solids in the settled sludge were adjusted to a nominal concentration of approximately 4000 mg/L by dilution with municipal water. 50 mL of synthetic sludge was added to each liter of adjusted sludge. |

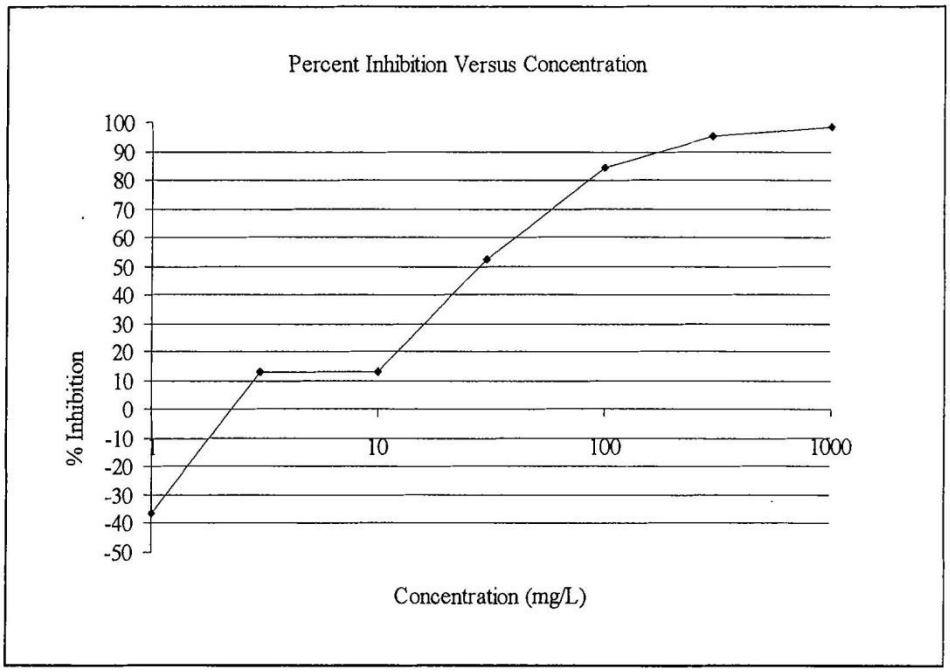
**Table A7.4.1.4/01-3: Test system**

| Criteria | Details |
|---|---|
| Culturing apparatus | 500 mL plastic Erlenmeyer flasks were used for the 3 h incubation period then placed into BOD bottles |
| Number of culture flasks/concentration | 2 controls and 1 for each reference substance and test substance concentration |
| Aeration device | vessels were aerated for 3 h using pressurized laboratory air |
| Measuring equipment | dissolved oxygen was measured with YSI Model 50B dissolved oxygen meter |
| Test performed in closed vessels due to significant volatility of TS | No |

**Table A7.4.1.4/01-4: Test conditions**

| Criteria | Details |
|---|---|
| Test temperature | 20 $\pm$ 2 °C |
| pH | 7.8 at test initiation |
| Aeration of dilution water | Not described |
| Suspended solids concentration | 4327 mg/L at test initiation |

**Figure A7.4.1.4/01-1: Percent Inhibition versus Concentration for 1,2-Benzisothiazolin-3-one**



| | | |
|---|---|---|
| **Section A7**<br><br>**Subsection A7.4**<br>**Subsection A7.4.2**<br>**Annex Point IIA7.5** | **Ecotoxicological Profile Including Environmental Fate and Behaviour**<br><br>**Fate and Behaviour in the Environment**<br>**Estimation of bioconcentration** | |
| | **JUSTIFICATION FOR NON-SUBMISSION OF DATA** | **Official use only** |
| **Other existing data [ x ]** | **Technically not feasible [ ] Scientifically unjustified [ ]** | |
| **Limited exposure [ ]** | **Other justification [ ]** | |
| **Detailed justification:** | A waiver is requested for performing a fish bio-accumulation study for BIT. This request is based on the log P (log octanol:water partition coefficient) for this compound.<br>• Log P < 1.5<br>This value indicates that the potential for BIT to bioaccumulate will be minimal. Many regulatory schemes have established that a log P of less than 3 precludes the need to perform a bio-accumulation study. Additionally using the US EPA's EPIWIN software suite the calculated Bioconcentration Factor (BCF) is 1.646 (Log BCF = 0.216).<br>Therefore, based on the log P values, bio-accumulation studies would not provide any necessary additional data but would result in the unnecessary sacrifice of animals. | |
| **Undertaking of intended data submission [ ]** | No. | |
| | | |
| | | |
| | **Evaluation by Competent Authorities** | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | *January 2011.* | |
| **Evaluation of applicant's justification** | *Applicant's justification is accepted.* | |
| **Conclusion** | *Applicant's justification is accepted.* | |
| **Remarks** | | |

| | | |
|---|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** | |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** | |
| **Subsection A7.4.3** | **Effects on aquatic organisms, further studies** | |
| **Subsection A7.4.3.1** | **Prolonged toxicity to an appropriate species of fish** | |
| **Annex Point IIIA XIII.2.1** | | |
| | **JUSTIFICATION FOR NON-SUBMISSION OF DATA** | **Official use only** |
| **Other existing data [ x]** | **Technically not feasible [ ] Scientifically unjustified [ ]** | |
| **Limited exposure [ ]** | **Other justification [ ]** | |
| **Detailed justification:** | As outlined in the "Technical guidance document in support of the directive 98/8/EC concerning the placing of biocidal products on the market", this test is not required as it does not add information as needed in the risk assessment. The existing guidelines are not sufficient. Other studies are available under section A7.4.3.2. | |
| **Undertaking of intended data submission [ ]** | No. | |
| | | |
| | | |
| | **Evaluation by Competent Authorities** | |

| | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *January 2011* |
| **Evaluation of applicant's justification** | *Applicant's justification is accepted* |
| **Conclusion** | *Applicant's justification is accepted.* |
| **Remarks** | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.3** | **Effects on aquatic organisms, further studies** |
| **Section A7.4.3.2.a/01** | **Effects on reproduction and growth rate of fish- Early life stage test, Fresh water fish, Fathead minnow** |
| **Annex Point IIIA XIII.2.2** | |

| | | | Official use only |
|---|---|---|---|
| | | **1 REFERENCE** | |
| **1.1** | **Reference** | **A7.4.3.2.a/01** [redacted] (2007b). 1,2-Benzisothiazolin-3-one: An early life-stage toxicity test with the fathead minnow (Pimephales promelas), [redacted] Rohm and Haas Report N° 06RC-090 (January 16, 2007), Unpublished. | |
| **1.2** | **Data protection** | Yes | |
| 1.2.1. | Data owner | Rohm and Haas Company | |
| 1.2.2. | Companies with letter of access | | |
| 1.2.3. | Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into Annex I/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **Guideline study** | Yes, OECD 210 and US EPA OPPTS 850.1400 | |
| **2.2** | **GLP** | Yes | |
| **2.3** | **Deviations** | No | |
| | | **3 Method** | |
| **3.1** | **Test material** | 1,2-Benzisothiazolin-3-one (BIT) | |
| 3.1.1 | Lot/Batch number | 2005-051 | |
| 3.1.2 | Specification | As given in section 2 | |
| 3.1.3 | Purity | 89.8% BIT | |
| 3.1.4 | Composition of Product | Not applicable | |

| | | | |
|---|---|---|---|
| 3.1.5 | Further relevant properties | Not applicable | |
| 3.1.6 | Method of analysis | High performance liquid chromatography with UV detection | |
| **3.2 Preparation of TS solution for poorly soluble or volatile test substances** | | see Table A7.4.3.2.a/01-1 | |
| **3.3 Reference substance** | | No | |
| **3.4 Testing procedure** | | | |
| 3.4.1 | Dilution water | see Table A7.4.3.2.a/01-2 | |
| 3.4.2 | Test organisms | see Table A7.4.3.2.a/01-3 | |
| 3.4.3 | Handling of embryos and larvae (OECD 210/212) | Embryos were removed from 10 individual spawning substrates and examined under a dissecting microscope to select healthy, viable specimens at approximately the same stage of development. Embryos were added to incubation cups in the test chambers. After a 5-day embryo hatching period, the larvae were released into the test chambers where exposure to BIT continued during a 28-day post-hatch juvenile growth period. | |
| 3.4.4 | Test system | see Table A7.4.3.2.a/01-4 | |
| 3.4.5 | Test conditions | see Table A7.4.3.2.a/01-5 | **X** |
| 3.4.6 | Duration of the test | 33 days (5 day hatch and 28 day post-hatch) | |
| 3.4.7 | Test parameter(s) | Time to hatch, hatching success, growth and survival | |
| 3.4.8 | Examination / Sampling | During the first day of exposure, embryos were examined twice for mortality and eggs with fungus. Observations of embryo mortality and the removal of dead embryos were performed once daily during the hatching period. During the 28-day post-hatch period, the larvae were observed daily for mortality, clinical signs of toxicity and abnormal behavior. Total length, wet weight and dry weight were measured on surviving fish. | |
| 3.4.9 | Monitoring of | Samples were collected from each treatment group and control group on | |

| | | |
|---|---|---|
| | TS concentration | Days 0, 7, 14, 21, 28 and 33 (test termination) and processed immediately for analysis. |
| 3.4.10 | Statistics | Post-hatch survival was calculated as the number of larvae surviving to test termination divided by the total number of embryos hatched successfully. Time to hatch data were evaluated by visual interpretation of the data. Hatching success and survival were analysed using Chi-square and Fisher's Exact tests.growth data were evaluated for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test ($p = 0.01$). Those treatments that were significantly different from the control means were identified using Dunnett's t-test ($p = 0.05$). All statistical test were performed with SAS software (The SAS System for Windows. 2001. Version 8.2. SAS Institute, Inc., Cary, North Carolina, USA) |
| | | **4 RESULTS** |
| **4.1 Range finding test** | | |
| 4.1.1 | Concentrations | Not described in report |
| 4.1.2 | Number/ percentage of animals showing adverse effects | Not described in report |
| 4.1.3 | Nature of adverse effects | Not described in report |
| **4.2 Results test substance** | | |
| 4.2.1 | Initial concentrations of test substance | Nominal concentrations (mg BIT/L)<br>0.31, 0.63, 1.3, 2.5 and 5.0 |
| 4.2.2 | Actual concentrations of test substance | Mean measured concentrations (mg BIT/L): |

| Nominal concentration | Mean measured |
|---|---|
| Negative control | < LOQ |
| Solvent control | < LOQ |

| | |
|---|---|
| 0.31 | 0.28 |
| 0.63 | 0.59 |
| 1.3 | 1.2 |
| 2.5 | 2.4 |
| 5.0 | 4.8 |

4.2.3 Effect data

The majority of fish in the 0.28, 0.59 and 1.2 mg BIT/L treatment groups appeared normal throughout the test. Several fish in the 2.4 mg BIT/L group were surfacing between days 2 and 4 but the fish appeared normal from day 5 through test termination. Several fish in the 4.8 mg BIT/L group were weak, surfacing, swimming erractically or with morphological abnormalities such as crooked spines. Most of these 4.8 mg BIT/L weakened fish died prior to test termination.

4.2.4 Concentration / response curve

Not described in report

4.2.5 Other effects

| BIT concentration | Fish total length (mm) | Fish wet weight (mg) | Fish dry weight (mg) |
|---|---|---|---|
| Negative control | 22.7 | 84.7 | 15.8 |
| Solvent control | 23.0 | 93.8 | 16.6 |
| 0.28 mg BIT/L | 22.9 | 91.5 | 16.8 |
| 0.59 mg BIT/L | 22.3 * | 85.3 * | 15.4 |
| 1.2 mg BIT/L | 22.8 | 88.5 | 16.6 |
| 2.4 mg BIT/L | 22.1 | 81.3 | 15.6 |
| 4.8 mg BIT/L | 21.1 | 67.7 | 12.7 |

The 1.2, 2.4 and 4.8 mg BIT/L groups were excluded from analyses of growth due to significant effects on larval survival.

* statistically significantly different from the pooled control (total length and dry weight) or the solvent control (wet weight) using Dunnett's test ($p \leq 0.05$).

Day 28 post-hatch mortality:

| BIT concentration | Number dead / Number hatched |
|---|---|
| Negative control | 4 / 77 |
| Solvent control | 8 / 76 |
| 0.28 mg BIT/L | 10 / 80 |
| 0.59 mg BIT/L | 8 / 78 |
| 1.2 mg BIT/L | 13 / 79 |
| 2.4 mg BIT/L | 34 / 75 |
| 4.8 mg BIT/L | 50 / 79 |

| | | |
|---|---|---|
| **4.3 Results of controls** | | |
| 4.3.1 | Number/ percentage of animals showing adverse effects | Not applicable |
| 4.3.2 | Nature of adverse effects | Not applicable |
| **4.4 Test with reference substance** | | Not performed |
| | **5 APPLICANT'S SUMMARY AND CONCLUSION** | |
| **5.1 Materials and methods** | | OECD 210 and US EPA OPPTS 850.1400, Early life stage toxicity study to fish under flow-through conditions with analytical confirmation of TS concentrations. |
| **5.2 Results and discussion** | | All environmental conditions were within acceptable limits during the test. Test solutions appeared clear and colorless in all test chambers with no precipitates noted during the test. There were no treatment-related effects on time to hatch or hatching success. All surviving fish appeared normal at 28 days post-hatch. The most sensitive end point was growth. The Maximum Acceptable Toxicant Concentration (MATC) = 0.41 mg BIT/L. |
| 5.2.1 | NOEC | 0.28 mg BIT/L, based on growth-related effects |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.3** | **Effects on aquatic organisms, further studies** |
| **Section A7.4.3.2.a/01** | **Effects on reproduction and growth rate of fish- Early life stage test, Fresh water fish, Fathead minnow** |
| **Annex Point IIIA XIII.2.2** | |

| | | | |
|---|---|---|---|
| 5.2.2 | LOEC | 0.59 mg BIT/L, based on growth-related effects | |
| **5.3 Conclusion** | | see Table A7.4.3.2.A/01-6 | |
| 5.3.1 | Other Conclusions | Not applicable | |
| 5.3.2 | Reliability | (1), reliable without restriction | |
| 5.3.3 | Deficiencies | No | |

| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *December 2010.* |
| **Materials and Methods** | ***3.4.5****: The water temperature differ more than ±1.5 ºC* |
| **Results and discussion** | *Applicant's version adopted* |
| **Conclusion** | *Applicant's version adopted* |
| **Reliability** | *2* |
| **Acceptability** | *Acceptable* |
| **Remarks** | |

**Table A7.4.3.2.a/01-1: Preparation of TS solution for poorly soluble or volatile test substances**

| Criteria | Details |
|---|---|
| Dispersion | Yes, sonicated and mixed by inversion |
| Vehicle | Dimethyl formamide (DMF) |
| Concentration of vehicle | 0.1 mL/L in solvent control and in all BIT treatment groups |
| Vehicle control performed | Yes |
| Other procedures | Not applicable |

**Table A7.4.3.2.a/01-2: Dilution water**

| Criteria | Details |
|---|---|
| Source | Filtered and sterilized freshwater obtained from a well approximately 40 meters deep located on the Wildlife International Limited site, Easton, Maryland, USA |
| Salinity | Not applicable |
| Hardness | 136 to 144 mg/L as $CaCO_3$ |
| pH | 8.1 |
| Oxygen content | 8.3 to 8.4 mg/L |
| Conductance | 340 to 350 µmhos/cm |
| Alkalinity | 180 to 185 mg/L as $CaCO_3$ |
| Holding water different from dilution water | No |

**Table A7.4.3.2.a/01-3: Test organisms**

| Criteria | Details |
|---|---|
| Species/strain | Fathead minnow (*Pimephales promelas*) |
| Source | Chesapeake Cultures, Inc., Hayes, Virgina, USA |
| Wild caught | no |
| Age/size | Embryos < 24 h old |
| Kind of food | Live brine shrimp nauplii (*Artemia* species) |
| Amount of food | *Ad libitum* |
| Feeding frequency | 3 times per day during first 7 days post-hatch. 3 times per day on weekdays and two times per day on weekends for the next 19 days. Fish were not fed for the 48 h prior to study termination to allow for clearance of the digestive tracts before weight measurements were made. |
| Post-hatch transfer time | 5 days post-hatch |
| Time to first feeding | 7 days post-hatch |
| Feeding of animals during test | yes |
| Treatment for disease within 2 weeks preceding test | No |

**Table A7.4.3.2.a/01-4: Test system**

| Criteria | Details |
|---|---|
| Test type | Flow-through |
| Renewal of test solution | A continuous-flow diluter and syringe pump were used to deliver the controls and BIT solutions into mixing chambers where the controls and BIT solutions were diluted with water and delivered to the test chambers. The diluter flow rate was adjusted to provide 10 volume additions of test solutions in each test chamber per day. |
| Volume of test vessels | 9 liter glass aquaria containing 7 liters of test solution |
| Volume/animal | 0.35 liters |
| Number of animals/vessel | 20 |
| Number of vessels/ concentration | 4 |
| Test performed in closed vessels due to significant volatility of TS | Not applicable |

**Table A7.4.3.2.a/01-5: Test conditions**

| Criteria | Details |
|---|---|
| Test temperature | 24.0 – 25.7 °C |
| Dissolved oxygen | ≥ 6.9 mg/L (84% of saturation) |
| pH | 8.0 – 8.2 |
| Adjustment of pH | Not described |
| Aeration of dilution water | Yes |
| Intensity of irradiation | Fluorescent light bulbs, 442 lux |
| Photoperiod | 16 h daylight and 8 h darkness with a 30 minute transition period of low light intensity |

**Table A7.4.3.2.a/01-6: Validity criteria for fish tests according to OECD Guidelines 210**

| | fulfilled | Not fullfilled |
|---|---|---|
| Concentration of dissolved oxygen > 60% saturation throughout the test | **yes** | |
| Difference of water temperature < 1.5% between test chambers or successive days at any time during test; temperature within range for specific test species | **yes** | |
| Overall survival of fertilized eggs in controls (and solvent controls) ≥ value, specified for the specific test species | **yes** | |
| Test substance concentrations maintained within ± 20% of mean measured values | **yes** | |
| No effect on survival nor any other adverse effect found in solvent control | **yes** | |
| Further criteria for poorly soluble test substances | **yes** | |

| | | |
|---|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** | |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** | |
| **Subsection A7.4.3** | **Effects on aquatic organisms, further studies** | |
| **Subsection A7.4.3.3** | **Bio-accumulation in aquatic organisms** | |
| | **JUSTIFICATION FOR NON-SUBMISSION OF DATA** | **Official use only** |
| **Other existing data [X]** | **Technically not feasible [ ]** **Scientifically unjustified [ ]** | |
| **Limited exposure [ ]** | **Other justification [ ]** | |
| **Detailed justification:** | 7.4.3.3.1 Bioaccumulation in fish<br>A waiver is requested for performing a fish bio-accumulation study for BIT. This request is based on the log P (log octanol:water partition coefficient) for this compound.<br>• Log P < 1.5<br>This value indicates that the potential for BIT to bioaccumulate will be minimal. Many regulatory schemes have established that a log P of less than 3 precludes the need to perform a bio-accumulation study. Additionally using the US EPA's EPIWIN software suite the calculated Bioconcentration Factor (BCF) is 1.646 (Log BCF = 0.216).<br>Therefore, based on the log P values, bio-accumulation studies would not provide any necessary additional data but would result in the unnecessary sacrifice of animals.<br>7.4.3.3.2 Bioaccumulation in invertebrates<br>A waiver is requested for performing a fish bio-accumulation study for BIT. This request is based on the log P (log octanol:water partition coefficient) for this compound.<br>• Log P < 1.5<br>This value indicates that the potential for BIT to bioaccumulate will be minimal. Many regulatory schemes have established that a log P of less than 3 precludes the need to perform a bio-accumulation study. Additionally using the US EPA's EPIWIN software suite the calculated Bioconcentration Factor (BCF)is 1.646 (Log BCF = 0.216).<br>Therefore, based on the log P values, bio-accumulation studies would not provide any necessary additional data but would result in the unnecessary sacrifice of animals. | |
| **Undertaking of intended data submission [ ]** | No studies are planned. | |
| | | |
| | | |
| | **Evaluation by Competent Authorities** | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |

| | | |
|---|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** | |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** | |
| **Subsection A7.4.3** | **Effects on aquatic organisms, further studies** | |
| **Subsection A7.4.3.3** | **Bio-accumulation in aquatic organisms** | |

| | |
|---|---|
| **Date** | *December 2010* |
| **Evaluation of applicant's justification** | *Applicant's justification is accepted.* |
| **Conclusion** | *Applicant's justification is accepted.* |
| **Remarks** | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.3** | **Effects on aquatic organisms, further studies** |
| **Subsection A7.4.3.4** | **Effects on reproduction and growth rate with an appropriate invertebrate species** |
| **Subsection A7.4.3.4.a/01** | **Effects on reproduction and growth rate with an invertebrate species-Freshwater, *Daphnia magna*** |
| **Annex Point IIIA XIII.2.4** | |

| | | | |
|---|---|---|---|
| | | **1 REFERENCE** | **Official use only** |
| **1.1** | **Reference** | **A7.4.3.4.a/01** [redacted] (2007c) 1,2-Benzisothiazolin-3-one: A flow-through life-cycle toxicity test with the cladoceran (*Daphnia magna*), [redacted], Rohm and Haas Report N° 06RC-091 (January 17, 2007), GLP, Unpublished. | |
| **1.2** | **Data protection** | Yes | |
| 1.2.1. | Data owner | Rohm and Haas Company | |
| 1.2.2. | Companies with letter of access | | |
| 1.2.3. | Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into Annex I/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **Guideline study** | Yes, OECD 211 and US EPA OPPTS 850.1300 | |
| **2.2** | **GLP** | Yes | |
| **2.3** | **Deviations** | No | |
| | | **3 METHOD** | |
| **3.1** | **Test material** | 1,2-Benzisothiazolin-3-one (BIT) | |
| 3.1.1 | Lot/Batch number | 2005-051 | |
| 3.1.2 | Specification | As given in section 2 | |
| 3.1.3 | Purity | 89.8% BIT | |
| 3.1.4 | Composition of Product | Not applicable | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.3** | **Effects on aquatic organisms, further studies** |
| **Subsection A7.4.3.4** | **Effects on reproduction and growth rate with an appropriate invertebrate species** |
| **Subsection A7.4.3.4.a/01** | **Effects on reproduction and growth rate with an invertebrate species-Freshwater, *Daphnia magna*** |
| **Annex Point IIIA XIII.2.4** | |

| | | | |
|---|---|---|---|
| 3.1.5 | Further relevant properties | Not applicable | |
| 3.1.6 | Method of analysis | High performance liquid chromatography | |
| **3.2 Preparation of TS solution for poorly soluble or volatile test substances** | | see Table A7.4.3.4.a/01-1 | |
| **3.3 Reference substance** | | No | |
| **3.4 Testing procedure** | | | |
| 3.4.1 | Dilution water | see Table A7.4.3.4.a/01-2 | **X** |
| 3.4.2 | Test organisms | see Table A7.4.3.4.a/01-3 | |
| 3.4.3 | Handling of offspring | Following the onset of reproduction, the numbers of second-generation daphnids were counted three times per week and at test termination. | |
| 3.4.4 | Test system | see Table A7.4.3.4.a/01-4 | |
| 3.4.5 | Test conditions | see Table A7.4.3.4.a/01-5 | **X** |
| 3.4.6 | Duration of the test | 21 days | |
| 3.4.7 | Test parameter | Mortality, immobility, sublethal signs of toxicity, onset of reproduction, mean lengths and dry weights in the first generation daphnids. First day of brood production and number of neonates indicated reproduction effects. | |
| 3.4.8 | Examination / Sampling | First-generation daphnids were observed daily. The numbers of second generation daphnids were counted three times per week and at test termination (day 21). Body lengths and dry weights of the surviving first generation daphnids were measured at the end of the exposure period. | |
| 3.4.9 | Monitoring of TS concentration | Yes, days –2, 0, 7, 14, 21. All samples were collected mid-depth, placed in glass scintillation vials and processed immediately for analysis. | |
| 3.4.10 | Statistics | Survival data were anlyzed using Chi-square and Fisher's Exact tests. | |

Reproduction and growth data were evaluated for normality using Shapiro-Wilk's test and for homogeneity using Levenes or Bartlett's tests ($p = 0.01$). Analysis of Variance (ANOVA) was used to determine if statistically significant differences existed among the BIT treatment groups ($p = 0.05$). The BIT treatments that were significantly different from the pooled control means were identified using Bonferroni's t-test ($p \leq 0.05$). All statisitical tests were performed using TOXSTAT (West, Inc. and D.D. Gulley. 1996. TOXSTAT® Version 3.5. Western EcoSystems Technology, Inc., Cheyenne, Wyoming, USA) or SAS (The SAS system for Windows. 1999-2001 Version 8.2, Cary, North Carolina, USA) software.

**4 RESULTS**

**4.1 Range finding test** Not described

**4.2 Results test substance**

4.2.1 Initial concentrations of test substance: Nominal (mg BIT/L): 0.25, 0.50, 1.0, 2.0, and 4.0

4.2.2 Actual concentrations of test substance: mg BIT/L

| Nominal concentration | Mean measured concentration | Percent of nominal |
|---|---|---|
| 0.25 | 0.21 | 84 |
| 0.50 | 0.46 | 92 |
| 1.0 | 0.91 | 91 |
| 2.0 | 1.9 | 95 |
| 4.0 | 3.8 | 95 |

4.2.3 Effect data: See Table A7.4.3.4.a/01-6. One dpahnid was lethargic and discoloured (pale) in the 3.8 mg BIT/L group **X**

4.2.4 Concentration / response curve: See Figure A7.4.3.4.a/01-1

4.2.5 Other effects: Not applicable

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.3** | **Effects on aquatic organisms, further studies** |
| **Subsection A7.4.3.4** | **Effects on reproduction and growth rate with an appropriate invertebrate species** |
| **Subsection A7.4.3.4.a/01**<br>**Annex Point IIIA XIII.2.4** | **Effects on reproduction and growth rate with an invertebrate species-Freshwater, *Daphnia magna*** |

| | | | |
|---|---|---|---|
| **4.3 Results of controls** | | After 21 days survival in the negative and solvent control groups was 95% and 100%, respectively. The first day of brood production in the negative and solvent control groups was Day 8 of the test. | |
| **4.4 Test with reference substance** | | Not performed | |
| | | **5 APPLICANT'S SUMMARY AND CONCLUSION** | |
| **5.1 Materials and methods** | | OECD 211 and US EPA OPPTS 850.1300, Aquatic invertebrate life-cycle study with analytical confirmation of TS concentrations. | |
| **5.2 Results and discussion** | | Since no significant differences between the control groups were found for any parameter tested ($p > 0.05$) the control data were pooled for comparison with the BIT treatment groups. After 21 days survival in the negative and solvent control groups was 95% and 100%, respectively. The control data was pooled for comparisons with the BIT treatment groups. The first day of brood production in the negative control, solvent control and the BIT treatment groups groups was Day 8 of the test indicating there was no apparent delay in the onset of production at any BIT concentration tested. | |
| 5.2.1 | NOEC | 0.91 mg BIT/L | |
| 5.2.2 | LOEC | 1.9 mg BIT/L | |
| 5.2.3 | $EC_{50}$ | 2.5 mg BIT/L, 21-day mortality/immobility (95% C.I.: 1.9 to 3.8 mg BIT/L)<br>> 3.8 mg BIT/L, reproduction | |
| 5.2.4 | MATC | 1.3 mg BIT/L | |
| **5.3 Conclusion** | | see Table A7.4.3.4.a/01-7 | |
| 5.3.1 | Reliability | (1), reliable without restrictions | |
| 5.3.2 | Deficiencies | No | |

| | |
|---|---|
| **Evaluation by Competent Authorities** | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.3** | **Effects on aquatic organisms, further studies** |
| **Subsection A7.4.3.4** | **Effects on reproduction and growth rate with an appropriate invertebrate species** |
| **Subsection A7.4.3.4.a/01**<br>**Annex Point IIIA XIII.2.4** | **Effects on reproduction and growth rate with an invertebrate species-Freshwater, *Daphnia magna*** |

| | |
|---|---|
| **Date** | *December 2010.* |
| **Materials and Methods** | *Applicant's version is accepted with the following remarks:*<br>▪ *3.4.1: It is recommended by the OECD Guideline 211 to estimate the TOC levels in the medium*<br>▪ *3.4.5: The light intensity was lower than the recommended by the OECD Guideline (15-20 µE*m$^2$/s).* |
| **Results and discussion** | *Applicant's version is accepted with the following remarks:*<br>▪ *4.2.3: The following results are missing in the report:*<br>o *Coefficient of variation for control fecundity (based on total number of living offspring per parent animal alive)*<br>o *The plot of total number of living offspring per parent animal (for each replicate) alive at the end of the test vs concentration,* |
| **Conclusion** | *Applicant's version adopted* |
| **Reliability** | *2* |
| **Acceptability** | *Acceptable* |
| **Remarks** | |

**Table A7.4.3.4.a/01-1: Preparation of TS solution for poorly soluble or volatile test substances**

| Criteria | Details |
|---|---|
| Dispersion | Yes, sonicated and mixed by inversion |
| Vehicle | Yes, dimethyl formaide (DMF) |
| Concentration of vehicle | 0.1 mL/L |
| Vehicle control performed | Yes |
| Other procedures | Not applicable |

**Table A7.4.3.4.a/01-2: Dilution water**

| Criteria | Details |
|---|---|
| Source | Fresh well water collected at the Wildlife International Limited site, Easton, Maryland, USA |
| Alkalinity | 178 to 182 mg/L as $CaCO_3$ |
| Hardness | 128 to 138 mg/L as $CaCO_3$ |
| TOC | Not described |
| Holding water different from dilution water | No |

**Table A7.4.3.4.a/01-3: Test organisms**

| Criteria | Details |
|---|---|
| Strain / Clone | *Daphnia magna* |
| Source | in-house culture |
| Age | less than 24 h old at test initiation |
| Breeding method | Not described |
| Kind of food | A mixture of yeast, cereal grass media and trout chow (YCT) as well as a suspension of *Pseudokirchneriella subcapitata* |
| Amount of food | At each feeding, each test chamber initially was fed 0.75 mL of YCT and 1.5 mL of algae. The amounts were increased to 1.0 mL YCT and 2.0 mL of algae on Day 16 of the test after dilution water flow rates were increased. |
| Feeding frequency | 3 times per day through Day 7 and 4 times per day until the last day of the test |
| Pretreatment | Adult daphnids were cultured in water from the same source and at approximately the same temperature as used during the test. |
| Feeding of animals during test | Yes |

**Table A7.4.3.4.a/01-4: Test system**

| Criteria | Details |
|---|---|
| Test type | Flow-through |
| Renewal of test solution | The diluter flow rate was adjusted to provide approximately 5 volume additions of test water in each test chamber per day until Day 15 of the test. On Day 15, the flow rate was increased to aid in maintaining dissolved oxygen concentrations and provided approximately 8 volume additions of test water in each test chamber per day through test termination. |
| Volume of test vessels | Two 300 mL glass beakers suspended in 25 L stainless steel aquaria filled with approximately 22 L test solution |
| Volume/animal | 27 mL |
| Number of animals/vessel | 10/beaker |
| Number of vessels/ concentration | 2 |
| Test performed in closed vessels due to significant volatility of TS | No |

**Table A7.4.3.4.a/01-5: Test conditions**

| Criteria | Details |
|---|---|
| Test temperature | 19.6 to 20.2 °C |
| Dissolved oxygen | ≥ 6.2 mg/L (≥ 69% saturation) |
| pH | 8.0 to 8.2 |
| Adjustment of pH | Not described |
| Conductivity | 300 to 320 µmhos/cm |
| Aeration of dilution water | Yes |
| Quality/Intensity of irradiation | 219 lux |
| Photoperiod | 16 h light, 8 h dark with 30 minute transition period of low light intensity |

**Table A7.4.3.4.a/01-6: Effect data**

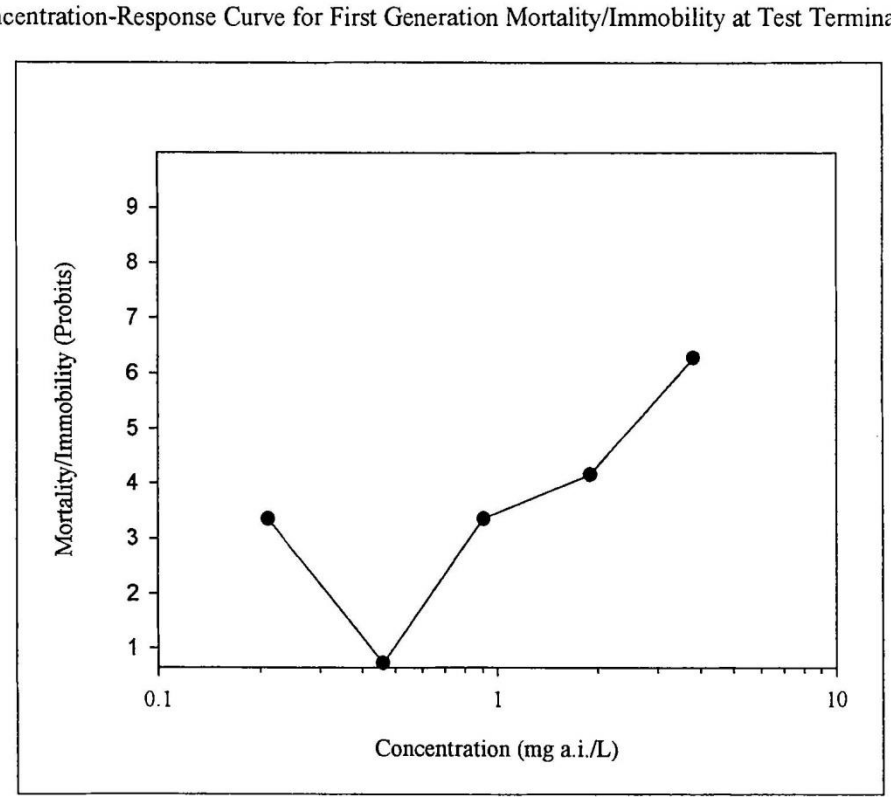
| Mean measured concentration (µg DCOIT/L) | % survival at 21 days | Mean no. of young produced per reproductive day | Day of first brood | Treatment mean length (mm) | Treatment mean dry weight (mg) |
|---|---|---|---|---|---|
| Negative control | 95 | 11.2 | 8 | 5.7 | 1.12 |
| DMF solvent control | 100 | 11.4 | 8 | 5.7 | 1.10 |
| 0.21 | 95 | 12.6 | 8 | 5.8 | 1.16 |
| 0.46 | 100 | 11.3 | 8 | 5.7 | 1.02 |
| 0.91 | 95 | 11.8 | 8 | 5.8 | 1.09 |
| 1.9 | 80 * | 10.5 | 8 | 5.5 | 0.99 |
| 3.8 | 10 * | 7.0 | 8 | 5.3 | 1.08 |

* Statistically significant decrease in survival in comparison to the pooled control (98%) using Fisher's Exact Test ($p \leq 0.05$)

**Table A7.4.3.4.a/01-7: Validity criteria for invertebrate reproduction test according**

| | fulfilled | Not fullfilled |
|---|---|---|
| Mortality of parent animals < 20% at test termination | **yes** | |
| Mean number of live offspring produced per parent animal surviving at test termination ≥ 60 | **yes** | |

**Figure A7.4.3.4.a/01-1: Concentration-response curve for First Generation Mortality/Immobility at Test Termination**



| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
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| **Subsection A7.4.3** | **Effects on aquatic organisms, further studies** |
| **Subsection A7.4.3.5** | **Effects on any other specific, non-target organisms (flora and fauna) believed to be at risk** |
| **Subsection A7.4.3.5.1a/01** | **Acute toxicity to sediment dwelling organisms-Freshwater, *Chironomus tentans*** |
| **Annex Point IIIA XIII.3.4** | |

| | | | Official use only |
|---|---|---|---|
| | | **1 REFERENCE** | |
| **1.1** | **Reference** | **A7.4.3.5.1a/01** [redacted] (2007) 1,2-Benzisothiazolin-3-one: A survival and growth sediment toxicity test with *Chironomus tentans* using spiked sediment, [redacted], Rohm and Haas Report N° 06RC-128 (March 9, 2007), Unpublished. | |
| **1.2** | **Data protection** | Yes | |
| 1.2.1. | Data owner | Rohm and Haas Company | |
| 1.2.2. | Companies with letter of access | | |
| 1.2.3. | Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into Annex I/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **Guideline study** | Yes, US EPA OPPTS 850.1735 | |
| **2.2** | **GLP** | Yes | |
| **2.3** | **Deviations** | No | |
| | | **3 MATERIALS AND METHODS** | |
| **3.1** | **Test material** | 1,2-Benzisothiazolin-3-one (BIT) | |
| 3.1.1 | Lot/Batch number | 2005-051 | |
| 3.1.2 | Specification | As given in section 2 | |
| 3.1.3 | Purity | 89.8% BIT | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
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| **Subsection A7.4.3** | **Effects on aquatic organisms, further studies** |
| **Subsection A7.4.3.5** | **Effects on any other specific, non-target organisms (flora and fauna) believed to be at risk** |
| **Subsection A7.4.3.5.1a/01** | **Acute toxicity to sediment dwelling organisms-Freshwater, *Chironomus tentans*** |
| **Annex Point IIIA XIII.3.4** | |

| | | | |
|---|---|---|---|
| 3.1.4 | Composition of Product | Not applicable | |
| 3.1.5 | Further relevant properties | Not applicable | |
| 3.1.6 | Method of analysis | High performance liquid chromatography with UV detection | |
| **3.2 Preparation of TS solution for poorly soluble or volatile test substances** | | see Table A7.4.3.5.1.a/01-1 | |
| **3.3 Reference substance** | | No | |
| **3.4 Testing procedure** | | | |
| 3.4.1 | Dilution water | see Table A7.4.3.5.1.a/01-2 | |
| 3.4.2 | Test organisms | see Table A7.4.3.5.1.a/01-3 | |
| 3.4.3 | Test system | see Table A7.4.3.5.1.a/01-4 | |
| 3.4.4 | Test conditions | see Table A7.4.3.5.1.a/01-5 | **X** |
| 3.4.5 | Duration of the test | 10 days | |
| 3.4.6 | Test parameter | survival, growth parameters | |
| 3.4.7 | Sampling | TS concentration was measured in the overlying water, pore water and sediment samples at test initiation and termination | |
| 3.4.8 | Monitoring of TS concentration | Yes, test initiation and termination | |
| 3.4.9 | Statistics | The ash-free dry weight data were analyzed using the computer program TOXSTAT version 3.5 (West, Inc. and D.D. Gulley. TOXSTAT version | |

3.5. Copyright 1996. Western Ecosystems Technology, Inc., Cheyenne, Wyoming, USA). The NOEC and LOEC were determined by visual interpretation of the dose-response pattern and statistical analyses of the survival and mean individual ash-free dry weight data. The ash-free dry weight (growth) data were evaluated for normality (Chi-Square) and homogeneity of variances (Levene's Test). The negative and solvent control growth data were compared using two-tailed t-test (p = 0.05). There were significant differences between the negative and solvent control groups, therefore treatment groups were compared to the solvent control.

**4 RESULTS**

**4.1 Limit Test** Not performed

**4.2 Results test substance**

4.2.1 Initial concentrations of test substance: 6.3, 13, 25, 50 and 100 mg BIT/kg (nominal)

4.2.2 Actual concentrations of test substance: Measured BIT concentrations in sediment samples:

| Nominal | Measured Day 0 | Measured Day 10 |
|---|---|---|
| Negative control | < LOQ | < LOQ |
| Solvent control | < LOQ | < LOQ |
| 6.3 mg BIT/kg | 3.38 | < LOQ |
| 13 mg BIT/kg | 6.13 | 2.85 |
| 25 mg BIT/kg | 15.4 | 5.91 |
| 50 mg BIT/kg | 32.8 | 13.0 |
| 100 mg BIT/kg | 45.9 | 22.2 |

Measured BIT concentrations in overlying water samples:

| Nominal | Measured Day 0 | Measured Day 10 |
|---|---|---|
| Negative control | < LOQ | < LOQ |
| Solvent control | < LOQ | < LOQ |
| 6.3 mg BIT/kg | < LOQ | < LOQ |
| 13 mg BIT/kg | < LOQ | < LOQ |
| 25 mg BIT/kg | < LOQ | < LOQ |
| 50 mg BIT/kg | < LOQ | < LOQ |
| 100 mg BIT/kg | 0.312 | < LOQ |

Measured BIT concentrations in pore water samples:

| Nominal | Measured Day 0 | Measured Day 10 |
|---|---|---|
| Negative control | < LOQ | < LOQ |
| Solvent control | < LOQ | < LOQ |
| 6.3 mg BIT/kg | 8.41 | 1.26 |
| 13 mg BIT/kg | 21.0 | 7.29 |
| 25 mg BIT/kg | 33.8 | 14.8 |
| 50 mg BIT/kg | 93.3 | 32.6 |
| 100 mg BIT/kg | 173 | 66.5 |

LOQ, limit of quantitation = 0.100 mg BIT/L

| | | |
|---|---|---|
| 4.2.3 | Effect data | see Table A7.4.3.5.1.a/01-6 and see Table A7.4.3.5.1.a/01-7 |

| | | |
|---|---|---|
| 4.2.4 | Concentration / response curve | Not described in report |
| 4.2.5 | Other effects | The organisms generally appeared normal and healthy throughout the study. A few organisms were observed on the surface of the sediment or climbing the walls of the test compartments in all BIT treatment groups and controls. |
| **4.3 Results of controls** | | see Table A7.4.3.5.1.a/01-6 |
| **4.4 Test with reference substance** | | Not performed |
| | | **5 APPLICANT'S SUMMARY AND CONCLUSION** |
| **5.1 Materials and methods** | | US EPA OPPTS 850.1735, Acute flow-through toxicity study in whole sediment to midge larvae with analytical confirmation of TS concentrations. BIT was added to the sediment. At test termination, midges were rinsed of excess sediment, placed into a pre-weighed crucible and dried for approximately 42 hours at 60 °C. The midges were weighed then placed into a furnace for approximately 2 hours at 550 °C to determine ash-free dry weights. |
| **5.2 Results and discussion** | | The overlying water appeared clear and colorless in all test compartments at test initiation and at test termination. All water quality parameters were within acceptable limits during the test. The organisms generally appeared normal and healthy throughout the study. Percent mortality at test termination was 0% in all treatment groups. One small midge in the 6.3 mg BIT/kg group and three small midges in the 25 mg BIT/kg group were noted. The NOEC was 50 mg BIT/kg and the LOEC was 100 mg BIT/kg, based on ash-free dry weights. |
| 5.2.1 | $EC_0$ | 50 mg BIT/kg |
| 5.2.2 | $EC_{50}$ | > 100 mg BIT/kg |
| 5.2.3 | $EC_{100}$ | Not applicable |
| **5.3 Conclusion** | | see Table A7.4.3.5.1.a/01-8 |
| 5.3.1 | Reliability | (1), reliable without restriction |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.3** | **Effects on aquatic organisms, further studies** |
| **Subsection A7.4.3.5** | **Effects on any other specific, non-target organisms (flora and fauna) believed to be at risk** |
| **Subsection A7.4.3.5.1a/01** | **Acute toxicity to sediment dwelling organisms-Freshwater, *Chironomus tentans*** |
| **Annex Point IIIA XIII.3.4** | |

| | | | |
|---|---|---|---|
| 5.3.2 | Deficiencies | No | |

| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *November 2012* |
| **Materials and Methods** | *Applicant's version is accepted with the following remarks:*<br>***3.4.4**: The light intensity used in the study is lower than those recommended by the OECD and OPPTS 850.1735 ( 500 to 1000 lux).* |
| **Results and discussion** | *Accept the applicant's version with the following remarks:*<br>▪ *Table A7.4.3.5.1-7 only give information about the total mass balance. Tables that consider recoveries for all compartments of the experiment (porewater, overlying water and sediment), can be obtained from the correspondent Doc. IV-A, and are included below:*<br>*Measured BIT concentrations in sediment samples:* |

| ***Nominal*** | ***Measured Day 0*** | ***Measured Day 10*** |
|---|---|---|
| *Negative control* | *< LOQ* | *< LOQ* |
| *Solvent control* | *< LOQ* | *< LOQ* |
| *6.3 mg BIT/kg* | *3.38* | *< LOQ* |
| *13 mg BIT/kg* | *6.13* | *2.85* |
| *25 mg BIT/kg* | *15.4* | *5.91* |
| *50 mg BIT/kg* | *32.8* | *13.0* |
| *100 mg BIT/kg* | *45.9* | *22.2* |

*Measured BIT concentrations in overlying water samples:*

| | |
|---|---|

| ***Nominal*** | ***Measured Day 0*** | ***Measured Day 10*** |
|---|---|---|
| *Negative control* | *< LOQ* | *< LOQ* |
| *Solvent control* | *< LOQ* | *< LOQ* |
| *6.3 mg BIT/kg* | *< LOQ* | *< LOQ* |
| *13 mg BIT/kg* | *< LOQ* | *< LOQ* |
| *25 mg BIT/kg* | *< LOQ* | *< LOQ* |
| *50 mg BIT/kg* | *< LOQ* | *< LOQ* |
| *100 mg BIT/kg* | *0.312* | *< LOQ* |

*Measured BIT concentrations in pore water samples:*

| ***Nominal*** | ***Measured Day 0*** | ***Measured Day 10*** |
|---|---|---|
| *Negative control* | *< LOQ* | *< LOQ* |
| *Solvent control* | *< LOQ* | *< LOQ* |
| *6.3 mg BIT/kg* | *8.41* | *1.26* |
| *13 mg BIT/kg* | *21.0* | *7.29* |
| *25 mg BIT/kg* | *33.8* | *14.8* |
| *50 mg BIT/kg* | *93.3* | *32.6* |
| *100 mg BIT/kg* | *173* | *66.5* |

*ECx values should consider the measured concentration at the beginning of the test (as recommended by the guidances) and not be based on nominals. Therefore:*

*$EC_0$ = 32.8 mg/kg; $EC_{50}$ > 45.9 mg/kg and $EC_{100}$ = Not applicable.*

| | |
|---|---|
| **Conclusion** | *Applicant's version is adopted* |
| **Reliability** | *2* |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
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| **Subsection A7.4.3** | **Effects on aquatic organisms, further studies** |
| **Subsection A7.4.3.5** | **Effects on any other specific, non-target organisms (flora and fauna) believed to be at risk** |
| **Subsection A7.4.3.5.1a/01** | **Acute toxicity to sediment dwelling organisms-Freshwater, *Chironomus tentans*** |

**Annex Point IIIA XIII.3.4**

| | | |
|---|---|---|
| **Acceptability** | *Acceptable* | |
| **Remarks** | | |

**Table A7.4.3.5.1.a/01-1: Preparation of TS solution for poorly soluble or volatile test substances**

| Criteria | Details |
|---|---|
| Dispersion | Yes |
| Vehicle | Acetone |
| Concentration of vehicle | Not applicable |
| Vehicle control performed | Yes |
| Other procedures | A primary stock solution was prepared by dissolving BIT in acetone at a nominal concentration of 10.0 mg BIT/ml. |

**Table A7.4.3.5.1.a/01-2: Dilution water**

| Criteria | Details |
|---|---|
| Source | Well fresh water, 40 meters deep |
| Alkalinity | 178-180 mg/L as $CaCO_3$ |
| Hardness | 136 mg/L as $CaCO_3$ |
| pH | 8.0 – 8.2 |
| Ca / Mg ratio | Not described |
| Na / K ratio | Not described |
| Oxygen content | Aerated, not measured |
| Conductance | 300-320 µmhos/cm |
| Holding water different from dilution water | No |

**Table A7.4.3.5.1.a/01-3: Test organisms**

| Criteria | Details |
|---|---|
| Strain | midge larvae (*Chironomus tentans*) |
| Source | Environmental Consulting and Testing, Superior, Wisconsin, USA |
| Age | 10 days |
| Breeding method | Not described |
| Kind of food | Flake food (TetraMin Flakes) |
| Amount of food | 1.5 mL of a 4 g/L suspension of flake food |
| Feeding frequency | Days 0 through 9 |
| Pretreatment | Midges were held for 3 days at approximately the same temperature of water used in the test |
| Feeding of animals during test | Yes, Days 0 through 9 |

**Table A7.4.3.5.1.a/01-4: Test system**

| Criteria | Details |
|---|---|
| Renewal of test solution | Flow-through. The diluter was adjusted so that approximately 786 mL of water was delivered every minute for 4 minutes to each splitting chamber 2 times per day resulting in approximately two volume additions in each test compartment per day. |
| Volume of test vessels | 300 mL glass beakers with 2 stainless steel mesh-covered holes on opposite to allow for the flow of water throught the test compartment. Each beaker contained approximately 100 ml of sediment and 150 mL of overlying water. |
| Volume/animal | 10 mL sediment per midge and 15 mL water per midge |
| Number of animals/vessel | 10 midges |
| Number of vessels/ concentration | 8 replicates with midge, 2 replicates for analytical purposes |
| Test performed in closed vessels due to significant volatility of TS | No |

**Table A7.4.3.5.1.a/01-5: Test conditions**

| Criteria | Details |
|---|---|
| Test temperature | 23 ±1 ºC |
| Dissolved oxygen | ≥ 5.6 mg/L (66% of saturation) |
| pH | 7.9 – 8.2 |
| Adjustment of pH | Not described |
| Aeration of dilution water | Yes |
| Quality/Intensity of irradiation | Fluorescent tubes that emit wavelengths similar to natural sunlight. Light intensity at test initiation was 219 lux at the surface of the water |
| Photoperiod | 16 hours daylight, 8 hours darkness with 30-minute transition period of low light intensity |

**Table A7.4.3.5.1.a/01-6: Effect and Mortality data**

| Test-Substance Concentration (effective) [1] [mg BIT/kg dry sediment] | | |
|---|---|---|
| | Percent Survival | Mean Individual Ash-Free Dry Weight (mg) |
| Negative control | 100 | 1.54 |
| Acetone control | 99 | 1.76 |
| 6.3 | 100 | 1.54 |
| 13 | 100 | 1.95 |
| 25 | 100 | 1.56 |
| 50 | 100 | 1.63 |
| 100 | 100 | 1.30 [2] |

[1] TS concentrations were nominal

[2] There was a statistically significant difference ($p < 0.05$) from the solvent control using Dunnett's test.

**Table A7.4.3.5.1.a/01-7: Effect data**

| | $EC_{50}$[1] | 95 % c.l. | $EC_0$[1] | $EC_{100}$[1] |
|---|---|---|---|---|
| **10 d [mg BIT/kg dry sediment]** | > 100 (n) | Not applicable | 50 (n) | Not applicable |

[1] effect data are based on nominal (n) concentrations

**Table A7.4.3.5.1.a/01-8: Validity criteria**

| | fulfilled | Not fullfilled |
|---|---|---|
| Mortality of control animals < 10% | **yes** | |
| Concentration of test substance ≥ 80% of initial concentration during test | **yes** | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
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| **Subsection A7.4.3** | **Effects on aquatic organisms, further studies** |
| **Subsection A7.4.3.5** | **Effects on any other specific, non-target organisms (flora and fauna) believed to be at risk** |
| **Subsection A7.4.3.5.1a/02**<br>**Annex Point IIIA XIII.3.4** | **Chronic toxicity to sediment dwelling organisms-Freshwater, *Chironomus riparius*** |

| | | | Official use only |
|---|---|---|---|
| | | **1 REFERENCE** | |
| **1.1** | **Reference** | **A7.4.3.5.1a/02** [redacted]. (2007) 1,2-Benzisothiazolin-3-one: A prolonged sediment toxicity test with *Chironomus riparius* using spiked sediment, [redacted] Rohm and Haas Report N° 06RC-094 (March 6, 2007), Unpublished. | |
| **1.2** | **Data protection** | Yes | |
| 1.2.1 | Data owner | Rohm and Haas Company | |
| 1.2.2 | Companies with letter of access | | |
| 1.2.3 | Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into Annex I/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **Guideline study** | Yes, OECD Guideline 218 | |
| **2.2** | **GLP** | Yes | |
| **2.3** | **Deviations** | No | |
| | | **3 MATERIALS AND METHODS** | |
| **3.1** | **Test material** | 1,2-Benzisothiazolin-3-one (BIT) | |
| 3.1.1 | Lot/Batch number | 2005-051 | |
| 3.1.2 | Specification | As given in section 2 | |
| 3.1.3 | Purity | 89.8% BIT | |
| 3.1.4 | Composition of Product | Not applicable | |
| 3.1.5 | Further relevant properties | Not applicable | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
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| **Subsection A7.4.3** | **Effects on aquatic organisms, further studies** |
| **Subsection A7.4.3.5** | **Effects on any other specific, non-target organisms (flora and fauna) believed to be at risk** |
| **Subsection A7.4.3.5.1a/02** | **Chronic toxicity to sediment dwelling organisms-Freshwater, *Chironomus riparius*** |
| **Annex Point IIIA XIII.3.4** | |

| | | |
|---|---|---|
| 3.1.6 Method of analysis | High performance liquid chromatography with UV detection | |
| **3.2 Preparation of TS solution for poorly soluble or volatile test substances** | see Table A7.4.3.5.1.a/02-1 | |
| **3.3 Reference substance** | No | |
| **3.4 Testing procedure** | | |
| 3.4.1 Dilution water | see Table A7.4.3.5.1.a/02-2 | |
| 3.4.2 Test organisms | see Table A7.4.3.5.1.a/02-3 | |
| 3.4.3 Test system | see Table A7.4.3.5.1.a/02-4 | **X** |
| 3.4.4 Test conditions | see Table A7.4.3.5.1.a/02-5 | **X** |
| 3.4.5 Duration of the test | 28 days | |
| 3.4.6 Test parameter | Development rates, development times, emergence rates and toal number of adults emerged | |
| 3.4.7 Sampling | overlying pore water, pore water and sediment samples. | |
| 3.4.8 Monitoring of TS concentration | test initiation, day 7, and test termination | |
| 3.4.9 Statistics | The 28-day $EC_{50}$ was calculated using TOXSTAT version 3.5 using the mortality data at the end of the study (West, Inc. and D.D. Gulley. TOXSTAT version 3.5. Copyright 1996. Western Ecosystems Technology, Inc., Cheyenne, Wyoming, USA). The NOEC and LOEC were determined by visual interpretation of the dose-response pattern and statistical analyses of the mean development times, emergence rates and development rates. The emergence rate and development rate were calculated for each replicate of each control and treatment group using SAS System for Windows version 8.2 (The SAS System for Windows. 1999-2001. Release 8.2 (TS2M0). SAS Institute, Inc., Cary, North Carolina, USA). The data were analyzed using an appropriate t-test to determine any statistical differences between solvent and negative control groups. The percent survival data were analyzed using a Bonferroni's t-test to identify those treatment levels that were statistically different ($p < 0.05$) from the pooled control group (D.J. Finney. 1971. Statistical Methods in Biological Assay. Second edition. Griffin Press, London)(W.R. Thompson. 1947. Bacteriological Reviews. Volume II, No. 2, pp. 115-145). A Chi-square test was performed to check normality and the homogeneity of variance was | |

checked using the Levene's test.

**4 RESULTS**

**4.1 Limit Test** Not performed

**4.2 Results test substance**

4.2.1 Initial concentrations of test substance: 6.3, 13, 25, 50, 100 mg BIT/kg

4.2.2 Actual concentrations of test substance: Measured BIT concentration in sediment samples:

| Nominal | Measured Day 0 | Measured Day 7 | Measured Day 28 |
|---|---|---|---|
| Negative control | < LOQ | < LOQ | < LOQ |
| Solvent control | < LOQ | < LOQ | < LOQ |
| 6.3 mg BIT/kg | 5.01 | < LOQ | < LOQ |
| 13 mg BIT/kg | 5.00 | < LOQ | < LOQ |
| 25 mg BIT/kg | 11.7 | 1.48 | < LOQ |
| 50 mg BIT/kg | 24.5 | 5.42 | < LOQ |
| 100 mg BIT/kg | 48.5 | 11.2 | 2.36 |

Measured BIT concentration in overlying pore water samples:

| Nominal | Measured Day 0 | Measured Day 7 | Measured Day 28 |
|---|---|---|---|

| | | | |
|---|---|---|---|
| Negative control | < LOQ | < LOQ | < LOQ |
| Solvent control | < LOQ | < LOQ | < LOQ |
| 6.3 mg BIT/kg | 0.565 | < LOQ | < LOQ |
| 13 mg BIT/kg | 1.28 | 0.336 | < LOQ |
| 25 mg BIT/kg | 2.32 | 0.413 | < LOQ |
| 50 mg BIT/kg | 5.13 | 4.32 | 0.152 |
| 100 mg BIT/kg | 9.88 | 6.59 | 3.74 |

Measured BIT concentration in pore water samples:

| Nominal | Measured Day 0 | Measured Day 7 | Measured Day 28 |
|---|---|---|---|
| Negative control | < LOQ | < LOQ | < LOQ |
| Solvent control | < LOQ | < LOQ | < LOQ |
| 6.3 mg BIT/kg | 8.59 | 0.248 | < LOQ |
| 13 mg BIT/kg | 12.7 | 1.13 | < LOQ |
| 25 mg BIT/kg | 40.5 | 4.80 | 0.251 |
| 50 mg BIT/kg | 59.6 | 17.3 | 0.613 |
| 100 mg BIT/kg | 111 | 28.1 | 4.56 |

LOQ, limit of quantitation = 0.100 mg BIT/L

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** |
| **Subsection A7.4.3** | **Effects on aquatic organisms, further studies** |
| **Subsection A7.4.3.5** | **Effects on any other specific, non-target organisms (flora and fauna) believed to be at risk** |
| **Subsection A7.4.3.5.1a/02** | **Chronic toxicity to sediment dwelling organisms-Freshwater, *Chironomus riparius*** |
| **Annex Point IIIA XIII.3.4** | |

| | |
|---|---|
| 4.2.3 Effect data | see Table A7.4.3.5.1.a/02-6 and see Table A7.4.3.5.1.a/02-7<br>Percent mortality at test termination was 10, 7.5, 6.3, 13, 15, 54 and 60 in the negative control, acetone control, 6.3, 13, 25, 50 and 100 mg BIT/kg treatment groups, respectively. |
| 4.2.4 Concentration / response curve | Not described in report |
| 4.2.5 Other effects | The organisms generally appeared normal and healthy throughout the study. During the study there were a few observations of organisms on the surface of the sediment in all treatment groups and controls. There were also a few observations of organisms swimming in the water column and climbing the walls of the test chamber. These observations were few in number and were not treatment related. |
| **4.3 Results of controls** | see Table A7.4.3.5.1.a/02-6 |
| **4.4 Test with reference substance** | |
| | **5 APPLICANT'S SUMMARY AND CONCLUSION** |
| **5.1 Materials and methods** | OECD Guideline 218, Chronic toxicity to sediment dwelling organisms with analytical confirmation of BIT concentrations. Midges were exposed to BIT concentrations for 28 days under static test conditions. Observations of mortality and abnormal behavior were made daily during the test. The total number of adults emerged at the end of the test period was recorded. Sediment samples were fortified with stock solution of BIT prepared in acetone. |
| **5.2 Results and discussion** | The overlying water appeared slightly tan and had a cloudy appearance in all test compartments at test initation and termination. The organisms generally appeared normal and healthy throughout the study. Percent mortality at test termination was 10, 7.5, 6.3, 13, 15, 54 and 60 in the negative control, acetone control, 6.3, 13, 25, 50 and 100 mg BIT/kg treatment groups, respectively. There were treatment related effects observed on development times in the 100 mg BIT/kg group and on mean emergence rates and development rates in the 50 and 100 mg BIT/kg treatment groups. Mean development time was 19.3, 21.5, 19.6, 19.6, 20.8, 22.7 and 25.1 days in the negative control, acetone control, 6.3, 13, 25, 50 and 100 mg BIT/kg treatment groups, respectively. The NOEC and LOEC for development time were based in the 100 mg BIT/kg values. |
| 5.2.1 LOEC | 100 mg BIT/kg, development time |

| | | | |
|---|---|---|---|
| | | 50 mg BIT/kg, emergence rate and development rate | |
| 5.2.2 | NOEC | 50 mg BIT/kg, development time<br>25 mg BIT/kg, emergence rate and development rate | |
| 5.2.3 | $EC_{50}$ | 52 mg BIT/kg (95% confidence interval of 40 – 95 mg BIT/kg), based on percent survival | |
| **5.3** | **Conclusion** | | |
| 5.3.1 | Reliability | (1), reliable without restriction | |
| 5.3.2 | Deficiencies | No | |

| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *December 2010.* |
| **Materials and Methods** | ***3.4.4**: The light intensity used in the study is lower than those recommended by the OECD (338 lux at water surface vs. 500 to 1000 lux).*<br>*According to the OECD 218 guidance, effect concentrations should be based on measured sediment concentrations at the beginning of the test.* |
| **Results and discussion** | *Applicant's version is adopted.*<br>*The final effect concentrations based on measurements are resulted as follows:*<br>*LOEC based on development time = 48.5 mg/kg*<br>*LOEC based on emergence rate and development time = 24.5 mg/kg*<br>*NOEC based on development time = 24.5 mg/kg*<br>*NOEC based on emergence rate and development time = 11.7 mg/kg*<br>*$EC_{50}$ = 32.79 mg/kg (19.39-55.46 mg/kg)* |
| **Conclusion** | *Applicant's version is adopted* |
| **Reliability** | *2* |
| **Acceptability** | *Acceptable* |
| **Remarks** | |

**Table A7.4.3.5.1.a/02-1: Preparation of TS solution for poorly soluble or volatile test substances**

| Criteria | Details |
|---|---|
| Dispersion | yes |
| Vehicle | Acetone |
| Concentration of vehicle | Not applicable |
| Vehicle control performed | Yes |
| Other procedures | The BIT primary stock solution was prepared by dissolving BIT in acetone at a nominal concentration of 10.0 mg BIT/ml. |

**Table A7.4.3.5.1.a/02-2: Dilution water**

| Criteria | Details |
|---|---|
| Source | Well freshwater, 40 meters deep |
| Alkalinity | 178 – 182 mg/L as $CaCO_3$ |
| Hardness | 136 mg/L as $CaCO_3$ |
| pH | 8.0 – 8.2 |
| Ca / Mg ratio | Not described |
| Na / K ratio | Not described |
| Dissolved Oxygen content | Not described |
| Conductance | 300-320 µmhos/cm |
| Holding water different from dilution water | No |

**Table A7.4.3.5.1.a/02-3: Test organisms**

| Criteria | Details |
|---|---|
| Strain | midge larvae (*Chironomus riparius)* |
| Source | Environmental Consulting and Testing, Superior, Wisconsin, USA |
| Age | 1-4 days |
| Breeding method | Not described |
| Kind of food | Hartz pet rabbit food |
| Amount of food | 10-30 mg |
| Feeding frequency | Approximately 3 times per week during the test. Organisms were not fed on day 27 due to the presence of fungal growth in the controls. |
| Pretreatment | Egg masses were held for four days prior to the start of the test at approximately the same temperature and water source as used during the test. |
| Feeding of animals during test | |

**Table A7.4.3.5.1.a/02-4: Test system**

| Criteria | Details |
|---|---|
| Renewal of test solution | No, static toxicity study |
| Volume of test vessels | Quart jars containing 2 cm of sediment and 600 mL of overlying water |
| Volume/animal | 30 mL/midge |
| Number of animals/vessel | 20 midges |
| Number of vessels/ concentration | 4 containing midges and 3 for analytical sampling |
| Sediment | < 1% humic acid and dolomite, 5% alpha-cellulose, 14% silt and clay (Kaolin clay) and 80% industrial quartz sand |
| Test performed in closed vessels due to significant volatility of TS | Loose plastic covers were placed over each test chamber during the test |

**Table A7.4.3.5.1.a/02-5: Test conditions**

| Criteria | Details |
|---|---|
| Test temperature | 20.4 – 21.0 ºC |
| Dissolved oxygen | ≥ 5.9 mg/L (66 % of saturation) |
| pH | 8.0 – 8.4 |
| Adjustment of pH | Not described |
| Total hardness | 136 mg/L as $CaCO_3$ |
| Ammonia | Not described |
| Aeration of dilution water | Aeration was applied to each test chamber through a glass pipette that extended no closer than 2 cm from the surface of the sediment |
| Quality/Intensity of irradiation | Fluorescent tubes that emitted wavelengths similar to natural sunlight. Light intensity = 338 lux at water surface |
| Photoperiod | 16 hours light and 8 hours darkness with 30-minute transition perion of low light intensity |

**Table A7.4.3.5.1.a/02-6: Effect and Mortality data**

| Test-Substance Concentration (nominal) [1] [mg BIT/kg dry sediment] | Percent emergence | Percent mortality | Mean development time (days) | Mean emergence rate | Mean development rate |
|---|---|---|---|---|---|
| Negative control | 93 | 10 | 19.3 | 0.93 | 0.0540 |
| Acetone control | 93 | 7.5 | 21.5 | 0.93 | 0.0486 |
| 6.3 | 96 | 6.3 | 19.6 | 0.96 | 0.0532 |
| 13 | 91 | 13 | 19.6 | 0.91 | 0.0531 |
| 25 | 85 | 15 | 20.8 | 0.85 | 0.0498 |
| 50 | 58 | 54 | 22.7 | 0.58 * | 0.0453 * |
| 100 | 61 | 60 | 25.1 | 0.61 * | 0.0409 * |

[1] TS concentrations were nominal

* Statistically significant ($p < 0.05$) differences from the pooled control using Dunnett's test

**Table A7.4.3.5.1.a/02-7: Effect data**

| | $EC_{50}$[1] | 95 % c.l. | $EC_0$[1] | $EC_{100}$[1] |
|---|---|---|---|---|
| **28 d [mg BIT/kg dry sediment]** | 52 (n) | 40 – 95 (n) | 25 (n) | Not applicable |

[1] effect data are based on nominal (n) concentrations

**Table A7.4.3.5.1.a/02-8: Validity criteria**

| | fulfilled | Not fullfilled |
|---|---|---|
| Mortality of control animals < 10% | **Yes** | |
| Concentration of test substance ≥ 80% of initial concentration during test | **yes** | |

| | | |
|---|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** | |
| **Subsection A7.4** | **Fate and Behaviour in the Environment** | |
| **Subsection A7.4.3** | **Effects on aquatic organisms, further studies** | |
| **Subsection A7.4.3.5** | **Effects on any other specific, non-target organisms (flora and fauna) believed to be at risk** | |
| **Subsection A7.4.3.5.2** | **Toxicity to aquatic plant** | |
| | **JUSTIFICATION FOR NON-SUBMISSION OF DATA** | **Official use only** |
| **Other existing data [ ]** | **Technically not feasible [ ] Scientifically unjustified [X]** | |
| **Limited exposure [X]** | **Other justification [ ]** | |
| **Detailed justification:** | Considering the environmental properties of BIT (not persistent, not accumulating, rapidly photolytically degradable, rapidly biodegraded), and the use pattern for BIT in the product type in question, which predicts low direct exposure to the aquatic and terrestrial environment, a long term exposure of the aquatic environment to high concentration of BIT is not expected. The environmental risk assessment included in Document II does not indicate a risk for the aquatic environment.<br>As a consequence, a test on aquatic plants is not considered necessary. | |
| **Undertaking of intended data submission [ ]** | No studies are planned. | |
| | | |
| | | |
| | **Evaluation by Competent Authorities** | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | *January 2010* | |
| **Evaluation of applicant's justification** | *Applicant's justification is accepted* | |
| **Conclusion** | *Applicant's justification is accepted* | |
| **Remarks** | | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.5** | **Effects on terrestrial organisms** |
| **Subsection A7.5.1** | **Terrestrial toxicity, initial tests** |
| **Subsection A7.5.1.1/01**<br>**Annex Point IIA7.4** | **Inhibition to microbial activity (terrestrial), carbon transformation** |

| | 1 REFERENCE | Official use only |
|---|---|---|
| **1.1 Reference** | **A7.5.1.1/01** [redacted] (2007a) 1,2-Benzisothiazolin-3-one: Soil microorganisms: carbon transformation test; [redacted] Rohm and Haas Report N° 06RC-097 (March 29, 2007), GLP, Unpublished. | |
| **1.2 Data protection** | Yes | |
| 1.2.1 Data owner | Rohm and Haas Company | |
| 1.2.2 Companies with letter of access | | |
| 1.2.3 Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into Annex I/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1 Guideline study** | Yes, OECD 217 | |
| **2.2 GLP** | Yes | |
| **2.3 Deviations** | No | |
| | **3 MATERIALS AND METHODS** | |
| **3.1 Test material** | 1,2-Benzisothiazolin-3-one (BIT) | |
| 3.1.1 Lot/Batch number | 2005-051 | |
| 3.1.2 Specification | As given in section 2 | |
| 3.1.3 Purity | 89.8% | |
| 3.1.4 Composition of Product | Not applicable | |
| 3.1.5 Further relevant properties | Not applicable | |
| 3.1.6 Method of analysis | High performance liquid chromatography (HPLC) | |

| | |
|---|---|
| **3.2 Reference substance** | No |
| 3.2.1 Method of analysis for reference substance | Not applicable |
| **3.3 Testing procedure** | |
| 3.3.1 Soil sample / inoculum/ test organism | see Table A7.5.1.1/01-1 |
| 3.3.2 Test system | see Table A7.5.1.1/01-2 |
| 3.3.3 Application of TS | see Table A7.5.1.1/01-3 |
| 3.3.4 Test conditions | see Table A7.5.1.1/01-4 |
| 3.3.5 Test parameter | Glucose-induced respiration |
| 3.3.6 Analytical parameter | $CO_2$ |
| 3.3.7 Duration of the test | 28 days |
| 3.3.8 Sampling | days 0, 7 and 28 for respiration |
| 3.3.9 Monitoring of TS concentration | No |
| 3.3.10 Controls | soil without test substance |
| 3.3.11 Statistics | The respiration rates were statistically analyzed using ANOVA and Bonferroni *t*-Test or Dunnett's test to determine the statistically significant differences from untreated controls at each sampling interval. |
| | **4 RESULTS** |
| **4.1 Range finding test** | Not performed |
| 4.1.1. Concentration | Not applicable |
| 4.1.2 Effect data | Not applicable |
| **4.2 Results test substance** | |
| 4.2.1 Initial concentrations of test | 0 (control), 10.7, 28.7, 100, 317 and 1000 mg BIT/kg soil |

| | | |
|---|---|---|
| substance | | |
| 4.2.2 Actual concentrations of test substance | Not applicable | |
| 4.2.3 Growth curves | Not applicable | |
| 4.2.4 Cell concentration data | Not applicable | |
| 4.2.5 Concentration/ response curve | see Figure A7.5.1.1/01-1 | |
| 4.2.6 Effect data | At the start of the test, respiration rates ranged from 17.3 to 23.3 mg $CO_2$ per kg dry soil per hour.<br>see Table A7.5.1.1/01-5 | |
| 4.2.7 Other observed effects | none | |
| **4.3 Results of controls** | see Table A7.5.1.1/01-5 | **X** |
| **4.4 Test with reference substance** | Not performed | |
| 4.4.1 Concentrations | Not applicable | |
| 4.4.2 Results | Not applicable | |
| | **5 APPLICANT'S SUMMARY AND CONCLUSION** | |
| **5.1 Materials and methods** | OECD 217, Effects on soil microflora respiration transformation.<br>One type of soil, a sandy loam, was used to prepare eighteen individual test chambers with 400 grams of dry soil. Soil moisture contents were adjusted to 22.7% water or 50% of the water holding capacity and acclimated in the dark at approximately 20 ºC for 28 days. Three replicates each were treated with BIT at calculated concentrations of 0, 10.7, 28.7, 100, 317 and 1000 mg a.i./kg. Soil samples were collected from each test chamber on Day 0, 7 and 28 for analyses of carbon dioxide production rates. | |
| **5.2 Results and discussion** | The long-term effects of BIT on carbon trams formation activity of soil microorganisms were minimal. After 28 days of exposure, the mean $CO_2$ production rates were 51% and 45% greater than the untreated controls at the two highest test concentrations. No significant adverse effects were observed. | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.5** | **Effects on terrestrial organisms** |
| **Subsection A7.5.1** | **Terrestrial toxicity, initial tests** |
| **Subsection A7.5.1.1/01**<br>**Annex Point IIA7.4** | **Inhibition to microbial activity (terrestrial), carbon transformation** |

| | | |
|---|---|---|
| 5.2.1 $EC_{10}$ | > 1000 mg BIT/kg | |
| 5.2.2 $EC_{25}$ | > 1000 mg BIT/kg | |
| 5.2.3 $EC_{50}$ | > 1000 mg BIT/kg | |
| **5.3 Conclusion** | The long term effects of BIT on carbon transformation activity of soil microrganisms were minimal. | |
| 5.3.1 Reliability | (1), reliable without restriction | |
| 5.3.2 Deficiencies | No | |

| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *March 2015* |
| **Materials and Methods** | *Applicant's version is accepted with the following remarks:*<br>▪ *The following deviations were noted:*<br>o *Variation among the controls on day 28 was not within the acceptable range (±15%). While two of the controls showed very similar results for its respiration rate (9.8 and 9.9 $CO_2$ mg/kg), the variability among the control results is mainly due to the respiration rate value of one single control (14.3 $CO_2$ mg/kg).*<br>o *Carbon content of microbial biomass is not specified.*<br>▪ *3.3. According to OECD 217, if the soil was stored, pre-incubation is recommended for a period between 2 and 28 days. For this test, soils were incubated only for one day prior the test.*<br>▪ *Application of the test substance was made by direct addition to the soils. Normally, the test substance is applied using a carrier.*<br>▪ *3.3.9. Test substance concentration was not monitored. Therefore, there is no evidence of the actual concentration of BIT during the test.* |

**Section A7** **Ecotoxicological Profile Including Environmental Fate and Behaviour**

**Subsection A7.5** **Effects on terrestrial organisms**

**Subsection A7.5.1** **Terrestrial toxicity, initial tests**

**Subsection A7.5.1.1/01** **Inhibition to microbial activity (terrestrial), carbon transformation**

**Annex Point IIA7.4**

| | |
|---|---|
| **Results and discussion** | *Applicant's version is accepted with the following remarks:*<br>▪ *4.1. Applicant should have performed a preliminary range-finding test, in order to determine the appropriate concentrations of the definitive test, including the $EC_{50}$ within the range of concentrations tested.*<br>▪ *4.2. There is a deviation: According to test report, on day 0 comparisons between treatments and controls were not possible due to missing replicates.*<br>*Data provided in test report correspond to calculated $CO_2$ production rates (Annex V of Doc. IV-A), calculated from raw data. Test report should include the raw data (decreases in pressure) used for these calculations.*<br>***"Table A7.5.1.1/01-5: Respiration rates",** second column title, should read "Measured (mg $CO_2$/kg dry soil/hour)" instead of "Measured (mg $O_2$/kg dry soil/hour)".* |
| **Conclusion** | *The test was considered valid. According to "**Table A7.5.1.1/01-5: Respiration rates**" and considering the increase in the respiration rates as an effect, the NOEC obtained is 100 mg/kg.* |
| **Reliability** | *2* |
| **Acceptability** | *Acceptable* |
| **Remarks** | *Although variability among the controls on day 28 was not within the acceptable range (±15%), variability among control replicates at previous intervals and of all other treatment groups was acceptable.* |

**Table A7.5.1.1/01-1: Microbial sample / Inoculum**

| Criteria | Details |
|---|---|
| Nature | loamy sand soil |
| Sampling site: | Grand Forks County, North Dakota |
| Geographical reference on the sampling site | Coordinates N 47º 48.166 – W 97º 37.264 |
| Data on the history of the site | Tree farm |
| Use pattern | Tree farm and no pesticides or fertilizers were applied in the previous year |
| Depth of sampling [cm] | Top 10-20 cm and sieved to 2 mm |
| Sand / Silt / Clay content [particle size distribution] | 66% sand, 16% silt and 18% clay |
| pH | 7.1 |
| Organic carbon content [% dry weight] | 1.4% |
| Nitrogen content [mg N/100 g] | Not described in report |
| Maximum water holding capacity [g/100 g dry soil] | 45.4% |
| Initial microbial biomass | 330 µg/g |
| Reference of methods | Soil content: USDA Textural Class hydrometer method<br>Microbial Biomass Carbon: Fumigation and Extraction Method by – Vance E.D. (1987) An extraction method for measuring soil microbial biomass C. Soil Biol. Biochem. Vol. 19, No. 6, pp.703-707. |
| Collection / storage of samples | The soil was transported to the laboratory and stored at refrigerated conditions for 80 days then transferred to a large plastic tray, covered with aluminium foil, and placed in a temperature-controlled room to incubate in the dark under aerobic conditions at approximately 20 ºC. |
| Preparation of inoculum for exposure | Not applicable |
| Pretreatment | Not applicable |

**Table A7.5.1.1/01-2: Test system**

| Criteria | Details |
|---|---|
| Culturing apparatus | 11 x 17 inch pyrexglass baking dishes containing 400 grams of dry soil |
| Number of vessels / concentration | 3 |
| Aeration device | Plastic lids had holes drilled into them to allow air circulation |
| Measuring equipment | OxiTop® measuring systems (WTW GmbH, Germany) included plastic cups filled with 40 mL of 1.5 N KOH solution to absorb $CO_2$ headspace gases. |
| Test performed in closed vessels | Plastic lids on the glass baking dishes |

**Table A7.5.1.1/01-3: Application of test substance**

| Criteria | Details |
|---|---|
| Application procedure | TS was applied to the soil by direct weight addition in the test chambers |
| Carrier | Not applicable |
| Concentration of liquid carrier [% v/v] | Not applicable |
| Liquid carrier control | Not applicable |
| Other procedures | Not applicable |

**Table A7.5.1.1/01-4: Test conditions**

| Criteria | Details |
|---|---|
| Organic substrate | Not applicable |
| Incubation temperature | 19.2 to 22.2 °C |
| Soil moisture | Maintained at 50% of maximum water holding capacity (43.0% to 51.8%) |
| Method of soil incubation | Individual sub samples |
| Aeration | Plastic lids had holes drilled into them to allow air circulation |

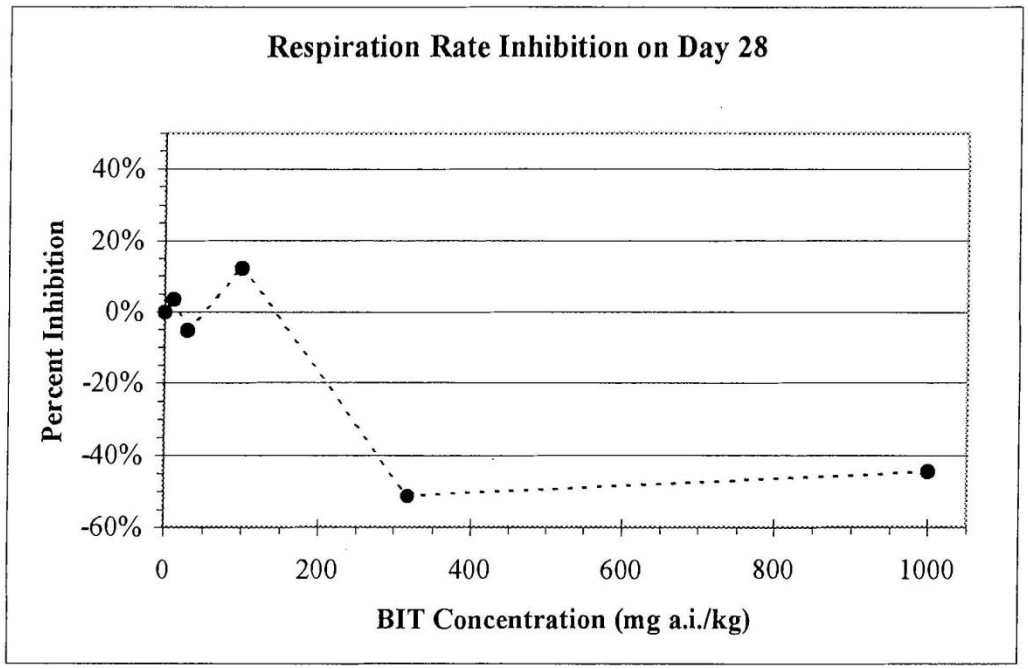
**Table A7.5.1.1/01-5: Respiration rates**

| Test Substance Concentration (nominal) [mg BIT/kg dry soil] | Measured ($mg\ O_2$/kg dry soil/hour) | | | % difference to control | | |
|---|---|---|---|---|---|---|
| | Day 0 | Day 7 | Day 28 | Day 0 | Day 7 | Day 28 |
| 0 (control) | 17.3 | 11.0 | 11.3 | -- | -- | -- |
| 10.7 | 18.3 | 14.3 | 10.9 | 106 | 130 | 96 |
| 28.7 | 18.8 | 11.9 | 11.9 | 109 | 108 | 105 |
| 100 | 23.3 | 12.9 | 10.0 | 135 | 117 | 88 |
| 317 | 20.2 | 10.5 | 17.1* | 117 | 95 | 151* |
| 1000 | 20.2 | 10.5 | 16.4* | 117 | 95 | 145* |

-- not applicable

* denotes statistically significant differences from respective controls

**Figure A7.5.1.1/01-1: Glucose induced short term respiration**



| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.5** | **Effects on terrestrial organisms** |
| **Subsection A7.5.1** | **Terrestrial toxicity, initial tests** |
| **Subsection A7.5.1.1/02**<br>**Annex Point IIA7.4** | **Inhibition to microbial activity (terrestrial), nitrogen transformation** |

| | **1 REFERENCE** | **Official use only** |
|---|---|---|
| **1.1 Reference** | **A7.5.1.1/02** [redacted] (2007b) 1,2-Benzisothiazolin-3-one: Soil microorganisms nitrogen transformation test; [redacted] Rohm and Haas Report N° 06RC-096 (March 29, 2007), GLP, Unpublished. | |
| **1.2 Data protection** | Yes | |
| 1.2.1. Data owner | Rohm and Haas Company | |
| 1.2.2. Companies with letter of access | | |
| 1.2.3. Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into Annex I/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1 Guideline study** | Yes, OECD 216 | |
| **2.2 GLP** | Yes | |
| **2.3 Deviations** | No | |
| | **3 MATERIALS AND METHODS** | |
| **3.1 Test material** | 1,2-Benzisothiazolin-3-one (BIT) | |
| 3.1.1 Lot/Batch number | 2005-051 | |
| 3.1.2 Specification | As given in section 2 | |
| 3.1.3 Purity | 89.8% | |
| 3.1.4 Composition of Product | Not applicable | |
| 3.1.5 Further relevant properties | Not applicable | |
| 3.1.6 Method of analysis | High performance liquid chromatography (HPLC) | |
| **3.2 Reference substance** | Nitrification Inhibitor Formula 2533, Lot Number A6251 contained 2-chloro-6(trichloromethyl) pyridine coated on a sodium sulfate | |

| | | |
|---|---|---|
| | substrate | |
| 3.2.1 Method of analysis for reference substance | Not described in report | |
| **3.3 Testing procedure** | | |
| 3.3.1 Soil sample / inoculum/ test organism | see Table A7.5.1.1/02-1 | |
| 3.3.2 Test system | see Table A7.5.1.1/02-2 | |
| 3.3.3 Application of TS | see Table A7.5.1.1/02-3 | |
| 3.3.4 Test conditions | see Table A7.5.1.1/02-4 | **X** |
| 3.3.5 Test parameter | Nitrogen transformation by soil microorganisms | |
| 3.3.6 Analytical parameter | Nitrite, nitrate and ammonia measurements | |
| 3.3.7 Duration of the test | 28 days | |
| 3.3.8 Sampling | days 0, 7 and 28 | |
| 3.3.9 Monitoring of TS concentration | No | |
| 3.3.10 Controls | Control without test substance | |
| 3.3.11 Statistics | The mean concentrations of ammonia, nitrite and nitrate were calculated for each test chamber at each sampling interval and each treatment mean was calculated from the three replicates. The mean concentrations were compared to appropriate controls and percent inhibition values were calculated. The mean concentrations were statistically analyzed using ANOVA Dunnett's Test and Tukey Method of Multiple Comparisons to determine statistically significant differences. | |
| | **4 RESULTS** | |
| **4.1 Range finding test** | | |
| 4.1.1 Concentration | Not described in report | **X** |
| 4.1.2 Effect data | 1000 mg BIT/kg was selected as the test concentration based on the results from the range-finding test | **X** |
| **4.2 Results test substance** | | |

| | | |
|---|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** | |
| **Subsection A7.5** | **Effects on terrestrial organisms** | |
| **Subsection A7.5.1** | **Terrestrial toxicity, initial tests** | |
| **Subsection A7.5.1.1/02**<br>**Annex Point IIA7.4** | **Inhibition to microbial activity (terrestrial), nitrogen transformation** | |
| 4.2.1Initial concentrations of test substance | 1000 mg BIT/kg, nominal | **X** |
| 4.2.2 Actual concentrations of test substance | Not applicable | |
| 4.2.3 Growth curves | Not applicable | |
| 4.2.4 Cell concentration data | Not applicable | |
| 4.2.5 Concentration/ response curve | See Figure 7.5.1.1/02-1 | |
| 4.2.6 Effect data | see Tables A7.5.1.1/02-5 | |
| 4.2.7 Other observed effects | see Tables A7.5.1.1/02-5 | |
| **4.3 Results of controls** | see Tables A7.5.1.1/02-5 | **X** |
| **4.4 Test with reference substance** | Performed: Nitrification Inhibitor Formula 2533, Lot Number A6251 contained 2-chloro-6(trichloromethyl) pyridine coated on a sodium sulfate substrate | |
| 4.4.1 Concentrations | 250 mg/kg, nominal | |
| 4.4.2 Results | Nitrification Inhibitor had higher concentrations of ammonia compared with controls but less than BIT; Day 0 = 12.5 mg $NH_4^+$/kg, Day 7 = 16.0 mg $NH_4^+$/kg and Day 28 = 5.8 mg $NH_4^+$/kg.<br>Nitrite concentrations in the Nitrification Inhibitor treated soils ranged from 4.8 to 6.9 mg $NO_2^-$/kg on Day 0 and were below the LOQ on Days 7 and 28.<br>Nitrate concentrations in the Nitrification Inhibitor treated soils were 69.4 mg $NO_3^-$/kg on Day 0, 96.9 mg $NO_3^-$/kg on Day 7, and 166.5 mg $NO_3^-$/kg on Day 28. | **X** |
| | **5 APPLICANT'S SUMMARY AND CONCLUSION** | |
| **5.1 Materials and methods** | OECD 216, Effects on soil microflora ammonia, nitrite and nitrate transformation. | |
| **5.2 Results and discussion** | Ammonia: At test day 0, concentrations of ammonia in all alfalfa-amended soils ranged from 11.0 to 18.9 mg $NH_4^+$/kg and concentrations in non-amended soils ranged from 0.3 to 1.2 mg $NH_4^+$/kg. On days 7 and 28, the soils treated with BIT had significantly higher levels of ammonia than the controls in both | |

| | | |
|---|---|---|
| | amended and non-amended soils.<br><br>Nitrite: At test day 0, concentrations of nitrite in alfalfa-amended controls ranged from 8.2 to 8.4 mg $NO_2^-$/kg. On days 7 and 28, none of the samples contained measureable amounts of nitrite. The limit of quantitation (LOQ) for nitrite was approximately 3 mg $NO_2^-$/kg.<br><br>Nitrate: At test day 0, concentrations of nitrate in all samples ranged from 55.0 to 92.0 mg $NO_3^-$/kg. There were no statisitically significant treatment related differences. At 1000 mg/kg, BIT transiently inhibited nitrate formation in soil on day 7 as evidenced by increased ammonia concentrations in both amended and non-amended soils and a significant decrease in nitrate concentration in alfalfa-amended soil. The non-amended soils did not show a significant decrease in nitrate concentration. The soil microrganisms recovered by day 28. In amended soils, the nitrate concentration was much less and nitrate concentrations were not statistically significant from the amended controls. | |
| $EC_{10}$ | alfalfa-amended soil = 833 mg BIT/kg<br><br>non-amended soil >1000 mg BIT/kg | |
| $EC_{25}$ | > 1000 mg BIT/kg | |
| $EC_{50}$ | > 1000 mg BIT/kg | |
| **5.3 Conclusion** | The long term effects of 1,2-Benzisothiazolin-3-one on nitrogen transformation activity of soil microorganisms were minimal. After 28 days of exposure, the mean nitrate concentrations in alfalfa-amended and non-amended soils treated at 1000 mg BIT/kg were 12% and 2% less than the respective untreated controls. The $EC_{10}$ in alfalfa-amended soil was 833 mg BIT/kg and > 1000 mg BIT/kg in non-amended soil. The $EC_{25}$ and $EC_{50}$ were estimated to be > 1000 mg BIT/kg. | |
| 5.3.1 Reliability | (1), reliable without restriction | |
| 5.3.2 Deficiencies | No | |

| **Evaluation by Competent Authorities** | | |
|---|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | *May 2013* | |
| **Materials and Methods** | *Applicant's version is accepted with the following remark:* | |

| | |
|---|---|
| | ▪ ***3.3.4*** *The moisture contents of the soils ranged from 19.3% to 24.8% (41.9% to 54.7% of WHC) throughout the test period, with one exception. The moisture content of test chamber 2 (untreated control) was calculated to be 17.7% (39.1% of WHC) on day 21, just prior to adding water.* |
| **Results and discussion** | *Applicant's version is accepted with the following remark:*<br>▪ ***4.1.1 and 4.1.2*** *On day 28 of the range-finding test, soils treated at nominal concentrations of 0.1, 1.0, 10, 100 and 1000 mg/kg exhibited inhibition of nitrate formation at 18%, 7%, 13%, 0%, and –127%, respectively, when compared with untreated control soil.. The increase in nitrate formation at the 1000 mg/kg treatment indicated the test substance may have been used as a nitrogen source; therefore, the study was conducted using both amended and non-amended soils.*<br>▪ ***4.2.1*** *A geometric series of at least five concentrations should have been used. In addition, these concentrations should have covered the range to determine ECx values.*<br>▪ ***4.3*** *The variation among the alfalfa-amended control replicates on days 0, 14 and 28 were 28.4%, 11.4% and 6.1%, respectively.* |
| **Conclusion** | *Applicant's version is adopted.* |
| **Reliability** | *2* |

Measured Concentrations of Nitrate in Soil Samples

| Treatment | Test Chamber ID (129E-119-) | Day 0 (mg $NO_3^-$/kg) | Day 7 (mg $NO_3^-$/kg) | Day 28 (mg $NO_3^-$/kg) |
|---|---|---|---|---|
| Control - Amended | 1 | 55.0 | 138.0 | 173.7 |
| | 2 | 66.6 | 157.7 | 190.7 |
| | 3 | 90.9 | 171.9 | 190.3 |
| | Means: | 70.8 | 155.8 | 184.9 |
| Reference Inhibitor - Amended | 4 | 59.4 | 82.6 | 131.4 |
| | 5 | 67.6 | 95.5 | 174.1 |
| | 6 | 81.2 | 111.6 | 193.9 |
| | Means: | 69.4 | 96.6 | 166.5 |
| BIT 1000 mg a.i./kg - Amended | 7 | 71.5 | 75.9 | 169.2 |
| | 8 | 76.5 | 78.8 | 169.0 |
| | 9 | 92.0 | 95.0 | 151.5 |
| | Means: | 80.0 | 83.2 | 163.3 |
| Control | 10 | 64.2 | 65.8 | 81.5 |
| | 11 | 68.3 | 80.2 | 94.0 |
| | 12 | 79.3 | 76.5 | 100.9 |
| | Means: | 70.6 | 74.2 | 92.1 |
| BIT 1000 mg a.i./kg | 13 | 65.4 | 68.5 | 82.9 |
| | 14 | 70.4 | 69.5 | 78.7 |
| | 15 | 78.8 | 81.9 | 108.7 |
| | Means: | 71.5 | 73.3 | 90.1 |

**Section A7** **Ecotoxicological Profile Including Environmental Fate and Behaviour**

**Subsection A7.5** **Effects on terrestrial organisms**

**Subsection A7.5.1** **Terrestrial toxicity, initial tests**

**Subsection A7.5.1.1/02** **Inhibition to microbial activity (terrestrial), nitrogen transformation**

**Annex Point IIA7.4**

| | |
|---|---|
| **Acceptability** | *Acceptable* |
| **Remarks** | *The variation among the alfalfa-amended controls was greater than the acceptable range specified in the protocol (±15% of the mean) on day 0; however, the variation among amended and non-amended controls was within the acceptable range at all other intervals during the study. The increased amount of variability at the start of the test did not affect the ability to determine differences between treatments and controls. This deviation had no impact on the results of the study.* |

**Table A7.5.1.1/02-1: Microbial sample / Inoculum**

| Criteria | Details |
|---|---|
| Nature | Sandy loam soil from Agvise Laboratories |
| Sampling site: | |
| Geographical reference on the sampling site | Grand Forks County, Northwood, North Dakota, USA, N 47º 48.166 and W 97º 37.264 |
| Data on the history of the site | tree farm |
| Use pattern | Tree farm with no pesticides or fertilizers applied in the previous year |
| Depth of sampling [cm] | 10-20 cm |
| Sand / Silt / Clay content [particle size distribution] | 66% sand, 16% silt and 18% clay |
| pH | 7.1 |
| Organic carbon content [% dry weight] | 1.4% |
| Nitrogen content [mg N/100 g] | Nitrite on day 0 < LOQ; Nitrate on Day 0 = 70.6 mg $NO_3^-$/kg; Ammonia on Day 0 = 0.3 to 1.2 mg $NH4^+$/kg. |
| Maximum water holding capacity | Mean = 45.4% |
| Initial microbial biomass | 330 µg/g |
| Reference of methods | Microbial biomass carbon based on a Fumigation and Extraction Method by: Vance, E.D. (1987) An Extraction Method for Measuring Soil Microbial Biomass C. Soil Biol. Biochem., Volume 19, No. 6, pp. 703-707. |
| Collection / storage of samples | Soil was collected from the top 10-20 cm and sieved to 2 mm and stored under refrigerated conditions for three months. |
| Preparation of inoculum for exposure | Not applicable |
| Pretreatment | Not applicable |

**Table A7.5.1.1/02-2: Test system**

| Criteria | Details |
|---|---|
| Culturing apparatus | 9 x 9 inch Pyrex glass baking dishes with plastic lids each filled with 245 grams of moist soil (equivalent to 200 grams of dry soil) |
| Number of vessels / concentration | 3 |
| Aeration device | Not described |
| Measuring equipment | Ammonia and nitrogen: Hach DR/890 colorimeter<br>Nitrate and Nitrite: Dionex DX-500 Ion Chromatography System |
| Test performed in closed vessels | Holes were drilled in the lids to allow circulation of air. |

**Table A7.5.1.1/02-3: Application of test substance**

| Criteria | Details |
|---|---|
| Application procedure | BIT was added to finely gound quartz sand. |
| Carrier | Finely ground quartz sand |
| Concentration of liquid carrier [% v/v] | Not applicable |
| Liquid carrier control | Not applicable |
| Other procedures | Not applicable |

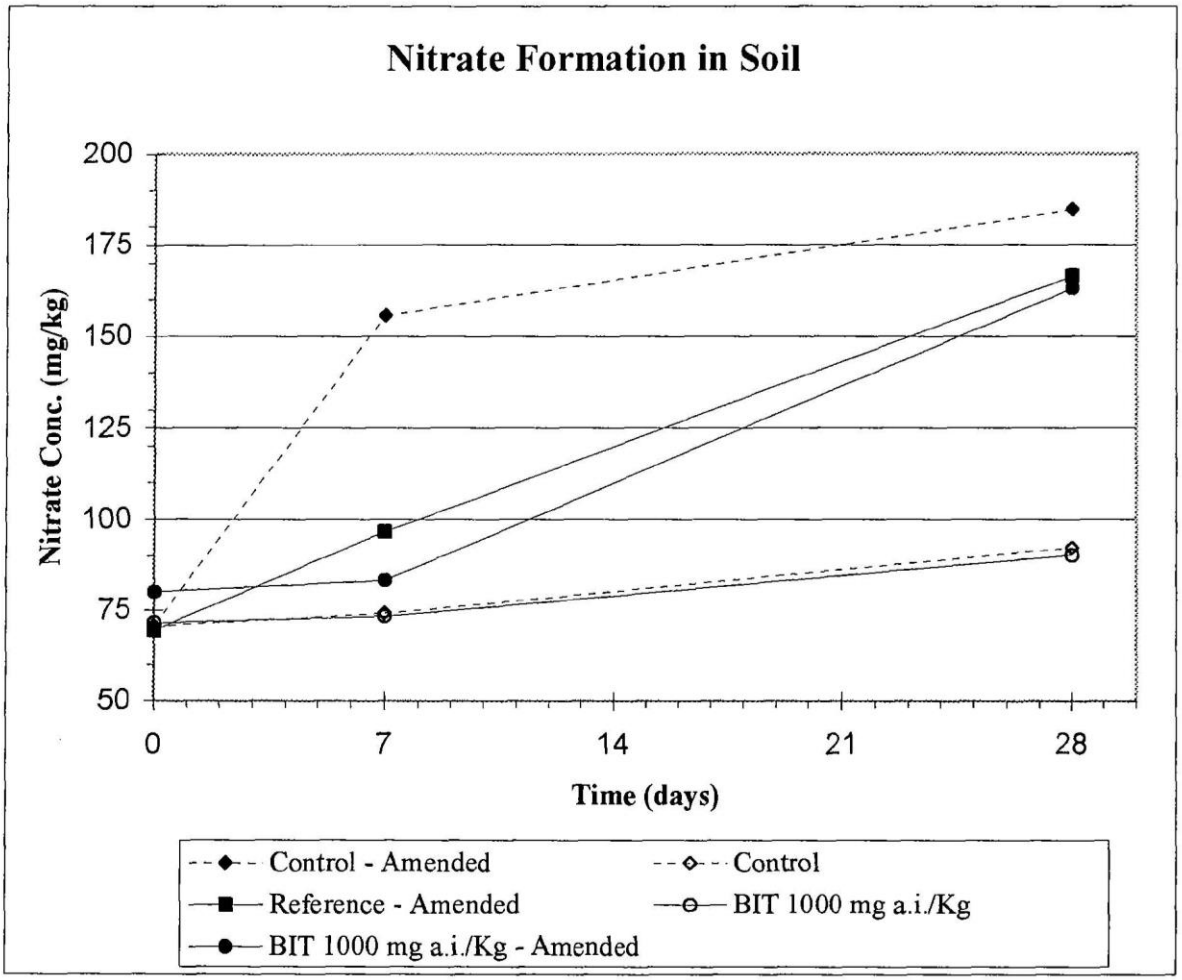
**Table A7.5.1.1/02-4: Test conditions**

| Criteria | Details |
|---|---|
| Organic substrate | Nine of the test chambers were amended with 5 g/kg of dried, ground alfalfa, while the other six were not amended. Three of the alfalfa-amended test chambers and three non-amended test chambers were untreated. Three of the alfalfa-amended test chambers and three non-amended test chambers were treated with the test substance at a nominal concentration of 1000 mg a.i./kg. Three of the alfalfa-amended test chambers were treated with a nitrification inhibitor at a nominal concentration of 250 mg/kg. |
| Incubation temperature | 20 ºC |
| Soil moisture | The moisture contents of the soils were adjusted to 22.7% water or 50% of the water holding capacity. |
| Method of soil incubation | All test chambers were incubated under aerobic conditions in the dark at approximately 20 ºC for two days prior to test initiation and throughout the 28-day test period. |
| Aeration | yes |

**Table A7.5.1.1/02-5: Ammonia**

| Test Substance Concentration (nominal) [mg BIT/kg soil] | Measured Ammonia (mg $NH_4^+$/kg dry soil/hour) | | | Measured Nitrate (mg $NO_3^-$/kg dry soil/day) | | |
|---|---|---|---|---|---|---|
| | Day 0 | Day 7 | Day 28 | Day 0 | Day 7 | Day 28 |
| 0, control - amended | 14.4 | 1.5 | 0.5 | 70.8 | 155.8 | 184.9 |
| Reference Inhibitor - amended | 12.5 | 16.0 | 5.8 | 69.4 | 96.6 | 166.5 |
| 1000 mg BIT/kg - amended | 14.0 | 48.6 | 45.2 | 80.0 | 83.2 | 163.3 |
| Control | 0.7 | 0.6 | 0.0 | 70.6 | 74.2 | 92.1 |
| 1000 mg BIT/kg | 0.5 | 13.3 | 25.3 | 71.5 | 73.3 | 90.1 |

**Figure 7.5.1.1/02-1**



**Section A7** **Ecotoxicological Profile Including Environmental Fate and Behaviour**

**Subsection A7.5** **Effects on terrestrial organisms**

**Subsection A7.5.1** **Terrestrial toxicity, initial tests**

**Subsection A7.5.1.2** **Earthworm, acute toxicity test**

**Annex Point IIIA XIII 3.2**

| | **1 Reference** | **Official use only** |
|---|---|---|
| **1.1 Reference** | **A7.5.1.2/01** [redacted] (2006) 1,2-Benzisothiazolin-3-one: An acute toxicity study with the earthworm in an artificial soil substrate, [redacted], Rohm and Haas Report N° 06RC-099 (August 17, 2006), GLP, Unpublished. | |
| **1.2 Data protection** | Yes | |
| 1.2.1. Data owner | Rohm and Haas Company | |
| 1.2.2. Companies with letter of access | | |
| 1.2.3. Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into Annex I/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1 Guideline study** | Yes, OECD Method 207 | |
| **2.2 GLP** | Yes | |
| **2.3 Deviations** | No | |
| | **3 METHOD** | |
| **3.1 Test material** | 1,2-Benzisothiazolin-3-one | |
| 3.1.1 Lot/Batch number | 2005-051 | |
| 3.1.2 Specification | As given in section 2 | |
| 3.1.3 Purity | 89.8% | |
| 3.1.4 Composition of Product | not applicable | |
| 3.1.5 Further relevant properties | not applicable | |

| | | | |
|---|---|---|---|
| 3.1.6 | Method of analysis | High performance liquid chromatography (HPLC) with UV detector | |
| **3.2 Reference substance** | | Yes, 2-chloracetamide, method of analysis not described. | **X** |
| **3.3 Testing procedure** | | | |
| 3.3.1 | Preparation of the test substance | see Table A7.5.1.2/01-1 | |
| 3.3.2 | Application of the test substance | Test soil was prepared by mixing BIT with reverse osmosis water and adding it to artificial soil. Moisture content was approximately 35% by weight. | |
| 3.3.3 | Test organisms | see Table A7.5.1.2/01-2 | |
| 3.3.4 | Test system | see Table A7.5.1.2/01-3 | |
| 3.3.5 | Test conditions | see Table A7.5.1.2/01-4 | |
| 3.3.6 | Test duration | 14 days | |
| 3.3.7 | Test parameter | Mortality and clinical signs | |
| 3.3.8 | Examination | Weight of worms was determined at the start and the end of the test. Time to burrow was observed at test initiation and on Day 7. On days 7 and 14, the contents of each test chamber were removed to determine the number of surviving earthworms. | |
| 3.3.9 | Monitoring of test substance concentration | No | |
| 3.3.10 | Statistics | The $LC_{50}$s and 95% confidence intervals were calculated using the Stephan computer program (Stephan, C.E., 1978. US EPA, Environmental Research Laboratory, Duluth, Minnesota, Personal Communication). The Day 7 $LC_{50}$ value was calculated by nonlinear interpolation and the Day 14 $LC_{50}$value was calculated by the Probit method. Body weights and change in body weights were statistically compared with Dunnett's 2-Tailed Test of Means ($\alpha = 0.05$) using SAS Version 8 (SAS Institute, Inc. 1999. SAS/STAT User's Guide, Version 8, Cary, North Carolina, USA) | |
| | | **4 RESULTS** | |
| **4.1 Filter paper test** | | Not performed | |
| **4.2 Soil test** | | | |

| | | |
|---|---|---|
| 4.2.1 Initial concentrations of test substance | 0 (negative control), 28.06, 56.13, 112.25, 224.5, 449 and 898 mg BIT/kg of soil on a dry weight basis | |
| 4.2.2 Effect data (Mortality) | see Table A7.5.1.2/01-5,A7.5.1.2/01-6 and Table A7.5.1.2/01-7 | |
| 4.2.3 Concentration / effect curve | No | **X** |
| 4.2.4 Other effects | Not applicable | **X** |
| **4.3 Results of controls** | | |
| 4.3.1 Mortality | There were no mortalities in the negative control. | |
| 4.3.2 Number/ percentage of earthworms showing adverse effects | All control worms were normal in appearance and behaviour throughout the test period. | |
| 4.3.3 Nature of adverse effects | Not applicable | |
| **4.4 Test with reference substance** | Performed | |
| 4.4.1 Concentrations | Nominal concentrations of 13, 25, and 50 mg chloroacetamide/kg dry soil. | |
| 4.4.2 Results | 14-day $LC_{50}$: 24.5 mg a.i./kg dry soil with 95% confidence interval of 13 and 50 mg a.i./kg dry soil | |
| | **5 APPLICANT'S SUMMARY AND CONCLUSION** | |
| **5.1 Materials and methods** | OECD Method 207, Acute toxicity to the earthworm | |
| **5.2 Results and discussion** | All control worms survived and were normal in appearance and behaviour throughout the test period. The earthworms showed a strong aversion to the test soils. On Day 0, worms in the negative control and the 28.06 mg BIT/kg treatment group burrowed within approximately ½ hour of being placed on the soil surface at test initiation. Worms in the 56.13 mg BIT/kg group were mostly burrowed at approximately one hour after test initiation. Worms in all of the other groups did not burrow and remained on the soil surface or on the sides of the test chamber above the soil surface. Worms in the 889 mg BIT/kg group were lethargic and some were dead ½ hour after test initiation. Body weights were not determined for the 224.5 mg BIT/kg and higher doses due to insufficient worms or no worms were available for final body weight comparisons in these groups. | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
| **Subsection A7.5** | **Effects on terrestrial organisms** |
| **Subsection A7.5.1** | **Terrestrial toxicity, initial tests** |
| **Subsection A7.5.1.2** | **Earthworm, acute toxicity test** |
| **Annex Point IIIA XIII 3.2** | |

| | | |
|---|---|---|
| 5.2.1 NOEC | 28.06 mg BIT/kg dry soil | |
| 5.2.2 $LC_0$ | 7-day and 14-day: 28.06 mg BIT/kg dry soil | |
| 5.2.3 $LC_{50}$ | 7-day: 278 mg BIT/kg dry soil<br>14-day: 114 mg BIT/kg dry soil | |
| 5.2.4 $LC_{100}$ | 7-day: 449 mg BIT/kg dry soil<br>14-day: 449 mg BIT/kg dry soil | |
| **5.3 Conclusion** | see Table A7.5.1.2/01-7 and see Table A7.5.1.2/01-8 | |
| 5.3.1 Other Conclusions | | |
| 5.3.2 Reliability | (1), reliable without restriction | |
| 5.3.3 Deficiencies | No | |

| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *January 2011* |
| **Materials and Methods** | *Applicant's version is accepted with the following remark:*<br>▪ *3.2 At Wildlife International, Ltd., reference toxicity tests with a reference toxicant, chloroacetamide, are conducted periodically to assess the sensitivity of the test species and test procedures. These studies are conducted under separate protocols, as independent studies. A summary of the results from the most current reference toxicity test is presented in this report.* |

## Section A7 Ecotoxicological Profile Including Environmental Fate and Behaviour

## Subsection A7.5 Effects on terrestrial organisms

## Subsection A7.5.1 Terrestrial toxicity, initial tests

## Subsection A7.5.1.2 Earthworm, acute toxicity test

**Annex Point IIIA XIII 3.2**

| | |
|---|---|
| **Results anddiscussion** | *Applicant's version is accepted with the following remarks:*<br>▪ *4.2.4 There were statistically significant effects on final body weight and the change in body weight at the 112.25 mg a.i./kg level when compared to the control group.*<br>▪ *$LC_{50}$ values should include the correspondent confidential limit intervals:*<br>o *$LC_{50}$-7-day: 278 mg BIT/kg dry soil (224.5 - 449 mg BIT/kg dry soil)*<br>o *$LC_{50}$-14-day: 114 mg BIT/kg dry soil (98.1 - 132 mg BIT/kg dry soil)* |
| **Conclusion** | *Applicant's version is adopted.* |
| **Reliability** | *2* |
| **Acceptability** | *Acceptable* |
| **Remarks** | |

**Table A7.5.1.2/01-1: Preparation of TS solution**

| Criteria | Details |
|---|---|
| Type and source of dilution water | Reverse osmosis water prepared at laboratory |
| Holding water different from dilution water | Not applicable |
| **In case of the use of an organic solvent** | |
| Dispersion | Not applicable |
| Vehicle | Not applicable |
| Concentration of vehicle | Not applicable |
| Vehicle control performed | Not applicable |
| Other procedures | Not applicable |

**Table A7.5.1.2/01-2: Test organisms**

| Criteria | Details |
|---|---|
| Species/strain | *Eisenia fetida* |
| Source of the initial stock | University of Maryland, Queenstown, Maryland, USA |
| Culturing techniques | Not applicable |
| Age/weight | adult worms with clitella, 0.57 to 0.66 grams |
| Pre-treatment | 24 hours prior to test initiation, worms were placed into artificial soil substrate adjusted to 35% by weight moisture content for the acclimation period. |

**Table A7.5.1.2/01-3: Test system**

| Criteria | Details |
|---|---|
| Artificial soil test substrate | Composition of the test substrate was 20% kaolin clay, 70% sand, 10% sphagnum peat. pH was adjusted to 5.9 by the addition of calcium carbonate. 35% moisture |
| Test mixture | Test soil was prepared by mixing BIT with reverse osmosis water and adding it to bulk artificial soil with 35% moisture content. |
| Size, volume and material of test container | One liter glass beakers covered with plastic wrap that was perforated for air exchange |
| Amount of artificial soil (kg)/ container | 750 grams of prepared soil |
| Nominal levels of test concentrations | 0 (negative control), 28.06, 56.13, 112.25, 224.5, 449 and 898 mg BIT/kg of soil on a dry weight basis |
| Number of replicates/concentration | 4 |
| Number of earthworms/test concentration | 40 |
| Number of earthworms/container | 10 |
| Light source | not described |
| Test performed in closed vessels due to significant volatility of test substrate | No |

**Table A7.5.1.2/01-4: Test conditions**

| Criteria | Details |
|---|---|
| Test temperature | 20 $\pm$ 2 °C |
| Moisture content | Day 0: 33.8 to 34.8%, Day 14: 32.8 to 34.0% |
| pH | Day 0 = 7.0 to 7.4; Day 14 = 7.2 to 7.5 |
| Adjustment of pH | Yes, pH was adjusted to 5.9 by the addition of calcium carbonate. |
| Light intensity / photoperiod | 400-800 lux, 24 h light and 0 h dark |
| Relevant degradation products | Not applicable |

**Table A7.5.1.2/01-5: Mortality data**

| Test Substance<br>Concentration (nominal)<br>[mg BIT/kg artificial soil] | Mortality | | | |
|---|---|---|---|---|
| | Number Dead or Missing | | Percentage | |
| | 7 d | 14 d | 7 d | 14 d |
| 0 (negative control) | 0/40 | 0/40 | 0 | 0 |
| 28.06 | 0/40 | 0/40 | 0 | 0 |
| 56.13 | 7/40 | 8/40 | 17.5 | 20 |
| 112.25 | 7/40 | 13/40 | 17.5 | 33 |
| 224.5 | 8/40 | 37/40 | 20 | 93 |
| 449 | 40/40 | 40/40 | 100 | 100 |
| 898 | 40/40 | 40/40 | 100 | 100 |
| **Temperature [°C]** | Day 0: 20.5-21.5 | Day 14: 20.2-21.0 | | |
| **pH** | Day 0: 7.0-7.4 | Day 14: 7.2-7.5 | | |
| **Moisture content** | Day 0: 33.8-34.8 | Day 14: 32.8-34.0 | | |

**Table A7.5.1.2/01-6: Number affected data**

| Test Substance<br>Concentration (nominal)<br>[mg BIT/kg artificial soil] | Number Affected | | | |
|---|---|---|---|---|
| | Number affected | | Percentage | |
| | 7 d | 14 d | 7 d | 14 d |
| 0 (control) | 0/40 | 0/40 | 0 | 0 |
| 28.06 | 0/40 | 0/40 | 0 | 0 |
| 56.13 | 2/40 not found | 8/40 not found | 5 | 20 |
| 112.25 | 5/40 not found | 13 not found, 6 reduced reaction to mechanical stimuli | 12.5 | 32.5 not found, 15 reduced reaction to mechanical stimuli |
| 224.5 | 8/40 not found, 12 reduced reaction to mechanical stimuli, 8/40 thin | 30/40 not found, 1 reduced reaction to mechanical stimuli, 2/40 thin | 20 not found, 30 reduced reaction to mechanical stimuli, 20 thin | 75 not found, 2.5 reduced reaction to mechanical stimuli, 5 thin |
| 449 | 32/40 not found | 40/40 not found | 80 | 100 |

| | | | | |
|---|---|---|---|---|
| 898 | 40/40 not found | 40/40 not found | 100 | 100 |

**Table A7.5.1.2/01-7: Effect data**

| | 14 d [mg BIT/kg dry soil][1] | 95 % C.I. |
|---|---|---|
| **$LC_0$** | 28.06 | Not described |
| **$LC_{50}$** | 114 | 98.1 – 132 |
| **$LC_{100}$** | 449 | Not described |

[1] effect data are based on measured concentrations

**Table A7.5.1.2/01-8: Validity criteria for acute earthworm test according to OECD 207**

| | fulfilled | Not fulfilled |
|---|---|---|
| Mortality of control animals < 10% | **yes** | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
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| **Subsection A7.5.1.3** | **Seedling emergence an growth** |
| **Subsection A7.5.1.3/01** | |
| **Annex Point IIIA XIII 3.4** | |

| | 1 REFERENCE | Official use only |
|---|---|---|
| **1.1 Reference** | **A7.5.1.3/01** [redacted] (2006) 1,2-Benzisothiazolin-3-one: A toxicity test to determine the effects of the test substance on seedling emergence of six species of plants; [redacted], Rohm and Haas Report N° 06RC-098 (December 13, 2006), GLP, Unpublished. | |
| **1.2 Data protection** | Yes | |
| 1.2.1. Data owner | Rohm and Haas Company | |
| 1.2.2. Companies with letter of access | | |
| 1.2.3. Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into Annex I/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1 Guideline study** | Yes, OECD Proposal for Revision of Guideline 208 | |
| **2.2 GLP** | Yes | |
| **2.3 Deviations** | No | |
| | **3 METHOD** | |
| **3.1 Test material** | 1,2-Benzisothiazolin-3-one (BIT) | |
| 3.1.1 Lot/Batch number | 2005-051 | |
| 3.1.2 Specification | As given in section 2 | |
| 3.1.3 Purity | 89.8% | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
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| | **Terrestrial plant toxicity** |
| **Subsection A7.5.1.3** | **Seedling emergence an growth** |
| **Subsection A7.5.1.3/01** | |
| **Annex Point IIIA XIII 3.4** | |

| | | |
|---|---|---|
| 3.1.4 Composition of Product | Not applicable | |
| 3.1.5 Further relevant properties | Not applicable | |
| 3.1.6 Method of analysis | High performance liquid chromatography | |
| **3.2 Preparation of TS solution for poorly soluble or volatile test substances** | see Table A7.5.1.3/01-1 | |
| 3.2.1 TS Concentrations | Nominal (mg BIT/kg dry soil): 0 (negative and solvent controls), 11.1, 33.3, 99.8, 299 and 898 mg for all species and<br>Nominal (mg BIT/kg dry soil): 0 (negative and solvent controls), 0.411, 1.23, 3.70, 11.1 and 33.3 for lettuce (*L. sativa*)<br>Day 0 measured BIT concentrations in stock solutions used to prepare the 11.1, 33.3, 99.8, 299 and 898 mg BIT/kg test soils were 102, 98, 95, 84 and 80% of nominal, respectively. | |
| **3.3 Reference substance** | No | |
| 3.3.1 Method of analysis for reference substance | Not applicable | |
| **3.4 Testing procedure** | | |
| 3.4.1 Dilution water | see Table A7.5.1.3/01-2 | |
| 3.4.2 Test plants | see Table A7.5.1.3/01-3 | |
| 3.4.3 Test system | see Table A7.5.1.3/01-4 | **X** |
| 3.4.5 Test conditions | see Table A7.5.1.3/01-5 | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
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| **Subsection A7.5.1** | **Terrestrial toxicity, initial tests** |
| | **Terrestrial plant toxicity** |
| **Subsection A7.5.1.3** | **Seedling emergence an growth** |
| **Subsection A7.5.1.3/01** | |
| **Annex Point IIIA XIII 3.4** | |

| | | |
|---|---|---|
| 3.4.6 Test duration | 21 days | |
| 3.4.7 Test parameter | Seedling emergence, survival, growth (dry weight) and condition | |
| 3.4.8 Sampling | Observations on days 7, 14 and 21 were made to document seedling emergence, i.e., visible plant tissue at the surface of the soil. Observations on day 21 were made to determine the condition of individual seedlings, i.e., necrosis, leaf wrinkle, chlorosis, plant lodging or plant stunting. | |
| 3.4.9 Method of analysis of the plant material | Shoot dry weights were evaluated at test termination. Seedlings were clipped at soil level and the shoots of all living seedlings within a replicate were placed in a labelled bag. The shoots were then dried in an oven and the total dry weight of the replicate was determined. | |
| 3.4.10 Quality control | Yes | |
| 3.4.11 Statistics | Statistical analyses were used to evaluate effects of BIT application on seedling emergence, survival and dry shoot weight. Mean seedling emergence, survival and dry shoot weight of the control and BIT treatment groups were compared with a one-tailed Dunnett's t-test using the Dunnett option of the GLM (general linear model) procedure of SAS version 8 (SAS Institute, Inc. 1999, SAS/STAT User's Guide, version 8, Cary, North Carolina, USA). Dunnett's test was used to establish the LOEC and NOEC by determining which treatment group differed significantly ($p<0.05$) from the control group. | **X** |
| | **4 RESULTS** | |
| **4.1 Results test substance** | | |
| 4.1.1 Applied initial concentration | Nominal (mg BIT/kg dry soil): 0 (negative and solvent controls), 11.1, 33.3, 99.8, 299 and 898 mg for all species and<br>Nominal (mg BIT/kg dry soil): 0 (negative and solvent controls), 0.411, 1.23, 3.70, 11.1 and 33.3 for lettuce (*L. sativa*) were incorporated into the soil. | |
| 4.1.2 Phytotoxicity rating | see Table A7.5.1.3/01-6 | |
| 4.1.3 Plant height | see Table A7.5.1.3/01-6 | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
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| | **Terrestrial plant toxicity** |
| **Subsection A7.5.1.3** | **Seedling emergence an growth** |
| **Subsection A7.5.1.3/01** | |
| **Annex Point IIIA XIII 3.4** | |

| | | |
|---|---|---|
| 4.1.4 Plant dry weights | see Table A7.5.1.3/01-6 | |
| 4.1.5 Root dry weights | Not applicable | |
| 4.1.6 Root length | Not applicable | |
| 4.1.7 Number of dead plants | see Table A7.5.1.3/01-6 | |
| 4.1.8 Effect data | see Table A7.5.1.3/01-6 | |
| 4.1.9 Concentration / response curve | See Figures A7.5.1.3/01-1 onions, A7.5.1.3/01-2 oats, A7.5.1.3/01-3 turnips, A7.5.1.3/01-4 cucumber, A7.5.1.3/01-5 lettuce (initial test), A7.5.1.3/01-6 lettuce (final test) and A7.5.1.3/01-7 tomatoes | |
| 4.1.10 Percent emergence | see Table A7.5.1.3/01-6 | |
| 4.1.11 Other effects | The most sensitive parameter for all six species was dry weight.<br>See Table A7.5.1.3/01-6 | **X** |
| **4.2 Results of controls** | | |
| 4.2.1 Number/ percentage of plants showing adverse effects | No effects to onions, oats, turnips, cucumber, lettuce or tomatoes | **X** |
| 4.2.2 Nature of adverse effects | Not applicable | |
| **4.3 Test with reference substance** | Not performed | |
| 4.3.1 Concentrations | Not applicable | |
| 4.3.2 Results | Not applicable | |

| | |
|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** |
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| | **Terrestrial plant toxicity** |
| **Subsection A7.5.1.3** | **Seedling emergence an growth** |
| **Subsection A7.5.1.3/01** | |
| **Annex Point IIIA XIII 3.4** | |

| | | |
|---|---|---|
| | **5 APPLICANT'S SUMMARY AND CONCLUSION** | |
| **5.1 Materials and methods** | OECD Proposal for Revision of Guideline 208 growth test in terrestrial plants with analytical confirmation of dosing solutions. | |
| **5.2 Results and discussion** | Effects of soil incorporation of BIT were observed on seedling emergence, survival, growth and condition of the six plant species tested. The most sensitive parameter for all six species was dry weight with $EC_{50}$ values ranging from 18.4 mg BIT/kg for lettuce to 166 mg BIT/kg for oat. The NOEC for tomato dry weight in this study was determined to be less than 11.1 mg BIT/kg, which was the lowest test concentration. | **X** |
| 5.2.1 NOEC | NOEC for tomato dry weight was < 11.1 mg BIT/kg dry soil, the lowest BIT concentration. See Table A7.5.1.3/01-7 for other plant species NOEC values. | |
| 5.2.2 $EC_{25}$ | See table A7.5.1.3/01-7 | |
| 5.2.3 $EC_{50}$ | See table A7.5.1.3/01-7 | |
| **5.3 Conclusion** | | |
| 5.3.1 Reliability | (1), reliable without restriction | |
| 5.3.2 Deficiencies | No | |

| | | |
|---|---|---|
| | **Evaluation by Competent Authorities** | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | *May 2013* | |

**Section A7** **Ecotoxicological Profile Including Environmental Fate and Behaviour**

**Subsection A7.5** **Effects on terrestrial organisms**

**Subsection A7.5.1** **Terrestrial toxicity, initial tests**

**Subsection A7.5.1.3** **Terrestrial plant toxicity**

**Subsection A7.5.1.3/01** **Seedling emergence an growth**

**Annex Point IIIA XIII 3.4**

| | |
|---|---|
| **Materials and Methods** | *Applicant's version is accepted with the following remarks:*<br>▪ *3.4.3 Number of seeds used in the study is not optimal in the case of tomato (Lycopersicon esculentum) and cucumber (Cucumis sativa). According to OECD guidelines 208; for theses species one or two, instead of 10 seeds should have been used per container. However, since in all control samples, the validity criteria as stated in the guideline (e.g. seedling emergence, mean survival, exhibition of phytotoxic effects) are fulfilled, the higher number of seeds used for tomato and cucumber does not affect the validity of the study.*<br>▪ *3.4.4. Test conditions. The reported temperatures and relative humidity show a large variability throughout the test. However, in the control groups, the validity criteria with respect to emergence and survival are fulfilled, which indicates that the large temperature and humidity range did not affect the reliability of the results.* |
| **Results and discussion** | *Applicant's version is accepted with the following remarks:*<br>▪ *5.2. It is not easy to check the validity criteria concerning the control plant with the information provided in this document III. The following table show additional data concerning the negative control plant.* |

| | ***Emergence -Day 7*** | ***Emergence -Day 14*** | ***Emergence -Day 21*** | ***Survival-Day 21*** | ***Shoot Dry Weight*** |
|---|---|---|---|---|---|
| *Allium cepa* | *7.38±1.06* | *9.00±0.76* | *9.00±0.76* | *8.75±1.28* | *0.185±0.0170* |
| *Avena sativa* | *9.50±0.53* | *9.50±0.53* | *9.50±0.53* | *9.50±0.53* | *2.25±0.303* |
| *Brassica rapa* | *9.38±0.74* | *9.50±0.76* | *9.50±0.76* | *9.38±0.74* | *4.87±0.839* |
| *Cucumis sativa* | *9.88±0.35* | *9.88±0.35* | *9.88±0.35* | *9.88±0.35* | *10.7±0.55* |
| *Lactuca sativa* | *9.00±1.07* | *9.13±0.99* | *9.25±1.04* | *9.25±1.04* | *1.12±0.285* |
| *Lycopersicon esculentum* | *8.13±0.99* | *8.50±0.76* | *8.50±0.76* | *8.25±1.04* | *2.69±0.351* |

**Section A7** **Ecotoxicological Profile Including Environmental Fate and Behaviour**

**Subsection A7.5** **Effects on terrestrial organisms**

**Subsection A7.5.1** **Terrestrial toxicity, initial tests**

**Subsection A7.5.1.3** **Terrestrial plant toxicity**

**Subsection A7.5.1.3/01** **Seedling emergence an growth**

**Annex Point IIIA XIII 3.4**

| | |
|---|---|
| | *No observed sign of toxicity in these negative controls.* |
| **Conclusion** | *Based on the results of this study, it can be concluded that 1,2-Benzisothiazol-3(2H)-one may affect the emergence, survival, growth and condition of the six plant species tested. The most sensitive parameter for all six species was dry weight. The lowest $EC_{50}$ value was 18.4 mg/kg for lettuce (L. sativa) and the lowest NOEC was observed for lettuce (L. sativa) dry weight and was determined to be 3.7 mg/kg.* |
| **Reliability** | *1* |
| **Acceptability** | *Acceptable* |
| **Remarks** | *Key study.* |

**Table A7.5.1.3/01-1: Preparation of TS solution for poorly soluble or volatile test substances**

| Criteria | Details |
|---|---|
| Dispersion | Yes, stirring and sonication |
| Vehicle | Yes, acetone |
| Concentration of vehicle | 150 ml acetone / 60 kg soil, initial test<br>150 ml acetone / 30 kg soil, repeated test with *L. sativa* |
| Vehicle control performed | Yes, acetone |
| Other procedures | Not applicable |

**Table A7.5.1.3/01-2: Dilution water**

| Criteria | Details |
|---|---|
| Source | Not applicable |
| Alkalinity / Salinity | Not applicable |

| | |
|---|---|
| Hardness | Not applicable |
| pH | Not applicable |
| Oxygen content | Not applicable |
| Conductance | Not applicable |
| Holding water different from dilution water | Not applicable |

**Table A7.5.1.3/01-3: Test plants**

| | Family | Species | Common name | Source (seed) |
|---|---|---|---|---|
| **Monocotyledonae** | *Liliaceae* | *Allium cepa* | Onion | Wannamaker Seeds, Inc., Matthews South Carolina, USA |
| | *Poaceae* | *Avena sativa* | Oat | Johnny's Selected Seeds, Winslow, Maine, USA |
| **Dicotyledonae** | *Brassicaceae* | *Brassica rapa* | Turnip | Park Seed Wholesale, Inc., Greenwood, South Carolina, USA |
| | *Cucurbitaceae* | *Cucumis sativa* | Cucumber | Meyer Seed Company, Baltimore, Maryland, USA |
| | *Asteraceae* | *Lactuca sativa* | Lettuce | Johnny's Selected Seeds, Winslow, Maine, USA |
| | *Solanaceae* | *Lycopersicon esculentum* | Tomato | Meyer Seed Company, Baltimore, Maryland, USA |

**Table A7.5.1.3/01-4: Test system**

| Criteria | Details |
|---|---|
| Test type | greenhouse |
| Container type | Plastic pots (16 cm diameter by 12 cm deep) |
| Seed germination potential | provided by seed supplier |
| Identification of the plant species | provided by seed supplier |
| Number of replicates | 4 |
| Numbers of plants per replicate per dose | 10 seeds per replicate |
| Date of planting | August 25, 2006 and October 12, 2006 |
| Plant density | 10 plants/pot |
| Date of test substance application | Test substance was incorporated into the soil prior to seed planting |
| Height of plants at application | Not applicable |
| Date of phytotoxicity rating or harvest | 7, 14, and 21 days after planting seeds |
| Dates of analysis | The test was terminated 21 days after seeds were planted. |

**Table A7.5.1.3/01-5: Test conditions**

| Criteria | Details |
|---|---|
| Test type | greenhouse |
| Method of application | soil incorporation |
| Application levels | Nominal (mg BIT/kg dry soil): 0 (negative and solvent controls), 11.1, 33.3, 99.8, 299 and 898 mg for all species and<br>Nominal (mg BIT/kg dry soil): 0 (negative and solvent controls), 0.411, 1.23, 3.70, 11.1 and 33.3 for lettuce (*L. sativa*) were incorporated into the soil. |
| Dose rates | not applicable |
| Substrate characteristics | sandy loam soil consisting of 65% sand, 18% silt and 17% clay with an organic matter content of 2.2% (organic carbon 1.3%) |
| Watering of the plants | Seedlings were subirrigated |
| Temperature | 25.16°C (18.88 to 37.76 ºC), initial test<br>21.47°C (17.20 to 30.53 ºC), repeated test with *L. sativa* |
| Thermoperiod | Not applicable |
| Light regime | 14.2 (6.6 to 16.6) moles photosynthetically active radiation, initial test<br>12.9 (7.8 to 18.9) moles photosynthetically active radiation, repeated test with *L. sativa* |
| Relative humidity | 69.44% (28.22 to 91.60%), initial test<br>43.24% (13.63 to 88.60%), repeated test with *L. sativa* |
| Wind volatility | Not applicable |
| Observation periods and duration of test | Observation periods: 7, 14 and 21 days: the number of emerged plants and condition of emerged plants.<br>Duration: 21 days |
| Pest control | Seeds were not pretreated with insecticides, fungicides or repellants |
| Any other treatments and procedures | not applicable |

**Table A7.5.1.3/01-6:**

***Allium cepa* (onion):**

| Test Substance Concentration (nominal) [mg BIT/kg] | Number of Emerged Seedlings mean ± SD (% reduction) Day 7 | Day 14 | Day 21 | Seedling Survival mean ± SD (% reduction) | Shoot Dry Weight mean ± SD (% reduction) |
|---|---|---|---|---|---|
| Pooled controls | 7.38 ± 1.06 | 9.00 ± 0.76 | 9.00 ± 0.76 | 8.75 ± 1.28 | 0.132 ± 0.0372 |
| 11.1 | 7.25 ± 2.50 (2%) | 8.25 ± 1.71 (8%) | 8.50 ± 1.29 (6%) | 8.25 ± 1.71 (6%) | 0.152 ± 0.0340 (-15%) |
| 33.3 | 5.75 ± 2.22 (22%) | 7.75 ± 1.71 (14%) | 7.75 ± 1.71 (14%) | 7.25 ± 1.50 (17%) | 0.106 ± 0.0364 (20%) |
| 99.8 | 0.25 ± 0.50** (97%) | 1.00 ± 0.82** (89%) | 1.50 ± 1.29** (83%) | 1.25 ± 1.26** (86%) | 0.008 ± 0.0071** (94%) |
| 299 | 0.00 ± 0.00** (100%) | 2.00 ± 1.83** (78%) | 2.50 ± 1.29** (72%) | 1.50 ± 1.00** (83%) | 0.005 ± 0.0022** (96%) |
| 898 | 0.00 ± 0.00** (100%) | 0.00 ± 0.00** (100%) | 0.00 ± 0.00** (100%) | 0.00 ± 0.00** (100%) | 0.00 ± 0.0000** (100%) |

* Treatment group mean is significantly different from the control mean (Dunnett's test $p < 0.05$)

** Treatment group mean is significantly different from the control mean (Dunnett's test $p < 0.01$)

***Avena sativa* (oat):**

| Test Substance Concentration (nominal) [mg BIT/kg] | Number of Emerged Seedlings mean ± SD (% reduction) Day 7 | Day 14 | Day 21 | Seedling Survival mean ± SD (% reduction) | Shoot Dry Weight mean ± SD (% reduction) |
|---|---|---|---|---|---|
| Pooled controls | 9.50 ± 0.53 | 9.50 ± 0.53 | 9.50 ± 0.53 | 9.50 ± 0.53 | 2.25 ± 0.303 |
| 11.1 | 9.75 ± 0.50 (-3%) | 10.00 ± 0.00 (-5%) | 10.00 ± 0.00 (-5%) | 10.00 ± 0.00 (-5%) | 2.32 ± 0.893 (-3%) |
| 33.3 | 9.75 ± 0.50 (-3%) | 9.75 ± 0.50 (-3%) | 9.75 ± 0.50 (-3%) | 9.75 ± 0.50 (-3%) | 2.37 ± 0.191 (-5%) |
| 99.8 | 8.25 ± 1.50 (13%) | 8.75 ± 1.50 (8%) | 9.00 ± 1.15 (5%) | 9.00 ± 1.15 (5%) | 1.81 ± 0.089 (20%) |
| 299 | 6.00 ± 1.63** (37%) | 9.00 ± 0.82 (5%) | 9.00 ± 0.82 (5%) | 9.00 ± 0.82 (5%) | 0.46 ± 0.185** (80%) |
| 898 | 0.75 ± 0.96** (92%) | 6.75 ± 0.96** (29%) | 7.00 ± 0.82** (26%) | 6.75 ± 0.96** (29%) | 0.05 ± 0.032** (98%) |

* Treatment group mean is significantly different from the control mean (Dunnett's test $p < 0.05$)

** Treatment group mean is significantly different from the control mean (Dunnett's test $p < 0.01$)

***Brassica rapa* (turnip):**

| Test Substance Concentration (nominal) [mg BIT/kg] | Number of Emerged Seedlings mean ± SD (% reduction) | | | Seedling Survival mean ± SD (% reduction) | Shoot Dry Weight mean ± SD (% reduction) |
|---|---|---|---|---|---|
| | Day 7 | Day 14 | Day 21 | | |
| Pooled controls | 9.38 ± 0.74 | 9.50 ± 0.76 | 9.50 ± 0.76 | 9.38 ± 0.74 | 4.87 ± 0.839 |
| 11.1 | 9.25 ± 0.96 (1%) | 9.25 ± 0.96 (3%) | 9.25 ± 0.96 (3%) | 9.25 ± 0.96 (1%) | 4.73 ± 0.696 (3%) |
| 33.3 | 9.00 ± 0.00 (4%) | 9.50 ± 0.58 (0%) | 9.50 ± 0.58 (0%) | 9.50 ± 0.58 (-1%) | 3.18 ± 0.870** (35%) |
| 99.8 | 3.25 ± 1.71** (65%) | 3.50 ± 1.29** (63%) | 4.50 ± 2.08** (53%) | 3.00 ± 2.16** (68%) | 0.04 ± 0.048** (99%) |
| 299 | 0.75 ± 0.96** (92%) | 0.75 ± 0.96** (92%) | 1.00 ± 0.82** (89%) | 0.75 ± 0.96** (92%) | 0.01 ± 0.009** (100%) |
| 898 | 0.00 ± 0.00** (100%) | 0.00 ± 0.00** (100%) | 0.00 ± 0.00** (100%) | 0.00 ± 0.00** (100%) | 0.00 ± 0.000** (100%) |

* Treatment group mean is significantly different from the control mean (Dunnett's test $p < 0.05$)

** Treatment group mean is significantly different from the control mean (Dunnett's test $p < 0.01$)

***Cucumis sativa* (cucumber):**

| Test Substance Concentration (nominal) [mg BIT/kg] | Number of Emerged Seedlings mean ± SD (% reduction) | | | Seedling Survival mean ± SD (% reduction) | Shoot Dry Weight mean ± SD (% reduction) |
|---|---|---|---|---|---|
| | Day 7 | Day 14 | Day 21 | | |
| Pooled controls | 9.88 ± 0.35 | 9.88 ± 0.35 | 9.88 ± 0.35 | 9.88 ± 0.35 | 10.7 ± 0.55 |
| 11.1 | 9.75 ± 0.50 (1%) | 9.75 ± 0.50 (1%) | 9.75 ± 0.50 (1%) | 9.75 ± 0.50 (1%) | 11.0 ± 0.69 (-2%) |
| 33.3 | 10.00 ± 0.00 (-1%) | 10.00 ± 0.00 (-1%) | 10.00 ± 0.00 (-1%) | 10.00 ± 0.00 (-1%) | 9.9 ± 0.91* (8%) |
| 99.8 | 8.00 ± 0.82** (19%) | 8.75 ± 0.50 (11%) | 9.00 ± 0.00 (9%) | 8.75 ± 0.50** (11%) | 2.6 ± 0.53** (76%) |
| 299 | 5.00 ± 1.41** (49%) | 7.50 ± 1.73** (24%) | 7.50 ± 1.73** (24%) | 4.25 ± 1.26** (57%) | 0.2 ± 0.11** (98%) |
| 898 | 0.75 ± 1.50** (92%) | 3.25 ± 2.63** (67%) | 3.25 ± 2.63** (67%) | 0.00 ± 0.00** (100%) | 0.00 ± 0.00** (100%) |

* Treatment group mean is significantly different from the control mean (Dunnett's test $p < 0.05$)

** Treatment group mean is significantly different from the control mean (Dunnett's test $p < 0.01$)

***Lactuca sativa* (lettuce):**

| Test Substance Concentration (nominal) [mg BIT/kg] | Number of Emerged Seedlings mean ± SD (% reduction) | | | Seedling Survival mean ± SD (% reduction) | Shoot Dry Weight mean ± SD (% reduction) |
|---|---|---|---|---|---|
| | Day 7 | Day 14 | Day 21 | | |
| Pooled controls | 9.00 ± 1.07 | 9.13 ± 0.99 | 9.25 ± 1.04 | 9.25 ± 1.04 | 1.12 ± 0.285 |
| 0.411 | 825 ± 2.06 (8%) | 825 ± 2.06 (10%) | 825 ± 2.06 (11%) | 825 ± 2.06 (11%) | 0.90 ± 0.335 (20%) |
| 1.23 | 9.50 ± 1.00 (-6%) | 9.50 ± 1.00 (-4%) | 9.50 ± 1.00 (-3%) | 9.50 ± 1.00 (-3%) | 1.02 ± 0.202 (9%) |
| 3.70 | 9.50 ± 0.58 (-6%) | 9.50 ± 0.58 (-4%) | 9.50 ± 0.58 (-3%) | 9.50 ± 0.58 (-3%) | 0.89 ± 0.088 (21%) |
| 11.1 | 9.75 ± 0.50 (-8%) | 9.75 ± 0.50 (-7%) | 9.75 ± 0.50 (-5%) | 9.75 ± 0.50 (-5%) | 0.58 ± 0.135** (48%) |
| 33.3 | 9.50 ± 1.00 (-6%) | 9.50 ± 1.00 (-4%) | 9.50 ± 1.00 (-3%) | 9.50 ± 1.00 (-3%) | 0.45 ± 0.154** (60%) |

* Treatment group mean is significantly different from the control mean (Dunnett's test $p < 0.05$)

** Treatment group mean is significantly different from the control mean (Dunnett's test $p < 0.01$)

***Lycopersicon esculentum* (tomato):**

| Test Substance Concentration (nominal) [mg BIT/kg] | Number of Emerged Seedlings mean ± SD (% reduction) | | | Seedling Survival mean ± SD (% reduction) | Shoot Dry Weight mean ± SD (% reduction) |
|---|---|---|---|---|---|
| | Day 7 | Day 14 | Day 21 | | |
| Pooled controls | 8.13 ± 0.99 | 8.50 ± 0.76 | 8.50 ± 0.76 | 8.25 ± 1.04 | 2.69 ± 0.351 |
| 11.1 | 7.75 ± 0.96 (5%) | 8.25 ± 0.50 (3%) | 8.25 ± 0.50 (3%) | 8.25 ± 0.50 (0%) | 2.18 ± 0.209** (19%) |
| 33.3 | 5.00 ± 2.16** (38%) | 7.25 ± 1.50 (15%) | 7.50 ± 1.29 (12%) | 7.50 ± 1.29 (9%) | 1.73 ± 0.307** (36%) |
| 99.8 | 0.00 ± 0.00** (100%) | 3.00 ± 2.16** (65%) | 4.50 ± 2.08** (47%) | 3.50 ± 2.89** (58%) | 0.05 ± 0.044** (98%) |
| 299 | 0.00 ± 0.00** (100%) | 1.75 ± 0.50** (79%) | 3.25 ± 0.96** (62%) | 2.25 ± 0.96** (73%) | 0.01 ± 0.006** (100%) |
| 898 | 0.00 ± 0.00** (100%) | 0.00 ± 0.00** (100%) | 0.00 ± 0.00** (100%) | 0.00 ± 0.00** (100%) | 0.00 ± 0.000** (100%) |

* Treatment group mean is significantly different from the control mean (Dunnett's test $p < 0.05$)

** Treatment group mean is significantly different from the control mean (Dunnett's test $p < 0.01$)

**Table A7.5.1.3/01-7: Conclusions**

| Species | 21-Day Emergence (mg BIT/kg) | | | | 21-Day Survival (mg BIT/kg) | | | | 21-Day Growth (Dry Weight) (mg BIT/kg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Monocots:** | **NOEC** | **LOEC** | **$EC_{25}$** | **$EC_{50}$** | **NOEC** | **LOEC** | **$EC_{25}$** | **$EC_{50}$** | **NOEC** | **LOEC** | **$EC_{25}$** | **$EC_{50}$** |
| *Allium cepa* (onion) | 33.3 | 99.8 | 26.9 | 67.6 | 33.3 | 99.8 | 24.3 | 55.7 | 33.3[1] | 99.8[1] | 25.1[1] | 42.7[1] |
| *Avena sativa* (oats) | 299 | 898 | 825 | > 898 | 299 | 898 | 756 | > 898 | 33.3 | 99.8 | 98.5 | 166 |
| **Dicots:** | | | | | | | | | | | | |
| *Brassica rapa* (turnip) | 33.3 | 99.8 | 59.7 | 102 | 33.3 | 99.8 | 45.3 | 79.3 | 11.1 | 33.3 | 29.3 | 39.0 |
| *Cucumis sativa* (cucumber) | 99.8 | 299 | 297 | 585 | 33.3 | 99.8 | 272 | 294 | 11.1 | 33.3 | 40.9 | 65.1 |
| *Lactuca sativa*[2] (lettuce) | 33.3 | >33.3 | > 33.3 | > 33.3 | 33.3 | >33.3 | > 33.3 | > 33.3 | 3.70 | 11.1 | 3.70 | 18.4 |
| *Lycopersicon esculentum* (tomato) | 33.3 | 99.8 | 87.8 | 166 | 33.3 | 99.8 | 53.0 | 110 | < 11.1 | 11.1 | 28.3 | 40.0 |

[1] Based on comparison to the Solvent Control only.

[2] Based on results of second test.

**Table A7.5.1.3/01-9: Validity criteria for terrestrial plant toxicity according to OECD Guideline 208 adopted July 2006**

| | Fulfilled | Not fulfilled |
|---|---|---|
| Seedling emergence on control > 70% | **yes** | |
| Seedlings did not exhibit signs of phytotoxicity | **yes** | |
| Mean survival of emerged control seedlings > 90% | **yes** | |

**Figure A7.5.1.3/01-1: Day 21 Emergence, Survivors and Biomass in Onion exposed to BIT**

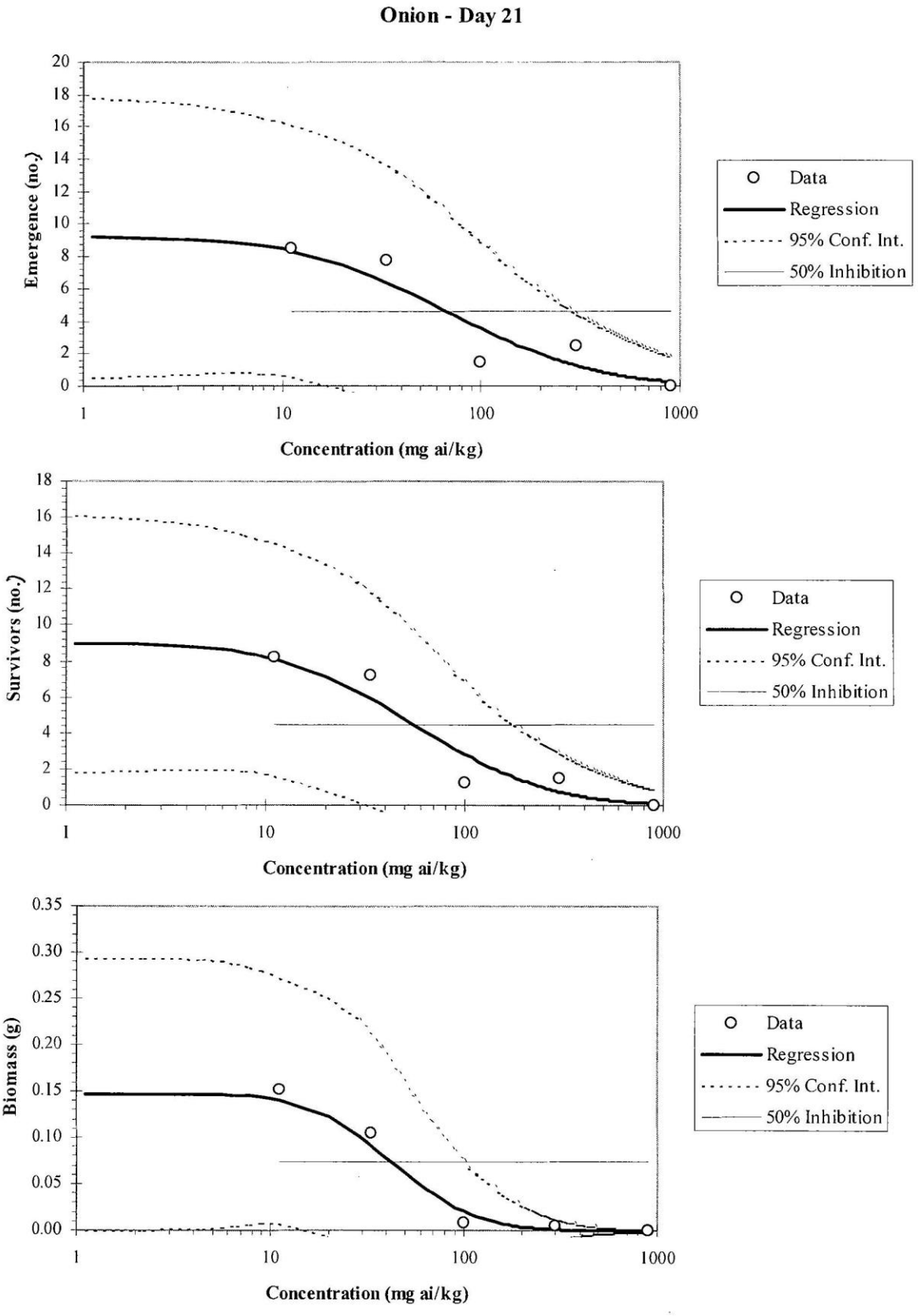


**Figure A7.5.1.3/01-2: Day 21 Emergence, Survivors and Biomass in Oats exposed to BIT**

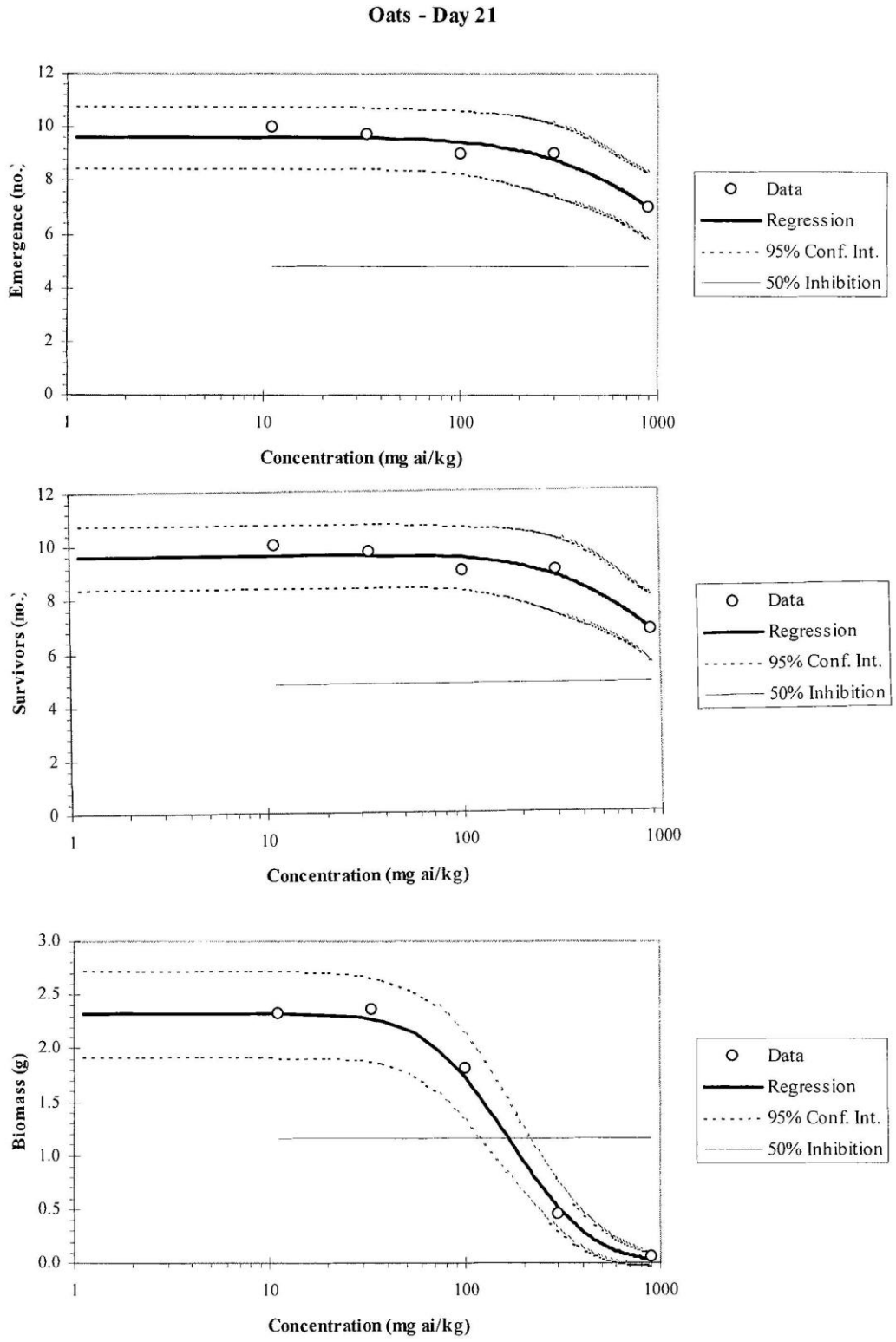
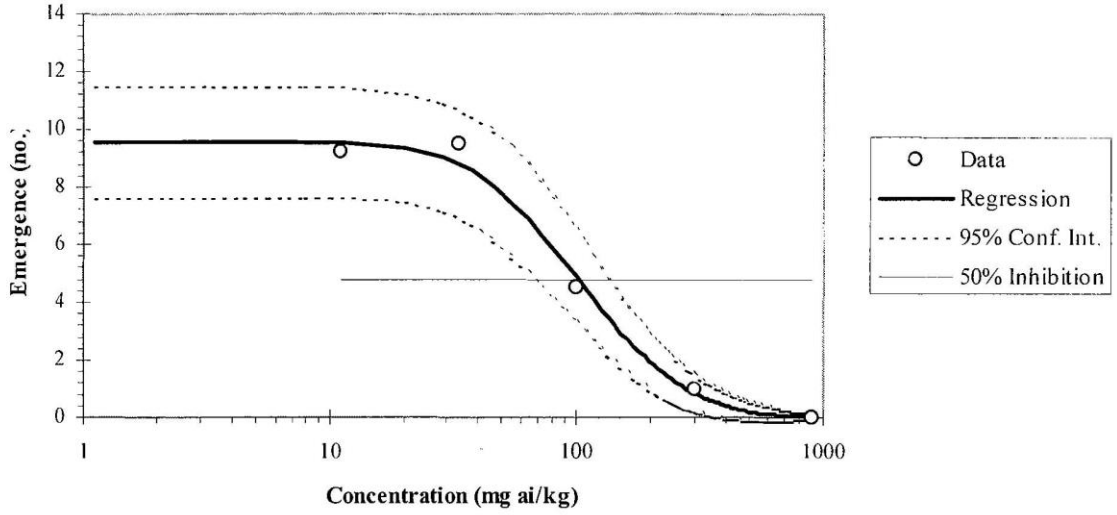
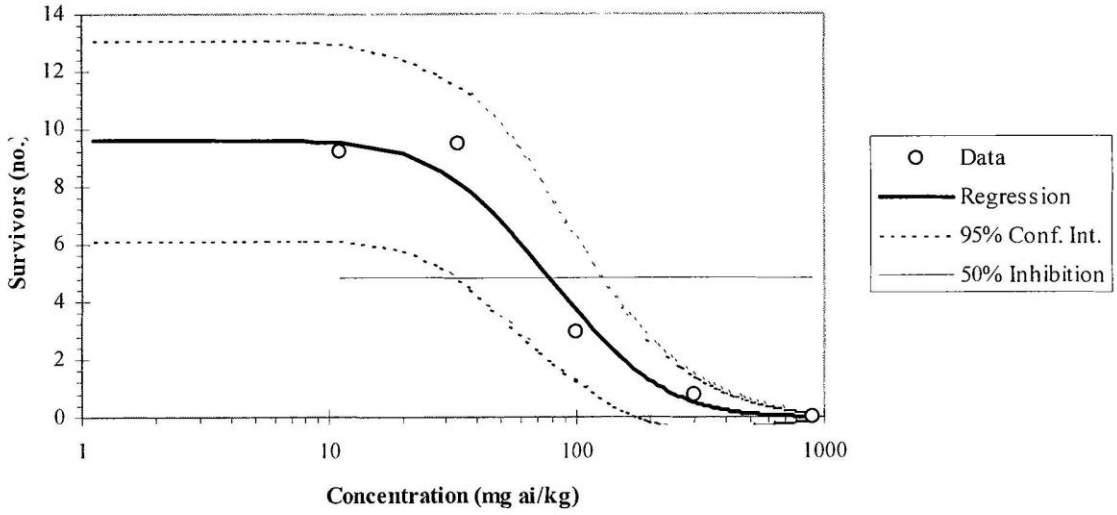



**Figure A7.5.1.3/01-3: Day 21 Emergence, Survivors and Biomass in Turnips exposed to BIT**

**Turnip - Day 21**



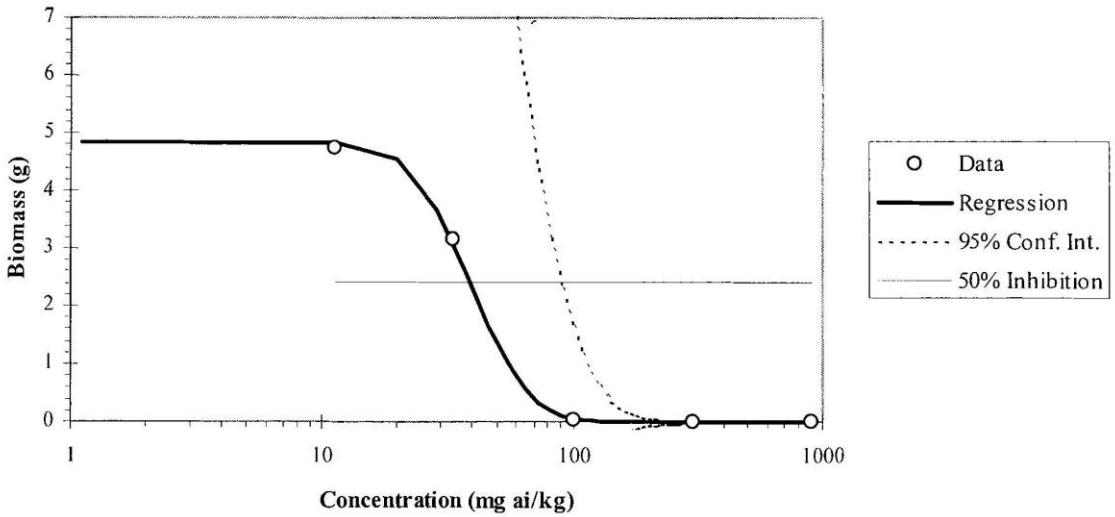



**Figure A7.5.1.3/01-4: Day 21 Emergence, Survivors and Biomass in Cucumber exposed to BIT**

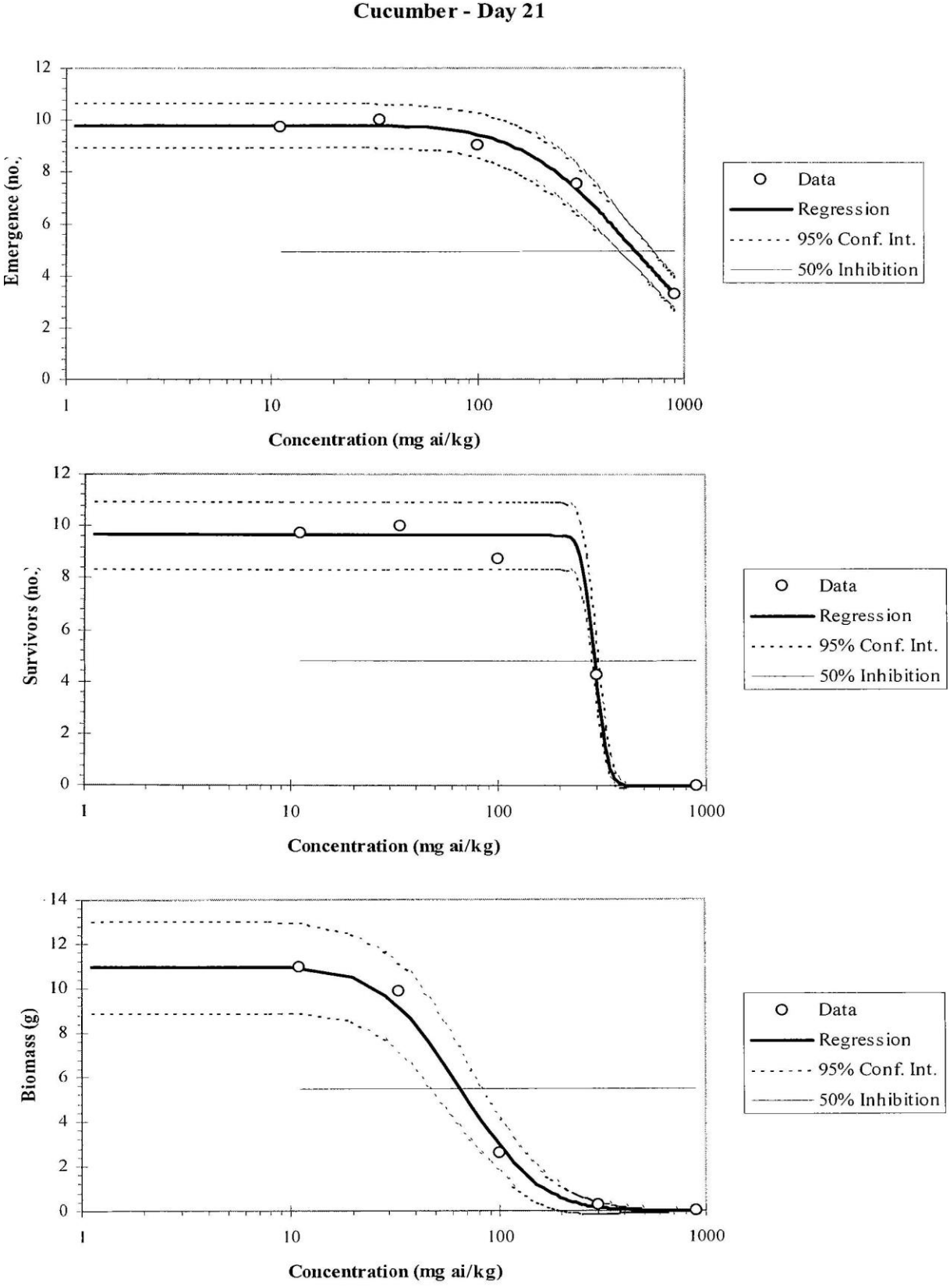


**Figure A7.5.1.3/01-5: Day 21 Emergence, Survivors and Biomass in Lettuce (initial trial) exposed to BIT**

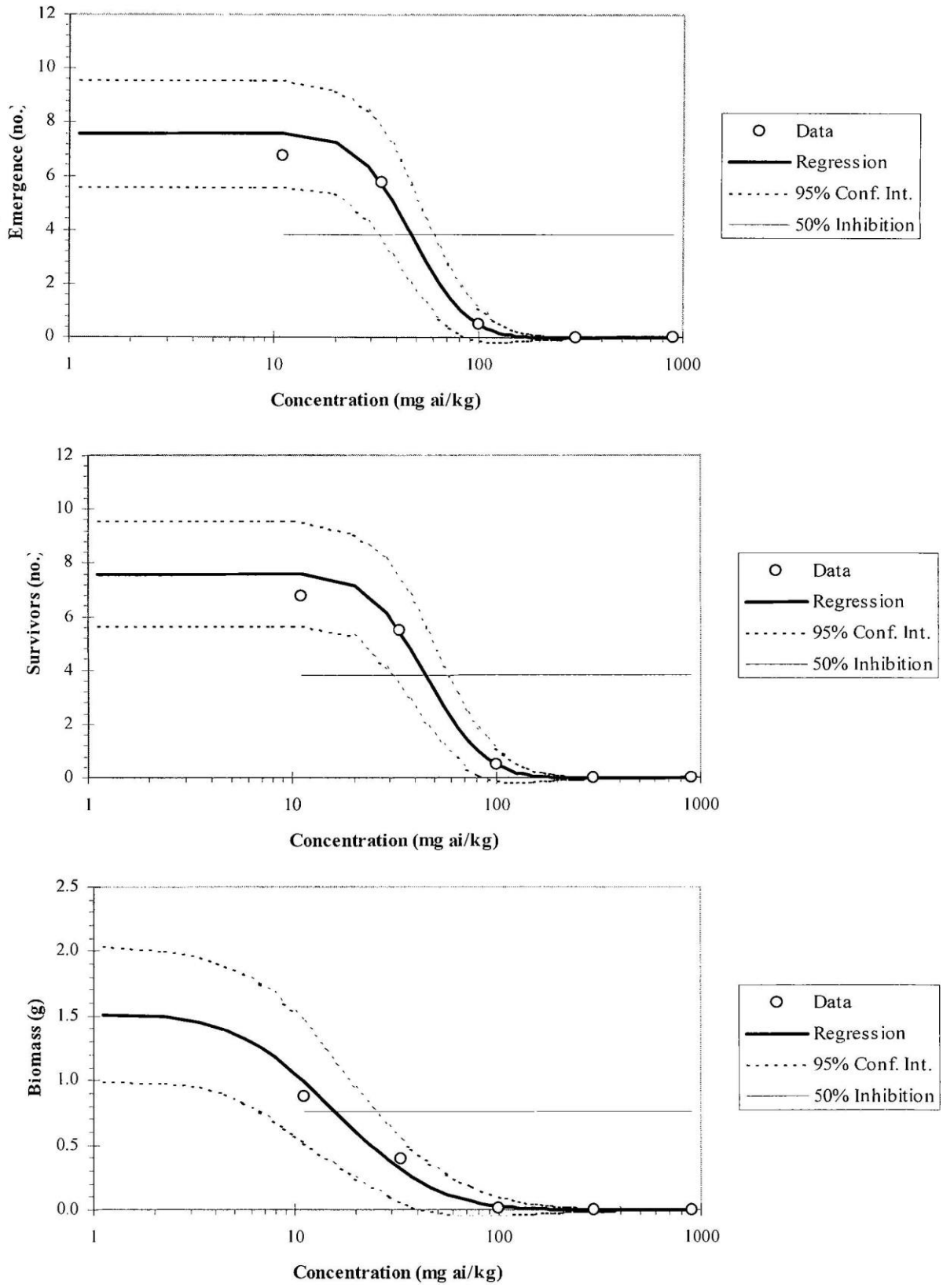


**Figure A7.5.1.3/01-6: Day 21 Emergence, Survivors and Biomass in Lettuce (final trial) exposed to BIT**

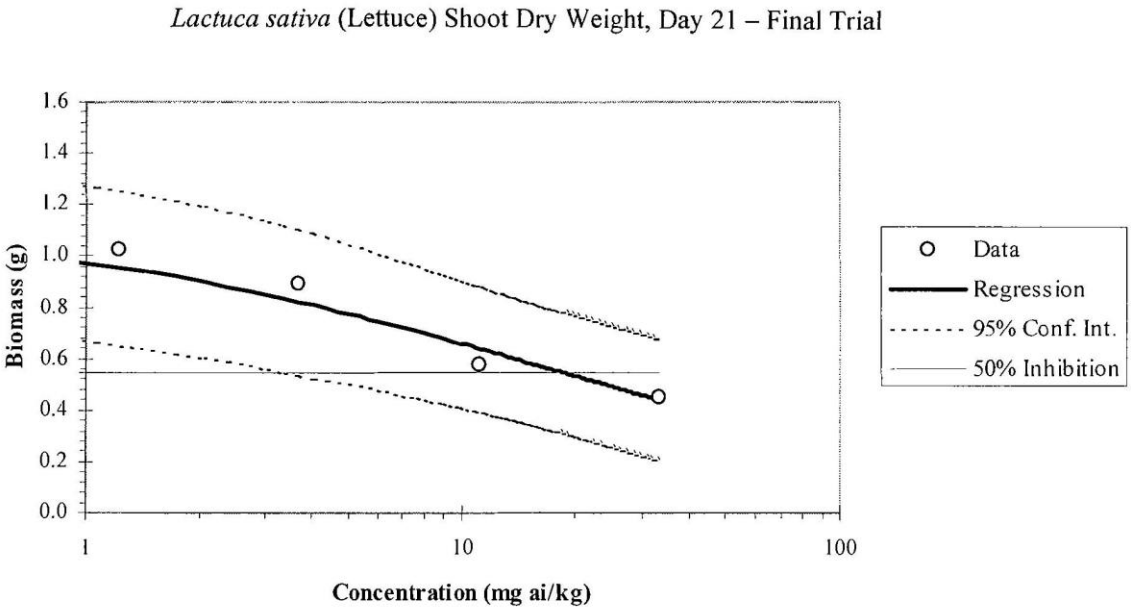
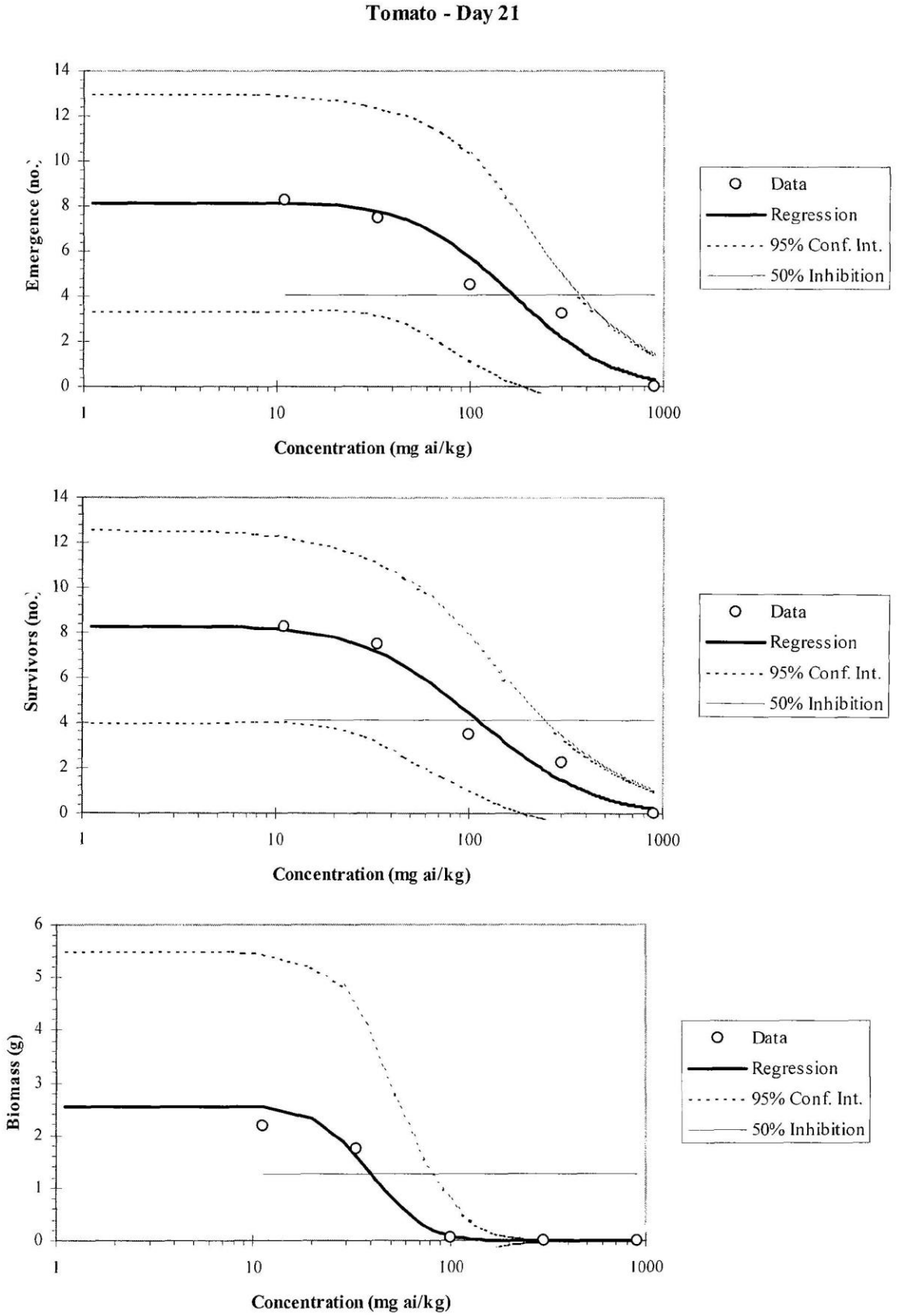


**Figure A7.5.1.3/01-7: Day 21 Emergence, Survivors and Biomass in Tomato exposed to BIT**



**Section A7** **Ecotoxicological Profile Including Environmental Fate and Behaviour**

**Subsection A7.5** **Effects on terrestrial organisms**

**Subsection A7.5.2** **Terrestrial tests, long-term tests**

**Subsection A7.5.2.1** **Earthworm, chronic toxicity test**

**Annex Point IIIA XIII.3.2**

| | **1 REFERENCE** | **Official use only** |
|---|---|---|
| **1.1 Reference** | **A7.5.2/01** [redacted] (2007) 1,2-Benzisothiazolin-3-one: A reproduction study with the earthworm in an artificial soil substrate, [redacted], Rohm and Haas Report N° 06RC-208 (January 15, 2007), Unpublished. | |
| **1.2 Data protection** | Yes | |
| 1.2.1 Data owner | Rohm and Haas Company | |
| 1.2.2 Companies with letter of access | | |
| 1.2.3 Criteria for data protection | Data on existing a.s. submitted for the first time in support of the first inclusion into Annex I/IA.<br>Data protection claimed in accordance with the Article 12.1 (c) (ii), as data generated after entry into force of the Directive. | |
| | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1 Guideline study** | Yes, OECD Method 222 and ISO 11268-2 | |
| **2.2 GLP** | Yes | |
| **2.3 Deviations** | No | |
| | **3 METHOD** | |
| **3.1 Test material** | 1,2-Benzisothiazolin-3-one (BIT) | |
| 3.1.1 Lot/Batch number | 2005-051 | |
| 3.1.2 Specification | As given in section 2 | |
| 3.1.3 Purity | 89.8 % | |
| 3.1.4 Composition of Product | Not applicable | |
| 3.1.5 Further relevant properties | Not applicable | |

| | | |
|---|---|---|
| 3.1.6 Method of analysis | High performance liquid chromatography (HPLC) | |
| **3.2 Reference substance** | No | **X** |
| **3.3 Testing procedure** | | |
| 3.3.1 Preparation of the test substance | See Table A7.5.1.2/01-1 | |
| 3.3.2 Application of the test substance | Test soil was prepared by mixing the appropriate amount of BIT in deionised water with dry artificial soil to which cow manure was added. Additional deionized water was added to the dry artificial soil to achieve a moisture content of approximately 35% by weight. Test soil components were mixed for a total of 20 minutes in order to achieve a homogeneous state. Negative control soil was prepared in the same manner as the treated soil but with only the addition of water. | |
| 3.3.3 Test organisms | See Table A7.5.2.1/01-2 | |
| 3.3.4 Test system | see Table A7.5.2.1/01-3 | |
| 3.3.5 Test conditions | see Table A7.5.2.1/01-4 | |
| 3.3.6 Test duration | 56 days: adult exposure for 28 days and cocoons / juveniles exposure for 28 days | |
| 3.3.7 Test parameter | Mortality, growth and reproduction | |
| 3.3.8 Examination | After 28 days of adult exposure, mortality and growth (percent weight change) were evaluated. After 56 days, the number of juvenile worms was assessed (reproduction). | |
| 3.3.9 Monitoring of test substance concentration | No | |
| 3.3.10 Statistics | Differences between the BIT treatment groups and the control group were evaluated to assess potential effects on body weight and change in body weight using the Dunnett's 2-tailed test (p = 0.05) in SAS version 8.2 (SAS Institute, Inc. 1999. SAS/STAT User's Guide, Version 8.2, Cary, North Carolina, USA). Prior to conducting Dunnett's test, the data were tested for homogeneity of variance and normal distribution. Differences between the mean numbers of juveniles produced in the treatment groups and the control group were determined using Dunnett's 1-tailed test (p = 0.05). The Jonckheere-Terpstra Test for Trend (p = 0.05) was also used to evaluate the numbers of juveniles produced. | |
| | **4 RESULTS** | |

| | | |
|---|---|---|
| **4.1 Filter paper test** | Not performed | |
| **4.2 Soil test** | | |
| 4.2.1 Initial concentrations of test substance | 0 (control), 1.3, 2.5, 5.0, 10, 20 and 40 mg BIT/kg dry soil. | |
| 4.2.2 Effect data (Mortality) | see Table A7.5.2.1/01-5, there was no treatment-related mortality of adult earthworms | **X** |
| 4.2.3 Concentration / effect curve | None | |
| 4.2.4 Other effects | No effects upon adult earthworm weights. There were no statistically significant effects on numbers of juveniles produced in the 1.3, 2.5, 5.0, 10, 20 and 40 mg BIT/kg dry soil treatment groups, however, the decrease in the mean number of juveniles at the 40 mg BIT/kg level indicated a possible treatment-related effect. | |
| **4.3 Results of controls** | | |
| 4.3.1 Mortality | 1.3% | |
| 4.3.2 Number/ percentage of earthworms showing adverse effects | See Table A7.5.2.1/01-6, one earthworm was not found and was presumed dead | |
| 4.3.3 Nature of adverse effects | None | |
| **4.4 Test with reference substance** | Not performed | |
| 4.4.1 Concentrations | Not applicable | |
| 4.4.2 Results | Not applicable | |
| | **5 APPLICANT'S SUMMARY AND CONCLUSION** | |
| **5.1 Materials and methods** | OECD Method 222 and ISO 11268-2, Earthworm reproduction test | |
| **5.2 Results and discussion** | BIT did not affect mortality and adult earthworm weight. There were no statistically significant effects on numbers of juveniles produced in the 1.3, 2.5, 5.0, 10, 20 and 40 mg BIT/kg dry soil treatment groups, however, the decrease in the mean number of juveniles at the 40 mg BIT/kg level indicated a possible treatment-related effect. | **X** |
| NOEC | 20 mg BIT/kg dry soil (NOEC of reproduction) | |

| | | |
|---|---|---|
| $LC_{10}$ | > 40 mg BIT/kg dry soil | |
| $EC_{50}$ | > 40 mg BIT/kg dry soil ($EC_{50}$ of reproduction) | |
| $LC_{100}$ | no concentration caused 100% mortality | |
| **5.3 Conclusion** | see Table A7.5.2.1/01-7 and see Table A7.5.2.1/01-8 | |
| 5.3.1 Other Conclusions | | |
| 5.3.2 Reliability | (1), reliable without restriction | |
| 5.3.3 Deficiencies | No | |

| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | *January 2011* |
| **Materials and Methods** | *Applicant's version is accepted with the following remark:*<br>▪ ***3.2*** *A reference toxicity test was conducted with carbendazim in 2005 (as cited in Doc IV-A). The $LC_{50}$ value for the mortality of the adult earthworms exposed to carbendazim for 28 days was 5 (4-8) mg a.i./kg dry soil. The $EC_{50}$ value for reproduction was calculated to be 1.85 (1.792-1.913) mg a.i./kg dry soil. The NOEC was determined to be 1 mg a.i./kg dry soil, and the LOEC, 2 mg a.i./kg dry soil.* |
| **Results and discussion** | *Applicant's version is accepted with the following remark:*<br>***4.2.2*** *and* ***5.2*** *The test concentrations should also included the $EC_{50}$ value.* |
| **Conclusion** | *Applicant's version is adopted.* |
| **Reliability** | *2* |
| **Acceptability** | *Acceptable* |
| **Remarks** | |

**Table A7.5.2.1/01-1: Preparation of TS solution**

| Criteria | Details |
|---|---|
| Type and source of dilution water | deionized water prepared at laboratory |
| Holding water different from dilution water | Not applicable |
| Dispersion | BIT was mixed with artificial soil for 20 minutes to assure homogeneity |
| Vehicle | Not applicable |
| Concentration of vehicle | Not applicable |
| Vehicle control performed | Not applicable |
| Other procedures | Not applicable |

**Table A7.5.2.1/01-2: Test organisms**

| Criteria | Details |
|---|---|
| Species/strain | *Eisenia fetida* |
| Source of the initial stock | Wildlife International Limited culture established with earthworms obtained from the University of Maryland Wye Research and Education Center, Queenstown, Maryland, USA |
| Culturing techniques | Earthworms were from synchronous cultures (individuals not differing in age by more than four weeks) maintained in moist peat moss and fed saturated alfalfa and/or cow manure |
| Age/weight | 0.48 to 0.63 grams weight at initiation, worms had well developed clitella |
| Pre-treatment | Eight days prior to test initiation, earthworms (with clitellum) were selected and placed in a glass container containing bedding. The worms were held under the environmental conditions to be used during testing. Two days prior to the test, the earthworms were removed from the container and divided into five one-liter beakers containing artificial soil substrate adjusted to a moisture content of approximately 35% by weight for the acclimation period. Earthworms were fed cow manure throughout the acclimation period |

**Table A7.5.2.1/01-3: Test system**

| Criteria | Details |
|---|---|
| Artificial soil test substrate | Composition of the artificial soil was 20% kaolin clay, 70% sand, 10% sphagnum peat moss and 35% moisture. One gram of cow manure/100 g soil was added to the mixture. |
| Test mixture | 1.3, 2.5, 5.0, 10, 20, 40 mg BIT/kg dry soil |
| Size, volume and material of test container | 1 L glass beakers |
| Amount of artificial soil (kg)/ container | 750 g prepared artificial soil |
| Nominal levels of test concentrations | 1.3, 2.5, 5.0, 10, 20, 40 mg BIT/kg dry soil |
| Number of replicates/concentration | 4 for BIT groups and 8 for negative control |
| Number of earthworms/test concentration | 40 for BIT groups and 80 for negative control |
| Number of earthworms/container | 10 |
| Light source | fluorescent bulbs |
| Test performed in closed vessels due to significant volatility of test substrate | No |

**Table A7.5.2.1/01-4: Test conditions**

| Criteria | Details |
|---|---|
| Test temperature | 20 ± 2 °C |
| Moisture content | Day 0 = 33.6 to 34.0 %<br>Day 56 = 34.6 to 36.2 % |
| pH | Day 0 = 7.0 to 7.2; Day 56: 7.1 to 7.3 |
| Adjustment of pH | Not applicable |
| Light intensity / photoperiod | 400 to 800 lux, 16 h light and 8 h dark |
| Relevant degradation products | Not applicable |

**Table A7.5.2.1/01-5: Mortality data**

| Test Substance<br>Concentration (nominal)<br>[mg BIT/kg artificial soil] | Mortality | |
|---|---|---|
| | Number Dead or Missing<br>Day 28 | Percentage<br>Day 28 |
| 0 (control) | 1/80 | 1.25 |
| 1.3 | 0/40 | 0 |
| 2.5 | 0/40 | 0 |
| 5.0 | 0/40 | 0 |
| 10 | 1/40 | 2.5 |
| 20 | 0/40 | 0 |
| 40 | 0/40 | 0 |
| **Temperature [°C]** | 20 ± 2 °C | |
| **pH** | 7.1 to 7.3 | |
| **Moisture content** | 34.6 to 36.2 % | |

**Table A7.5.2.1/01-6: Number affected data**

| Test Substance<br>Concentration (nominal)<br>[mg BIT/kg artificial soil] | Number Affected | | |
|---|---|---|---|
| | Adult worm weights (grams/replicate)<br>Day 28<br>Mean change | % change | Mean Replicate Reproduction<br>Day 56<br>Mean number of juvenile worms* |
| 0 (control) | 0.101 | 10.1 | 104 |
| 1.3 | 0.095 | 9.5 | 98.8 |
| 2.5 | 0.090 | 9.0 | 99.8 |
| 5 | 0.080 | 8.0 | 108 |
| 10 | 0.111 | 11.1 | 100 |
| 20 | 0.070 | 7.0 | 102 |
| 40 | 0.093 | 9.3 | 87.5 |
| **Temperature [°C]** | 20 ± 2 °C | | |
| **pH** | 7.1 to 7.3 | | |
| **Moisture content** | 34.6 to 36.2 % | | |

* The number of juveniles was not statistically significantly different ($p > 0.05$) for any group when compared to the control using a one-tailed Dunnett's Test. However, the p value for the 40 mg a.i./kg group was 0.0537.

**Table A7.5.2.1/01-7: Effect data[1]**

| | |
|---|---|
| LOEC (number of juveniles) | 40 mg BIT/kg dry soil |
| NOEC (number of juveniles) | 20 mg BIT/kg dry soil |
| $EC_{50}$ (reproduction) | > 40 mg BIT/kg dry soil |

[1] all effect data are based on nominal concentrations

**Table A7.5.2.1/01-8: Validity criteria for acute earthworm test according to OECD 222**

| | fulfilled | Not fulfilled |
|---|---|---|
| Number of juveniles in each of the control replicates > 30 | **yes** | |
| Coefficient of variation of reproduction ≤ 30 % | **yes** | |
| Mortality of control animals < 10% | **yes** | |

| | | |
|---|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** | |
| **Subsection A7.5** | **Effects on terrestrial organisms** | |
| **Subsection A7.5.2** | **Terrestrial tests, long-term tests** | |
| **Subsection A.7.5.2.2**<br>**Annex Point IIIA XII.2.1** | **Biological Sewage Treatment – Anaerobic biodegradation** | |
| | **JUSTIFICATION FOR NON-SUBMISSION OF DATA** | **Official use only** |
| **Other existing data [ ]** | **Technically not feasible [ ] Scientifically unjustified [ ]** | |
| **Limited exposure [ X]** | **Other justification [X]** | |
| **Detailed justification:** | For the in can application (PT 6), as well as for Metal working fluid preservatives (PT 13), a long term toxicity of BIT to terrestrial plants is not required as the terrestrial compartment is not the major compartment of concern. | |
| **Undertaking of intended data submission [ ]** | No further studies planned | |
| | | |
| | | |
| | **Evaluation by Competent Authorities** | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | *January 2011* | |
| **Evaluation of applicant's justification** | *Applicant's justification is accepted* | |
| **Conclusion** | *Applicant's justification is accepted* | |
| **Remarks** | *Applicant's justification is accepted* | |

| | | |
|---|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** | |
| **Subsection A7.5** | **Effects on terrestrial organisms** | |
| **Subsection A7.5.3** | **Effects on birds** | |
| **Annex Point IIIA XII.2.1** | | |
| | **JUSTIFICATION FOR NON-SUBMISSION OF DATA** | **Official use only** |
| **Other existing data [ ]** | **Technically not feasible [ ] Scientifically unjustified [ ]** | |
| **Limited exposure [ X]** | **Other justification [X]** | |
| **Detailed justification:** | For the in can application or the metalworking fluid preservatives, an acute or 8-day study on birds is not required because the terrestrial organisms are not target organisms. | |
| **Undertaking of intended data submission [ ]** | No further studies planned | |
| | | |
| | | |
| | **Evaluation by Competent Authorities** | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | *January 2011* | |
| **Evaluation of applicant's justification** | *Applicant's justification is accepted* | |
| **Conclusion** | *Applicant's justification is accepted* | |
| **Remarks** | *Applicant's justification is accepted* | |

| | | |
|---|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** | |
| **Subsection A7.5** | **Effects on terrestrial organisms** | |
| **Subsection A7.5.3** | **Effects on birds** | |
| **Subsection A7.5.3.1.3** | **Bird reproduction** | |
| **Annex Point IIIA XII.2.1** | | |
| | **JUSTIFICATION FOR NON-SUBMISSION OF DATA** | **Official use only** |
| **Other existing data [ ]** | **Technically not feasible [ ] Scientifically unjustified [ ]** | |
| **Limited exposure [ X]** | **Other justification [X]** | |
| **Detailed justification:** | For the in can application or the metalworking fluid preservatives, a reproduction study on birds is not required because the terrestrial organisms are not target organisms. | |
| **Undertaking of intended data submission [ ]** | No further studies planned | |
| | | |
| | | |
| | **Evaluation by Competent Authorities** | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | *January 2011* | |
| **Evaluation of applicant's justification** | *Applicant's justification is accepted* | |
| **Conclusion** | *Applicant's justification is accepted* | |
| **Remarks** | *Applicant's justification is accepted* | |

| | | |
|---|---|---|
| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** | |
| **Subsection A7.5** | **Effects on terrestrial organisms** | |
| **Subsection A7.5.4** | **Effects on honeybees** | |
| **Annex Point IIIA XII.2.1** | | |
| | **JUSTIFICATION FOR NON-SUBMISSION OF DATA** | **Official use only** |
| **Other existing data [ ]** | **Technically not feasible [ ] Scientifically unjustified [ ]** | |
| **Limited exposure [ X]** | **Other justification [X]** | |
| **Detailed justification:** | Tests on honeybees are not required for the in can application. or the metalworking fluid preservatives. | |
| **Undertaking of intended data submission [ ]** | No further studies planned | |
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| | **Evaluation by Competent Authorities** | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | *January 2011* | |
| **Evaluation of applicant's justification** | *Applicant's justification is accepted* | |
| **Conclusion** | *Applicant's justification is accepted* | |
| **Remarks** | *Applicant's justification is accepted* | |

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| **Section A7**<br>**Subsection A7.5**<br>**Subsection A7.5.5**<br>**Annex Point IIIA XII.2.1** | **Ecotoxicological Profile Including Environmental Fate and Behaviour**<br>**Effects on terrestrial organisms**<br>**Bioconcentration, terrestrial** | |
| | **JUSTIFICATION FOR NON-SUBMISSION OF DATA** | **Official use only** |
| **Other existing data [ ]** | **Technically not feasible [ ] Scientifically unjustified [ ]** | |
| **Limited exposure [ X]** | **Other justification [X]** | |
| **Detailed justification:** | Section A7.5.5.1 Bioconcentration in earthworms<br>The potential of BIT bioconcentration in earthworms is very low. based on the partition coefficient. | |
| **Undertaking of intended data submission [ ]** | No further studies planned | |
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| | **Evaluation by Competent Authorities** | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | *January 2011* | |
| **Evaluation of applicant's justification** | *Applicant's justification is accepted* | |
| **Conclusion** | *Applicant's justification is accepted* | |
| **Remarks** | *Applicant's justification is accepted* | |

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| **Section A7**<br>**Subsection A7.5**<br>**Subsection A7.5.6**<br>**Annex Point IIIA XII.2.1** | **Ecotoxicological Profile Including Environmental Fate and Behaviour**<br>**Effects on terrestrial organisms**<br>**Effects on other terrestrial non-target organisms** | |
| | **Justification for non-submission of data** | **Official use only** |
| **Other existing data [ ]** | **Technically not feasible [ ] Scientifically unjustified [ ]** | |
| **Limited exposure [ X]** | **Other justification [X]** | |
| **Detailed justification:** | For the in can application or the metalworking fluid preservatives, further tests are not required as the terrestrial compartment is not the major compartment of concern. | |
| **Undertaking of intended data submission [ ]** | No further studies planned | |
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| | **Evaluation by Competent Authorities** | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | *January 2011* | |
| **Evaluation of applicant's justification** | *Applicant's justification is accepted* | |
| **Conclusion** | *Applicant's justification is accepted* | |
| **Remarks** | *Applicant's justification is accepted* | |

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| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** | |
| **Subsection A7.5** | **Effects on terrestrial organisms** | |
| **Subsection A7.5.7** | **Effects on mammals** | |
| **Annex Point IIIA XII.2.1** | | |
| | **Justification for non-submission of data** | **Official use only** |
| **Other existing data [ ]** | **Technically not feasible [ ] Scientifically unjustified [ ]** | |
| **Limited exposure [ X]** | **Other justification [X]** | |
| **Detailed justification:** | Tests with mammals are summarised in the Toxicological section (Section A6). The summaries are not repeated in the current section, please refer to section A6. | |
| **Undertaking of intended data submission [ ]** | No further studies planned | |
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| | **Evaluation by Competent Authorities** | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | *January 2011* | |
| **Evaluation of applicant's justification** | *Applicant's justification is accepted* | |
| **Conclusion** | *Applicant's justification is accepted* | |
| **Remarks** | *Applicant's justification is accepted* | |

## Section A8 Measures necessary to protect man, animals and the environment

| Subsection (Annex Point) | | | Official use only |
|---|---|---|---|
| **8.1** | **Recommended methods and precautions concerning handling, use, storage, transport or fire** | Precautions during handling:<br>Avoid dust, keep packing tightly closed and clean.<br>Precautions during storage:<br>Must be marked, palletised and shrink-wrapped for transportation. In case of fire remove product. Store in such a way that the material is prevented from drying out.<br>Packaging for use:<br>HM-HDPE open top drums, with polythene liner.<br>Suitable extinguishing media: | |

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| | | Use foam, carbonic acid, powder or water mist.<br>Special protective equipment:<br>Firefighters should be equipped with breathing apparatus.<br>Control Limits:<br>Material corrodes with metals such as steel, copper and zinc.<br>Other Information:<br>The compound should not be in contact with oxidising agents. Avoid contact with oxidising materials and acids.<br>Respiratory Protection:<br>Dust respirator P2.<br>Hand Protection:<br>PVC/synthetic (nitrile) rubber gloves.<br>Eye Protection:<br>Always use goggles or face visor etc.<br>Skin Protection:<br>Disposable dress on top of normal working clothes. Always use gloves/and boots made of nitrile rubber.<br>General Protection:<br>Keep workplace clean. Replace drum lids promptly after use, to avoid excess moisture loss to remaining contents. Material must not get too dry. |
| **8.2** | **In case of fire, nature of reaction products, combustion gases, etc.** | By fire CO and $CO_2$ are developed and harmful or poisonous gases like SOX, NOX, $NH_3$ could be generated. |
| **8.3** | **Emergency measures in case of an accident** | First Aid Measures:<br>Inhalation: Symptoms are sneezing and coughing. Risk of allergy by prolonged inhalation. Remove the affected person to fresh air and seek medical attention.<br>Skin contact: Wash skin immediately with water, using soap if available. Remove contaminated clothing. Seek medical attention if symptoms persist. Risk of sensitisation.<br>Eye contact: Wash immediately with eye wash solution and/or water. Seek medical attention.<br>Ingestion: Immediately rinse mouth, give litre of water or milk to drink. Do not induce vomiting. Seek medical attention. |
| **8.4** | **Possibility of destruction or** | Do not contaminate any lakes, streams, ponds, groundwater or soil. |

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| | **decontamination following release in or on the following:** | | |
| | (a) air | No environmental hazards have to be specially mentioned. No special measures are proposed. | |
| | (b) water, including drinking water | The contaminated water may be neutralised (detoxified) by applying alkaline 5% sodium bisulphite solution. Take care to dispose of wash water appropriately. | |
| | (c) soil | The contaminated area may be treated by washing with alkaline sodium bisulphate solution. | |
| **8.5** | **Procedures for waste management of the active substance for industry or professional users** | | |
| 8.5.1 | possibility of re-use or recycling | No specific information given | |
| 8.5.2 | possibility of neutralisation of effects | Collected waste may be neutralised (detoxified) by applying alkaline 5% sodium bisulphite solution. | |
| 8.5.3 | conditions for controlled discharge including leachate qualities on disposal | Disposal of product:<br>Sweep up and place in suitable containers for subsequent decontamination. Collected waste may be neutralised (detoxified) by applying alkaline 5% sodium bisulphite solution. The contaminated area may also be treated by washing with alkaline sodium bisulphate solution – take care to dispose of wash water appropriately. Follow relevant local, state, provincial, federal or national laws and regulations. Do not contaminate any lakes, streams, ponds, groundwater or soil. Keep unnecessary people away, isolate hazard area and deny entry. The compound should not be in contact with oxidising agents. Avoid contact with oxidising materials and acids.<br>Disposal of containers:<br>Treat polythene liners containing residues of product as waste preferably for incineration. The drums may be recycled after first rinsing with alkaline 5% sodium bisulphite solution and then water. | |
| 8.5.4 | conditions for controlled incineration | No specific information given | |

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| **Section A7** | **Ecotoxicological Profile Including Environmental Fate and Behaviour** | |
| **Subsection A7.5** | **Effects on terrestrial organisms** | |
| **Subsection A7.5.7** | **Effects on mammals** | |
| **Annex Point IIIA XII.2.1** | | |

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| **8.6** | **Observations on undesirable or unintended side-effects, e.g. on beneficial and other non-target organisms** | No specific information given | |
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| **Evaluation by Competent Authorities** | |
|---|---|
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | |
| **Materials and methods** | |
| **Conclusion** | |
| **Reliability** | |
| **Acceptability** | *Accepted* |
| **Remarks** | |

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| **Section A9**<br>**Annex Point IIA IX** | **Classification and labelling** | |
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| | **1 CLASSIFICATION AND LABELLING** | **Official use only** |
| **Classification** | Xn; R22 – harmful if swallowed,<br>Xi; R38, R41 – irritant to skin, risk of serious damage to eyes<br>R43 - may cause sensitization by skin contact<br>N; R50 – very toxic to aquatic organisms | X |
| **Symbols** |  | X |
| **R phrases** | R22, R38, R41, R43, R50 | X |
| **S phrases** | S2, S24, S26, S37/39, S61 | X |
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| | **Evaluation by Competent Authorities** | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | *May 2020* | |
| **Materials and Methods** | | |
| **Results and discussion** | | |
| **Conclusion** | *The proposed classification according to Regulation (EC) 1272/2008 should be: Danger; GHS06, GHS05; Acute toxicity 4 (oral), Acute toxicity 3 (inhalation), Skin irritation 2, Serious eye damage 1, Skin sensitization 1B: H302, H331, H318, H317, H400, H410.*<br>*We propose the following P-phrases: P102, P262, P305+P351+P338, P280, P273+P502.* | |
| **Reliability** | | |
| **Acceptability** | | |
| **Remarks** | | |