Section A7.1.1.1.1-01 Annex Point IIA VII.7.6.2.1		Hydrolysis as a function of pH and identification of breakdown products	
		1 REFERENCE	**Official use only**
1.1	**Reference**	Lucas T. (1998): [^{14}C]-[redacted]OIT: Hydrolytic Stability. [redacted]	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Thor GmbH	
1.2.2	Companies with letter of access	None	
1.2.3	Criteria for data protection	Data submitted on existing a.s. for the purpose of its entry into Annex I.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes; US EPA Pesticide Assessment Guidelines, Subdivision N, Paragraph 161-1 (October 1982)	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material (radiolabelled)**	[^{14}C]-[redacted]OIT, 2-n-octyl-4-[4,5-^{14}C]-isothiazolin-3-one	
3.1.1	Lot/Batch number	[redacted]	
3.1.2	Specification	[redacted]	
3.1.3	Purity	[redacted]	
3.1.4	Further relevant properties	[redacted]	
3.2	**Test material (non-radiolabelled)**	[redacted]OIT (CAS 26530-20-1), 2-octyl-2H-isothiazol-3-one, empirical formula $C_{11}H_{19}NOS$, used as reference substance for cochromatography and for the determination of pH stability and sterility in control units	
3.2.1	Lot/Batch number	[redacted]	
3.2.2	Specification	As given in section 2	
3.2.3	Purity	[redacted]	
3.2.4	Further relevant properties	Water solubility: 0.525 g/L (at 22 °C); physical state: clear yellow-brown liquid; MW unlabelled: 213.33 g/mol	
3.3	**Test solution**	For the preparation and composition of the buffer solutions used [pH 5, 7 and 9 (± 0.2)]: see Table A7.1.1.1.1-1. All buffers were prepared in a water bath at a temperature of 25 ± 1 °C (i.e. at the definitive study temperature). A sufficient number of 100 mL aliquots of these buffer solutions was transferred into incubation glass vials, sealed with crimped PTFE-lined septa tops and sterilised by gamma irradiation. Sterility was confirmed via microbial analysis.	

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		Sterility and the pH of the irradiated buffer solutions were also checked prior to and following application of the test article and at the end of the incubation period (control units). For the description of the test solutions and experimental conditions: see Table A7.1.1.1.1-2.	
3.4	**Testing procedure**		
3.4.1	Test system	The sterile incubation flasks were maintained in a climate chamber in the dark at 25 ± 1 °C over a period of 30 days following application of the test article with a daily gentle mixing for at least 15 minutes on an orbital shaker. For further information see Table A7.1.1.1.1-3	
3.4.2	Temperature	25 ± 1 °C	
3.4.3	pH	The pH values of the three buffer solutions treated with non-radiolabelled [redacted]OIT (control units) did not vary significantly during the course of the study. Duplicate measurements showed that the (mean) pH values for the four timepoints 'post-irradiation', 'pre-treatment', 'post-treatment' and 'post-incubation'were: • 4.9, 5.0, 4.9, 5.1 for nominally pH 5 • 6.95, 7.1, 7.1, 6.95 for nominally pH 7 • 9.0, 9.1, 9.1, 8.95 for nominally pH 9	
3.4.4	Duration of the test	30 days	
3.4.5	Number of replicates	Two replicates per sampling interval and per pH level	
3.4.6	Sampling	Duplicate units were removed for analyses at intervals of 0 (immediately after dosing), 1, 3, 7, 15 and 30 days after test compound application. The samples were then immediately analysed.	
3.4.7	Analytical methods	The general method to analyse the samples of each treated buffer solution was as follows: At each sampling interval, 100 µL aliquots of the contents of the incubation vessels were removed and subjected to LSC after mixing with 10 mL of a scintillantion cocktail (e.g. Emulsifier-Safe-Cocktail or [redacted]Cocktail). Radioactivity was measured using a [redacted]liquid scintillation counter equipped with a quench corrected dpm (disintegrations per minute) computer program. Limit of detection was taken as 1.5 times the background radioactivity. The samples were routinely counted for five minutes. For HPLC analysis (and for selected samples also for TLC confirmation analysis), 25 µL aliquots of the sampled incubation flask were directly applied to the respective analytical system. HPLC was performed under the following conditions: Column: [redacted] Flow rate: [redacted] UV detection: [redacted] ^{14}C detection: [redacted] The eluting gradient solvent consisted of [redacted] and was applied according to the following scheme:	

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		Time (min) / % acid / % acetonitrile 0 / 100 / 0 20 / 0 / 100 30 / 0 / 100 32 / 100 / 0 35 / 100 / 0 The non-radiolabelled test compound was cochromatographed (20 µL) with each test sample injection. Column recoveries were checked occasionally based on the tototal column eluent submitted to LSC and ranged always between 95 and 100%. LOD and LOQ for HPLC were determined to be 0.04 and 0.08 ppm, respectively (amendment No. 1 to report). Selected samples were also analysed by one-dimensional TLC in order to confirm the results obtained by HPLC. The TLC system consisted of [redacted] (20x20 cm) and the solvent [redacted]. Again, cochromatography with the non-radiolabelled test substance was performed with each test sample. Visualisation was done by irradiation with UV light at [redacted], whereas the areas of radioactivity were located and quantified using a [redacted]	
		4 RESULTS	
4.1	**Recovery of applied radioactivity**	The total recovery of the applied radioactivity from each aquatic system is shown in Table A7.1.1.1.1-4. For the pH 5 system individual mass balances ranged from 102.9 to 106.6 % of the applied dose with an overall mean of 104.7%. For the pH 7 system individual mass balances ranged from 103.6 to 108.1 % of the applied dose with an overall mean of 105.0%. For the pH 9 system individual mass balances ranged from 103.7 to 107.7 % of the applied dose with an overall mean of 105.2%. As recoveries for all incubates were excellent, no check of adsorbed radioactivity to the inner surface of the incubation vessels was necessary.	
4.2	**Profile of components**	Since no hydrolysis of the test compound [redacted]OIT under the study conditions occurred, no identifiable transformation products emerged in the course of the study. The characterisation of radioactivity for each pH system by HPLC is presented in Table A7.1.1.1.1-5. In all cases (different pH levels and sampling intervals), HPLC revealed an amount > 97% of the applied dose representing the parent substance [redacted]OIT. These results were confirmed by TLC analyses.	
4.3	**Hydrolysis rate constant (k_h) or half-life (DT_{50})**	Since the test compound [redacted]OIT was found to be hydrolytically stable in sterile aqueous buffer solutions at pH 5, 7 and 9 over the study period of 30 days at 25 ± 1 °C in the dark, no hydrolysis degradation rate constant or degradation half-life can be given.	
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and**	The hydrolytic behaviour of [^{14}C]-[redacted]OIT-was investigated in	

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	methods	sterile aqueous buffer systems at pH 5, 7 and 9 at a temperature of 25 °C in the dark. The GLP study was conducted according to US EPA Guideline 161-1 (1982). Mass balances were established using LSC. Study samples were analysed by HPLC (and for confirmatory purposes for selected samples also by one-dimensional TLC)	
5.2	**Results and discussion**	The test substance [redacted]OIT is stable in sterile aqueous media at pH 5, pH 7 and pH 9 over a period of 30 days at 25 °C in the dark. Greater than 97% of the applied radioactivity were detected as parent compound at any pH and sampling point. Mean recoveries for pH systems 5, 7 and 9 turned out to range from 102.9 to 106.6%, from 103.6 to 108.1% and from 103.7 to 107.7% of the applied dose, respectively.	
5.2.1	k_H	Not applicable, since no hydrolytic degradation	
5.2.2	DT_{50}	Not applicable, since no hydrolytic degradation	
5.2.3	r^2	Not applicable, since no hydrolytic degradation	
5.3	**Conclusion**	In accordance with the guideline requirements, the sterility and pH of representative samples were confirmed prior to and following test article application and at the end of the incubation. Also, darkness was maintained throughout the study period of 30 days. The treatment rate did not exceed 250 ppm and was significantly below the aqueous solubility of the test compound. The incubation temperature was 25 ± 1 °C and the test solutions were buffered. Methanol used as diluting agent was by far below 1% (v/v). The material balance (> 90% and < 110% of the applied dose) and the characterisation data of radioactivity demonstrate that there was no loss of radioactivity and that the radioactivity detected consisted essentially of the parent compound (these results also qualify the fact that volatile traps were not installed during the study as little important). Hence, the conclusion that [redacted]OIT is a hydrolytically stable compound under environmentally relevant conditions, is considered to be reliable.	
5.3.1	Reliability	1	
5.3.2	Deficiencies	No	

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	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	27 Nov 2009	
Materials and Methods	The applicant's version is acceptable.	
Results and discussion	The applicant's version is acceptable.	
Conclusion	The applicant's version is acceptable.	
Reliability	1 Study conducted in compliance with agreed protocols, with no or minor deviations from standard test guidelines and /or minor methodological deficiencies, which do not affect the quality of relevant results.	
Acceptability	Acceptable	
Remarks	All endpoints and data presented have been checked against the original study and are correct.	
	COMMENTS FROM ...	
Date		
Materials and Methods		
Results and discussion		
Conclusion		
Reliability		
Acceptability		
Remarks		

Table A 7.1.1.1.1-1: Type and composition of buffer solutions

pH	Type of buffer (final molarity)	Composition
5	0.01 M sodium citrate buffer	Citric acid monohydrate and citric acid trisodium dihydrate were dissolved separately in HPLC grade water at 10 mmol/L. After filtration through a 0.22 µm [redacted], the citric acid solution was titrated to pH 4.4 with the trisodium citrate solution to produce the required buffer. The pH 5 buffer solution was prepared at a lower value to compensate for the slight increase in pH expected following sterilisation by gamma irradiation.
7	0.01 M TRIS-maleic acid buffer	TRIS (= tris(hydroxymethyl)aminomethane) was dissolved together with maleic acid in HPLC grade water, each at 10 mmol/L. Separately, sodium hydroxide was dissolved in HPLC grade water at 10 mmol/L. Both solutions were filtered through a 0.22 µm [redacted]. The TRIS-maleic acid solution was then titrated to pH 7.2 with the sodium hydroxide solution to produce the required buffer.

		The pH 7 buffer solution was prepared at a higher value to compensate for the slight decrease in pH expected following sterilisation by gamma irradiation.
9	0.01 M Borate-boric acid buffer	Boric acid and sodium tetraborate decahydrate were dissolved separately in HPLC grade water at 10 mmol/L. After filtration through a 0.22 µm [redacted], the boric acid solution was titrated to pH 8.9 with the sodium tetraborate solution to produce the required buffer. The pH 9 buffer solution was prepared at a lower value to compensate for the slight increase in pH expected following sterilisation by gamma irradiation.

Table A 7.1.1.1.1-2: Description of test solution

Criteria	Details
Purity of water	HPLC grade (acc. to the study plan water was purified in-house by a commercially available water purification system)
Preparation of test medium and test concentration	The supplied test article amount ([^{14}C]-[redacted]OIT, ca 814 MBq/105 mg) was diluted with methanol to a stock solution providing a concentration of 18.367 mg/mL corresponding to 143.35 MBq/mL. The treatment solution was then prepared by a 1:1 mixture (w/w) of this stock solution with methanol (3.99 g each), leading to a final concentration of 8.932 mg/mL or 69.71 MBq/mL, as measured by LSC. 70 µL of this treatment solution (containing 625.2 µg [^{14}C]-[redacted]OIT with 4.880 MBq) was applied dropwise to each sterilised incubation vessel (with 100 mL buffer) via the flask septa (17 to 18 incubation units per pH) yielding finally to a test concentration of 6.25 ppm.
Temperature (ºC)	25 ± 1 ºC
Controls	A solution of 9.094 mg/mL of the non-radiolabelled [redacted]OIT was prepared in methanol (totally 100 mL solution) for cochromatography purposes and for the treatment of the control units needed for pH and sterility testing at various stages of the study. 16 control vessels per pH level containing 100 mL of the respective buffer solution were treated with 72 µL of the non-radioactive solution (corresponding to a dose of 654.8 µg Acticide OIT, equivalent to a concentration of ca 6.55 ppm).
Identity and concentration of solvent	A certain dilution of [^{14}C]-[redacted]OIT-with methanol was done to achieve a reasonable test concentration. Finally, 70 µL of treatment solution were added to the 100 mL buffer incubation units, i.e. methanol as 'solvent' was used in a final concentration of less than 0.1% v/v (0.07/100 = 0.07%).
Replicates	Two replicates per sampling interval and per pH level

Table A 7.1.1.1.1-3: Description of test system

Glassware	Glass vials of at least 100 mL volume, sealed with crimped PTFE-lined septa tops
Other equipment	Climate chamber controlled at 25 ºC; orbital shaker [redacted]; pH/redox probe [redacted]
Method of sterilization	Gamma irradiation

Table A 7.1.1.1.1-4: Recovery of applied radioactivity from sterile aqueous buffer systems

Incubation time (days)	Recovery (% of the applied radioactivity)					
	pH 5		pH 7		pH 9	
	Replicates	Mean	Replicates	Mean	Replicates	Mean
0	106.6/105.9	**106.3**	108.1/107.2	**107.6**	106.7/107.7	**107.2**
1	103.7/102.9	**103.3**	104.6/104.5	**104.5**	104.4/106.3	**105.4**
3	104.5/104.3	**104.4**	105.2/103.6	**104.4**	103.7/104.4	**104.0**
7	104.6/105.1	**104.8**	104.1/104.5	**104.3**	104.4/105.5	**104.9**
15	104.1/104.8	**104.4**	104.5/103.6	**104.0**	105.4/105.5	**105.5**
30	105.3/105.0	**105.2**	105.6/104.2	**104.9**	104.4/104.3	**104.3**
	Total mean	**104.7**		**105.0**		**105.2**
	Min	**102.9**		**103.6**		**103.7**
	Max	**106.6**		**108.1**		**107.7**

Table A 7.1.1.1.1-5: HPLC analyses of the buffer solutions following [^{14}C]-[redacted]OIT application

Incubation time (days)	Recovery of parent compound (% of the applied radioactivity)[1]					
	pH 5		pH 7		pH 9	
	Replicates	[^{14}C]-[redacted]OIT	Replicates	[^{14}C]-[redacted]OIT	Replicates	[^{14}C]-[redacted]OIT
0	97.8/97.2	**97.5**	97.6/97.6	**97.6**	97.4/97.1	**97.3**
1	97.2/97.4	**97.3**	97.5/97.9	**97.7**	97.2/97.3	**97.3**
3	97.2/97.6	**97.4**	97.9/97.2	**97.6**	97.0/97.7	**97.4**
7	97.6/98.0	**97.8**	97.4/97.2	**97.3**	97.1/97.1	**97.1**
15	97.7/97.1	**97.4**	97.9/97.5	**97.7**	97.2/97.3	**97.3**
30	97.2/97.7	**97.5**	97.4/97.5	**97.5**	97.6/97.5	**97.6**

[1] As samples were directly applied to the HPLC column without any clean-up steps, the percentage of applied radioactivity present as parent compound (or degradation product) in the assayed sample was directly taken from the HPLC printout. Therefore, the amount of parent given above signifies the percentage of the applied dose recovered by the HPLC system (= 100%). The residual radioactivity (classified in the report as 'remainder') is

then obtained by: 100% radioactivity recovered minus %parent radioactivity. Since the occasional column recovery checks demonstrated 95 – 100% recovery, this normalisation procedure seems justified.