Annex I to the CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2

International Chemical Identification:

2-phenylhexanenitrile

EC Number: 423-460-8

CAS Number: 3508-98-3

Index Number: 608-039-00-0

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Not relevant for this dossier.

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Not relevant for this dossier.

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Not relevant for this dossier.

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Not relevant for this dossier.

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Not relevant for this dossier.

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Not relevant for this dossier.

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Not relevant for this dossier.

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Not relevant for this dossier.

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Not relevant for this dossier.

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A summary of the original study is included in the CLH dossier.

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Not relevant for this dossier.

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Not relevant for this dossier.

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Not relevant for this dossier.

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Not relevant for this dossier.

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Not relevant for this dossier.

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Not relevant for this dossier.

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Not relevant for this dossier.

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Not relevant for this dossier.

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Not relevant for this dossier.

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Not relevant for this dossier.

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Not relevant for this dossier.

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Not relevant for this dossier.

4. EVIRONMENTAL HAZARDS

4.1 Degradation

4.1.1 Ready biodegradability (screening studies)

Title: Salicynalva (2-phenylhexanenitrile) ready biodegradability (closed bottle test)

Author: Bell, G

Year: 1996

Reliability: Klimisch 1 (reliable without restriction).

Test type

A study was performed to assess the ready biodegradability of 2-phenylhexanenitrile. The study was conducted in accordance with EEC Methods for Determination of Ecotoxicity Annex to Directive 92/69/EEC (0.1. No. L383A, 29.12.92) Part C, Method 4-E "Determination of ready biodegradability, Closed Bottle Test" and the OECD Guideline for Testing of Chemicals No. 301D "Ready Biodegradability: Closed Bottle Test" and in compliance with GLP.

Test substance

- The test material used in the study is equivalent to the substance identified in the CLH dossier.
- Name of test material (as cited in study report): Salicynalva (2-phenylhexanenitrile)
- Substance type: Yellow liquid with pleasant odour
- Physical state: Liquid
- Analytical purity: > 95 %
- Batch No: 6162H
- Storage condition of test material: In the dark at ambient temperature

Materials and methods

- Inoculum: activated sludge, domestic (adaptation not specified). A sample of activated sewage sludge was collected from the sewage treatment farm on 1995-02-02.
- Source: Sewage treatment plant, Godmanchester, Cambridgeshire, United Kingdom, treating predominantly domestic sewage

Pre-treatment

- The sample was allowed to settle for approx. 30 mins and the supernatant filtered through Whatman GF A coarse filter paper (first 250 mL discarded). The filtrate was maintained under aeration and used within 24 hrs of collection.

Preparation of test solutions

- The test substance (purity >95%) was dissolved in chloroform to give a stock solution of 560 mg/10 mL. Then, 10 µL aliquots of stock solution were placed on individual pieces of glass filter paper and the solvent allowed to evaporate to dryness.
- One piece of paper was placed in each test bottle prior to filling with inoculated medium. Filter paper blanks were prepared in the same manner using solvent only. Sodium benzoate standards were prepared by dissolving the sample directly in nutrient medium.

Study design

- Oxygen conditions: aerobic
- Duration of test (contact time): 28 days

Test system

- Initial test concentration: 2 mg 2-phenylhexanenitrile/L.
- Parameter followed for biodegradation estimation: oxygen consumption

- The following treatments were prepared:
- (a) Inoculated nutrient medium.
- (b) Inoculated nutrient medium plus filter paper.
- (c) 2 mg/L test substance, plus filter paper.
- (d) 3 mg/L standard substance, sodium benzoate.
- (e) 2 mg/L test substance plus filter paper and 3 mg/L sodium benzoate (inhibition check).
- Inoculation of the stock nutrient medium with activated sewage sludge filtrate at a rate of 1 ml of inoculum per litre, was performed immediately prior to the preparation of treatments (a), (b), (c), (d), (e).

Standard nutrient medium
- Solution 1: KH_2P0_4 8.5 g/L, K_2HPO_4 21.75 g/L, $Na_2HPO_4.2H_2O$ 33.4 g/L, NH_4Cl 0.5 g/L
- Solution 2: $MgSO_4.7H_2O$ 22.5 g/L
- Solution 3: $CaCl_2$ 27.5 g/L
- Solution 4: $FeCl_3.6H_2O$ 0.25 g/L
- 1 mL of each of solutions 1 - 4 were added to each litre of aerated reverse osmosis purified water.
- The nutrient medium was left at room temperature (approximately 20 °C), under aeration, for approx. 24 hrs prior to use.

Procedure
- Biochemical Oxygen Demand bottles (280 mL) of darkened glass and fitted with ground glass stoppers were filled by siphon and firmly stoppered to exclude all air bubbles. Sufficient bottles were prepared in each test and control series to allow a single oxygen determination per bottle to be made at 0, 4, 7, 11, 14, 18, 21, 25, 28 d (duplicate bottles at each sampling time).
- Sufficient bottles for series (e) were prepared to provide information on inhibitory effects on 0, 14 and 28 d only.
- The bottles were incubated in a water bath at 20 ± 1 °C until removed for measurement of dissolved oxygen concentration by means of a Yellow Springs BOD Meter (Model 59).

Data evaluation
- Percent biodegradation was determined by comparing the oxygen depletion value with the corresponding Theoretical Oxygen Demand.
- The test substance would have been considered to have had an inhibitory effect on the bacteria if the oxygen demand in treatment (e) was 25 % less than the sum of oxygen demands in treatments (c) and (d) at 14 d.
- ThOD of the test substance was calculated to be 2.77 mg O_2/mg.
- ThOD of the reference substance (sodium benzoate) was calculated to be 1.67 mg O_2/mg.

Results
- 2-phenylhexanenitrile attained no biodegradation after 28 days and it was not found to be inhibitory to activated sewage sludge bacteria under the conditions of this test. Degradation (%) of test substance (O2 consumption): 0 after 4 d; 1 after 7 d; 3 after 14 d; 1 after 21 d and 0 after 28 d (see Table 1 for details).

Table 1 - Dissolved oxygen measurements (mg O2/L)

Culture medium		Day								
		0	4	7	11	14	18	21	25	28
Nutrient medium with inoculum	R1	8.9	8.65	8.69	8.22	8.49	8.18	8.1	8.09	7.99
	R2	8.9	8.66	8.67	8.17	8.35	8.23	8.13	8.05	8.05
	Mean	8.900	8.655	8.680	8.195	8.420	8.205	8.115	8.070	8.020

Nutrient medium with inoculum + filter paper	R1	8.9	8.62	8.62	8.27	8.01	8.12	8.05	8.11	8.10
	R2	8.9	8.65	8.74	8.10	7.99	7.99	8.00	8.07	8.08
	Mean	8.900	8.635	8.680	8.185	8.000	8.055	8.025	8.090	8.090
Test substance (2 mg/L) + filter paper	R1	8.9	8.63	8.62	8.15	8.25	8.22	8.12	8.08	8.05
	R2	8.9	8.67	8.68	8.27	8.07	8.24	8.02	8.07	8.08
	Mean	8.900	8.650	8.650	8.210	8.160	8.230	8.070	8.075	8.065
Standard substance (3 mg/L of sodium benzoate)	R1	8.9	5.51	5.04	4.63	4.39	4.37	4.12	4.08	4.09
	R2	8.9	5.41	5.12	4.64	4.49	4.76	4.19	4.07	4.04
	Mean	8.900	5.460	5.080	4.635	4.440	4.565	4.155	4.075	4.065
Test substance (2 mg/L) + filter paper and sodium benzoate (3 mg/L)	R1	8.9				4.97				4.49
	R2	8.9				4.26				4.53
	Mean	8.900				4.615				4.510

- Oxygen depletions in the inoculated control series were within the prescribed limits (≤ 1.5 mg O_2/L at 28 d).
- Sodium benzoate attained 80 % biodegradation within 28 d.

Bacterial inhibition
- Cultures containing both test and standard substances combined showed an oxygen depletion value 11 % lower than that anticipated on the basis of results from separate cultures at 14 d. Consequently 2-phenylhexanenitrile is not considered to have had an inhibitory effect on sewage bacteria under the conditions of this test.

- Statistical analysis: not performed.

Conclusion
- 2-phenylhexanenitrile was found to undergo no degradation under the conditions of the Closed Bottle Test.

4.1.2 BOD5/COD

No data available.

4.1.3 Aquatic simulation tests

Title: Salicynalva (2-phenylhexanenitrile) (test article 97-202-01): Determination of biodegradability by EPA OPPTS 835.3220 and modified OECD Test Guideline 303A, draft dated September, 1996

Author: Jordinson, GM and Morris DS

Year: 1998

Reliability: Klimisch 1 (reliable without restriction).

Test type

The biodegradation and/or removal of 2-phenylhexanenitrile within sewage treatment processes was investigated using the OECD Test Guideline 303A with appropriate modifications to ensure compliance with EPA "Public Draft" Guideline OPPTS 835.3220 and in compliance with GLP.

Test substance

- The test material used in the study is equivalent to the substance identified in the CLH dossier.
- Name of test material (as cited in study report): Salicynalva (2-phenylhexanenitrile) (Test article 97-202-01)
- Substance type: Yellow liquid with pleasant odour
- Physical state: Liquid
- Analytical purity: > 99 %
- Storage condition of test material: In darkness at room temperature

Materials and methods

- Inoculum: Mixture of sewage, predominantly domestic (adaptation not specified but assumed to be non-adapted) and synthetic sewage.
- Source: Buckland Sewage Treatment Works, Newton Abbot, Devon, United Kingdom

Pre-treatment

- Activated sludge was stored at 20 ± 3 °C under aeration until required for use (within 24 hrs of receipt).
- At the start of the test, the activated sludge solids concentration was determined and each porous pot was inoculated with 2500 mg/L Mixed Liquor Suspended Solids (MLSS). At the start of the test, the activated sludge solids concentration was determined and each porous pot was inoculated with 2500 mg 1-1 Mixed Liquor Suspended Solids (MLSS).
- Synthetic sewage is normally dosed into the porous pot system as a relatively dilute solution. Due to the low aqueous solubility of 2-phenylhexanenitrile, a larger than normal volume of water was dosed into the system from the test solution of 2-phenylhexanenitrile. Consequently, it was necessary to add synthetic sewage in a concentrated form. This was achieved by the addition of concentrated synthetic sewage to domestic sewage. This meant that reference substance porous pots and 2-phenylhexanenitrile porous pots were each connected to vessels containing 2400 mL of domestic sewage/synthetic sewage concentrate which was diluted by 80% (i.e. a 1 in 5 dilution) at a 'Y-piece' by the solution of either sodium benzoate or 2-phenylhexanenitrile solution, respectively. Each control pot was dosed with the same mixture of domestic and synthetic sewage diluted by tapwater to a total volume of 12 litres. The sewage mixture consisted of a ratio of 11:1 domestic:synthetic sewage and had a nominal concentration of 200 mg/L DNPOC after dilution.

Synthetic sewage

- Synthetic sewage was prepared as a concentrate and diluted daily. The following are the concentration of ingredients, per litre of tapwater, prior to dilution:
- Bacteriological peptone: 16 g
- Lab-lemco: 11 g
- Urea: 3 mg
- Sodium chloride: 0.7 mg

- Calcium chloride dihydrate: 0.4 mg
- Magnesium sulphate heptahydrate: 0.2 mg

The nominal DNPOC concentration of this concentrated stock solution was 12,000 mg/L.

Preparation of test solutions
- The limited aqueous solubility of 2-phenylhexanenitrile (approximately 38 mg/L) prevented the preparation of a concentrated stock solution. Thus, fresh test solutions were prepared daily by adding 640 mg 2-phenylhexanenitrile to 19.2 litres tapwater. These test solutions were stirred vigorously overnight prior to use. This addition of 2-phenylhexanenitrile gave a nominal concentration of 31 mg/L as test substance (using a Specific Gravity figure of 0.9434 as measured at Brixham Environmental Laboratory), and 26 mg/L as carbon (from the empirical formula supplied by the Sponsor, 2-phenylhexanenitrile was calculated to consist of 83% carbon).

Study design
- Oxygen conditions: aerobic
- Duration of test (contact time): 53 days

Test conditions
- Volume of test solution/treatment: 9.6 L
- Composition of medium: 2.4 L of domestic sewage at nominal DNPOC concentration of 1000 mg/L
- Test temperature: 22.5 °C ± 2 °C
- pH adjusted to 7 to 8.
- CEC (meq/100 g): No data

Test system
- Culturing apparatus: 3 L porous pots - cylindrical vessels made from PVC
- Number of culture flasks/concentration: 2 (treated pots numbered 13, 14)
- Method used to create aerobic conditions: Rotameter used to regulate airflow and ensure dissolved O_2 remained at > 2 mg/L
- Measuring equipment: Anglicon control unit
- Test performed in open system
- Initial test concentration: 31 mg 2-phenylhexanenitrile/L.
- Parameter followed for biodegradation estimation: dissolved non-purgeable organic carbon (DNPOC) removal

Sampling
- Sampling frequency: Porous pot feed and effluent 2 ×/wk until the definitive test period; during definitive test period every weekday.

Identification and quantification of test substance
- Gas chromatography determination using a flame ionisation detector. Transformation products were not measured.

Carbon analysis

- The instrument used was a Dohnnan DC 190 analyser in which carbon is oxidised at high temperature (680°C) with oxygen over a platinum catalyst. The resulting carbon dioxide was then measured by non-dispersive infra-red detection.
- DNPOC (dissolved non-purgeable organic carbon) was determined after centrifugation, acidification and gassing with nitrogen to remove inorganic carbon dioxide and suspended material.
- TOC (total organic carbon) was determined by measuring TC (total carbon) and IC (inorganic carbon) and subtracting the latter from the former.

Analysis for total oxidised nitrogen and ammonia
- Concentrations of total oxidised nitrogen and ammonia in the settled effluents from the porous pots were determined using "Palintest" reagents and kits. The Palintest method reduces nitrates to nitrite (using a zinc based reagent). Sulphanilic acid is used in a reaction with nitrite to form a diazo compound, this couples with N-(1-naphthyl)-ethylene diamine to form a red coloured solution, which is then measured at 570 nm.
- Ammonia analysis was based on an indophenol method; ammonia present in the sample would react with alkaline salicylate in the presence of chlorine to form a green-blue indophenol complex, the resulting colour absorbance was measured at 640 nm.

Results
- The test substance attained a level of primary biodegradation/removal in this study of 88% and 89%, as shown by the parent compound analytical data. These high levels of test substance removal was already attained on day 4 of the equilibrium period and remained as such during the whole test. The level of ultimate biodegradation/removal achieved, provided by an assessment of the level of dissolved organic carbon removed, was 87% (attained already on day 9 of the equilibrium period and remained as such during the whole test). Whilst this figure was shown to be statistically different to the levels of DOC removal observed in the control and reference substance pots, the statistical analyses also showed significant differences between duplicate control and reference substance pots. Therefore, including the small data-set, undue emphasis should not be placed on the statistical results obtained. The overall difference, in terms of percentage carbon removal, between the test pots and the control and reference substance pots, during the definitive period, was only 3%. Although there was apparent adsorption of the test substance to the test solution feed lines, there was no notable amount of adsorption to either the sludge solids or the porous pot apparatus. A final reasoning considering primary degradation and ultimate degradation is therefore not possible.

Carbon analysis
- The overall mean percentage removal values for the definitive test period were 89% and 91 % for the control pots 9 and 10, 89% and 92% for the reference pots 11 and 12 and 87% and 87% for the test substance pots 13 and 14, respectively.

Parent compound analysis
- During the definitive period, the mean measured values of test substance in the solutions being dosed to pots 13 and 14 (sampled from the test solution vessels and prior to dilution with sewage mixture) were 27 mg/L and 27 mg/L respectively (87% and 87% of the nominal value, respectively). In addition, the mean measured values of test substance in the feeds being dosed to pots 13 and 14 (sampled post V-piece and after dilution with sewage mixture) were 15 mg/L and 15 mg/L, respectively (60 % and 60 % of the nominal value, respectively).

- The differences between the percent nominal figures in the above solutions which cannot be accounted for by dilution are thought to be due to adsorption of the test substance to the test solution feed lines. The concentration of test substance entering the porous pots appeared to reach equilibrium during the course of the study.
- The occasional very low levels of test substance in the feeds to the control pots is thought to be due to contamination during the centrifugation procedure (confirmed by the extra samples taken on 1997-08-21). The presence of very low levels of test substance in the control pots did not adversely affect the results of the study.

Nitrogen analysis
- A substantial amount of nitrification was observed within all of the porous pots as shown by the analytical data for NH_4-N and total oxidised nitrogen. The test substance appeared to cause no detrimental effect to this important and sensitive environmental process.

Statistical analysis:
- Percentage removal of Carbon was calculated as: 100 × (1 - effluent concentration at t + 1 days)/Feed concentration at t days.
- Data were initially tested for normality. Normally distributed datasets could be tested using parametric tests. Data were analysed using paired t-tests to determine if there was significant mean difference in percentage carbon removal between pots. Unpaired t-tests, assuming unequal variance, were also carried out to test for a significant difference in mean carbon removal between pots. Tests between duplicate pots were 2-tailed as there was no preconception as to whether one would have a higher value than the other. All other tests were one-tailed, since only a reduction in the percentage carbon removal was being investigated. Paired t-tests take account of variation over time, whereas unpaired t-tests allow the use of all data values from each pot, even if there are no corresponding values in the pot being compared against, thus increasing the size of the available dataset.
- Results from comparisons of data from test pots and individual control and reference substance Pots show that, with the exception of the comparisons between pots 11 and 13 (both methods of analyses) and pots 11 and 14 (Matched Pairs analysis only), results obtained from the test substance pots are significantly different (at 5 % or less) to those obtained from the control and reference pots.

Conclusion

The test substance attained a level of primary biodegradation/removal in this study of 88% and 89%, as shown by the parent compound analytical data. In view of the similar removal between the reference substance, sodium benzoate, and the test substance it can be expected that a significant level of ultimate biodegradation/removal was obtained but no final conclusion on ultimate biodegradation can be made based on this study. It is stated in the OECD 303A Guideline that, if a high initial removal has taken place, the simulation test cannot differentiate between biological and abiotic elimination processes. In such cases, the ready biodegradation screening test is more appropriate to evaluate biological processes. Furthermore according to Annex II, section II.2.3.5 of the Guidance on the Application of the CLP Criteria (version 4.1, November 2015), results from tests simulating the conditions in a sewage treatment plant (STP) e.g. the OECD Test Guideline 303 cannot be used for assessing the degradation in the aquatic environment. The main reasons for this are that the microbial biomass in a STP is significantly different from the biomass in the environment, that there is a considerably different composition of substrates, and that the presence of rapidly mineralised organic matter in waste water may facilitate degradation of the test substance by co-metabolism.

4.1.4 Other degradability studies

Title: Salicynalva (2-phenylhexanenitrile) abiotic degradation: Hydrolysis as a function of pH

Author: Betteley, JMT

Year: 1996

Reliability: Klimisch 1 (reliable without restriction).

Test type

The hydrolysis of the substance has been tested using a protocol similar to OECD TG 111 and in compliance with EEC Methods for the determination of ecotoxicity, Directive 92/69/EEC (OJ No. L383A, 29. 12. 92), Part C, Method C7. This study was performed in compliance with GLP.

Test substance
- The test material used in the study is equivalent to the substance identified in the CLH dossier.
- Name of test material (as cited in study report): Salicynalva
- Chemical name: 2-Phenylhexanenitrile
- Appearance: A clear yellow liquid
- Storage conditions: In the dark at room temperature
- Date received: 28 September 1994
- Purity: > 95 %
- Lot/batch No.: 6162H

Materials and methods

Preparation of buffer solutions
- pH 4 buffer: Potassium dihydrogen citrate (ca. 23.0 g) was dissolved in 1 litre of distilled water. The solution was stirred and heated on a magnetic stirrer/hotplate. The pH was adjusted to pH 4. 0 with 1M aqueous sodium hydroxide solution with the temperature of the buffer solution at 50 ± 0.5 °C.
- pH 7 buffer: Potassium dihydrogen orthophosphate (ca. 26 g) was dissolved in 2 litres of distilled water. The solution was stirred and heated on a magnetic stirrer/hotplate. The pH was adjusted to pH 7. 0 with 1M aqueous sodium hydroxide solution with the temperature of the buffer solution at 50°C ± 0. 5°C.
- pH 9 buffer: Potassium chloride (ca. 1.5 g) and boric acid (ca. 11.2 g) were dissolved in 2 litres of distilled water. The solution was stirred and heated on a magnetic stirrer/hotplate. The pH was adjusted to pH 9. 0 with 1M aqueous sodium hydroxide solution with the temperature of the buffer solution at 50 ± 0.5 °C.

Note: For the tests at 60 ± 0.5 °C and 70 ± 0.5 °C, the buffer was heated to the appropriate temperature and then the pH adjusted with 1M aqueous sodium hydroxide solution

Number of replicates
- An aliquot (10 mL or 3 mL for test 1 Days 7, 8 and 11) was pipetted into a volumetric flask (20 mL or 5 mL for test 1 Days 7, 8 and 11) and made to volume with 80/20 v/v acetonitrile/water.

Positive control
- No positive control was used.

Degradation

- Abiotic degradation: hydrolysis as a function of pH. Hydrolysis was evaluated at 50 °C in aqueous solutions at pH 4, 7 and 9. The starting concentration was circa 15 mg/L. At each time point, including 0 hours, 2.4 hours and 5 days, an aliquot of test solution was diluted with mobile phase and the concentration of the substance determined by HPLC analysis.

Calculations
- The peak response of the test substance in each calibration solution chromatogram was measured and calibration curves constructed by linear regression of the standard response versus standard test substance concentration. The response of the peak observed at the characteristic retention volume for the test substance in the sample chromatograms was measured, and the concentration of test substance in test phases determined using the equation: kobserver = -S x 2.303 where kobserved = observed rate constant and S is the slope derived from regression data of log10 C versus time.

Results
Under the preliminary test condition, the substance was found to undergo hydrolysis at pH 4, 7 and 9, which indicated a half-life of between 1 day and 1 year at 25 °C and therefore a full study was carried out. Under the test conditions in the first test at 50 °C, the substance was found to undergo a pseudo first order reaction at pH 4, 7 and 9 and therefore the final test was performed at 60 and 70 °C. 2-Phenylhexanenitrile possesses half-life times in water of 25.9 days at pH 4, 15.4 days at pH 7 and 4.7 days at pH 9 at 25°C.

Note: The correlation coefficients show that a linear response of peak areas versus concentration was obtained on each occasion of analysis. Regression greater than 0.990.

Conclusion
2-phenylhexanenitrile possesses half-life times in water of 25.9 days at pH 4, 15.4 days at pH 7 and 4.7 days at pH 9 at 25°C. The substance is considered as hydrolytically stable under environmental conditions for classification purposes since the longest half-life is above 16 days at pH range 4-9 (see ECHA Guidance on the Application of the CLP Criteria, v. 4.1, June 2015).

4.2 Bioaccumulation

4.2.1 Bioaccumulation test on fish

No data available.

4.2.2 Bioaccumulation test with other organisms

No data available.

4.3 Acute toxicity

4.3.1 Short-term toxicity to fish

Title: Salicynalva (2-phenylhexanenitrile) acute toxicity for Rainbow Trout (*Oncorhynchus mykiss*)

Author: Bell, G; Thirkettle, KM and Smith, B

Year: 1996

Reliability: Klimisch 1 (reliable without restriction).

Test type

A study was performed to assess the acute toxicity of 2-phenylhexanenitrile to rainbow trout (*Oncorhynchus mykiss*) under semi-static conditions. The study was conducted in accordance with EEC Methods for Determination of Ecotoxicity Annex to Directive 92/69/EEC (O.J. No. L383A, 29. 12.92) Part C, Method 1 "Acute toxicity for fish" and the OECD Guideline for Testing of Chemicals No. 203 "Fish, Acute Toxicity Test" and in compliance with GLP.

Test substance

- The test material used in the study is equivalent to the substance identified in the CLH dossier.
- Name of test material (as cited in study report): Salicynalva
- Chemical name: 2-Phenylhexanenitrile
- Physical state: Light yellow liquid
- Analytical purity: > 95%
- Lot/batch No.: 6162H
- Storage condition of test material: In darkness at room temperature

Materials and methods

Test organism

- Common name: Rainbow trout (*Oncorhynchus mykiss*)
- Source: Westacre Trout Farm, Norfolk, UK.

Acclimation

- The stock of fish was obtained from the supplier on 28 March 1995 and acclimatised to test conditions immediately. Temperature remained within the range 16 ± 3°C and dissolved oxygen within the range 9.1 ± 0.7 mg O2/L in the 14 day period immediately prior to the study. Although the temperature was slightly outside the limits stated in the protocol (12 - 17°C), this was not considered to have affected the results of the study. The fish were fed daily to repletion with commercial fry pellet but food was not given during the 24 hour period immediately prior to exposure or during the exposure period itself. No medication was given during the acclimatisation period and no mortality was recorded in the seven days prior to the test.
- The size of the fish used in the test was determined by measuring the control fish at the end of the exposure period. The mean standard length was found to be 4.7 cm (SD = 0.36 cm) and the mean weight determined as 1.41 g (SD = 0.37 g).

Preparation of test solutions

- The test substance (purity > 95%) was dissolved in Tween 80 acetone (20: 80 v/v) to give an initial stock solution of 100 mg/mL. The test solutions were prepared by serial dilutions of this stock and the solvent control contained 100 µL solvent per liter.

Study design

- Test type: semi-static

- Water media type: Freshwater
- Total exposure duration: 96 hours

Test system
- Seven test concentrations plus one control and one solvent control (100 µL auxiliary solvent per litre). Nominal test concentrations: 0.10, 0.22, 0.46, 1.0, 2.2, 4.6 and 10 mg 2-phenylhexanenitrile/L.
- Ten animals per test concentration and ten animals per test vessel.
- Animals were placed at random in glass aquaria containing prepared test media or diluent water plus 100 µL auxiliary solvent per litre, as appropriate. Each vessel contained 20 litres of medium to a depth of 19 cm (approximate dimensions of vessel: 25 x 46 x 25 cm). This provided an initial loading of 0.71 g bodyweight/litre (static volume).
- Medium renewal: Animals were exposed to the test or control conditions for a period of 96 hours with daily batchwise renewal of the test media to ensure the maintenance of satisfactory environmental conditions and near nominal exposure levels.

Test water
- Laboratory tap water filtered, dechlorinated and softened by passage through an Elga® water purification system was used. The water passes in sequence through a high pressure sand filter to remove colloids and particulate matter and a high grade activated carbon filter to remove chlorine and any organic contaminants. A proportion of the supply then passes through a water softener before final reverse osmosis treatment to produce a highly purified water supply. The two grades of dechlorinated water are then remixed in the ratio of approximately 1 : 1 to give a supply with the desired water hardness.
- Chlorine levels remained below the limit of detection throughout the acclimatisation and exposure periods and the mean hardness level, calculated from daily measurements during the same period, was determined as 148 mg CaCO3/L (SD = 8.8 mg CaCO3/L).
- Total organic carbon: 3.7 mg/L (min, mean & max)
- Particulate matter: no data
- Metals:
Aluminium: < 10 µg/L (min, mean & max)
Iron: min < 10 µg/L; mean < 22 µg/L; max: < 120 µg/L
Copper: 13 µg/L (min, mean & max)
Zinc: 8.0 µg/L (min, mean & max)
Silver: < 1.0 µg/L (min, mean & max)
Mercury: < 10 µg/L (min, mean & max)
Nickel: 2.1 µg/L (min, mean & max)
Lead: 1.9 µg/L; mean 2.2 µg/L; max: 3.6 µg/L
- Pesticides: 0 µg/L; mean 0.0020 µg/L; max: 0.030 µg/L
- Chlorine: 62 mg/L; mean 63 mg/L; max: 64 mg/L
- Alkalinity: 230 mg/L (min, mean & max)
- Ca/mg ratio: 150 mg/L (min, mean & max)
- Conductivity: 630 µS/cm; mean 640 µS/cm; max: 650 µS/cm

Other test conditions

- Treatment and control groups were maintained at 14 ± 1 °C throughout the exposure period.
Supplementary aeration was provided via narrow bore glass tubes in this study. A photoperiod of 16 h light : 8 h dark was established and daily records of temperature, pH and dissolved oxygen were maintained for each control and test vessel. The measurements were made in freshly prepared solutions at time 0 and thereafter in the 24 hour old expired solutions. The fish were not fed during the 96 hour exposure period.

Effects parameters measured (with observation intervals if applicable)
- The criteria of death employed in this study were (i) absence of respiratory movement and (ii) absence of response to physical stimulation of the caudal peduncle.
In addition to observations on mortality at 3, 6, 24, 48, 72 and 96 hours, subjective assessments were also made on the type and incidence of sub-lethal effects as compared to control fish.

Identification and quantification of test substance
- High Performance Liquid Chromatography (HPLC) with UV detection.
Procedural recoveries were determined for the mid-test level and analysed concurrently with test samples as 90.8-93.2% of nominal at the start and 93.3-103% of nominal at 25 hours.
The LOD was calculated to be 0.004 mg 2-phenylhexanenitrile/L.

Results

- Mean measured test concentrations: 0.068, 0.15, 0.37, 0.79, 1.7, 3.7 and 8.6 mg 2-phenylhexanenitrile/L. Measured concentrations ranged from 76 to 102% of nominal in freshly prepared solutions and from 51 to 81 % of nominal in 24 hour old expired solutions. The greatest deviations from nominal concentration were observed at the lower concentrations which suggests this may be the result of adsorption of the compound. All results are expressed in terms of mean measured concentration. No mortality was observed at 0.79 mg 2-phenylhexanenitrile/L, while at 8.6 mg/L it was 100% (see Table 2 for details). The 24, 48 and 72h-LC50 were 4.3, 3.1 and 2.2 mg/L, respectively. The 96h-LC50 was 2.2 mg/L (95% confidence limits: 1.5 - 3.2 mg/L).

Table 2 - Cumulative mortality data for rainbow trout exposed for 96 hours to 2-phenylhexanenitrile

Nominal concentration (mg 2-phenylhexanenitrile/L)	Mean measured concentration (mg 2-phenylhexanenitrile/L)	Cumulative mortality (initial population: 10)					
		3 h	6 h	24 h	48 h	72 h	96 h
Control	Control	0	0	0	0	0	0
Solvent control	Solvent control	0	0	0	0	0	0
0.10	0.068	0	0	0	0	0	0
0.22	0.15	0	0	0	0	0	0
0.46	0.37	0	0	0	0	0	0
1.0	0.79	0	0	0	0	0	0
2.2	1.7	0	0	0	0	4	4
4.6	3.7	0	0	4	8	9	9
10	8.6	0	0	10	10	10	10

- In both controls no mortality was observed.
- Environmental parameters remained within acceptable limits throughout the duration of the study: pH (mean): 7.6 - 7.8; temperature: 14 - 15°C and dissolved oxygen: 9.2 -9.6 mg O2/L. This would result in c.a. 96%saturation value in air.
- Statistical analysis: LC50 values and 95% confidence limits were calculated according to the method of Thompson and Weil (Thompson, W. R. & Weil, C. S. , 1952, Biometrics 8; 51 - 54).

Conclusion

- The 96h-LC50 value for 2-phenylhexanenitrile with rainbow trout was 2.2 mg/L.

4.3.2 Short-term toxicity to aquatic invertebrates

Title: Salicynalva (2-phenylhexanenitrile) acute toxicity to *Daphnia magna*

Author: Bell, G; Thirkettle, KM and Smith, B

Year: 1996

Reliability: Klimisch 1 (reliable without restriction).

Test type

A study was performed to assess the acute toxicity of 2-phenylhexanenitrile to *Daphnia magna*. The study was conducted in accordance with EEC Methods for Determination of Ecotoxicity Annex to Directive 92/69/EEC (O.J. No. L383A, 29.12.92) Part C, Method 2 "Acute toxicity for Daphnia" and the OECD Guideline for Testing of Chemicals No. 202, Part I "Daphnia sp., Acute Immobilisation Test" and in compliance with GLP.
Note: During the acclimatisation period of the test organisms, the following deviation occurred: The brood stocks were cultured at 21 ± 2°C, which was slightly outside the temperature stated in the protocol, 18 - 22°C. This deviation was not considered to have affected the validity of the study.

Test substance

- The test material used in the study is equivalent to the substance identified in the CLH dossier.
- Name of test material (as cited in study report): Salicynalva
- Chemical name: 2 - Phenylhexanenitrile
- Physical state: Light yellow liquid
- Analytical purity: > 95%
- Lot/batch No.: 6162H
- Expiration date of the lot/batch: 1 October 1995
- Storage condition of test material: In darkness at room temperature

Materials and methods

Test organism
- Species: *Daphnia magna*
- Source: Laboratory culture originating from a strain supplied by the Institute National de Recherche Chimique Appliquee (lRChA), France.

Acclimation
- Brood stocks of *Daphnia magna* were cultured under a 16 h light : 8 h dark photoperiod at 21 ± 2°C in polypropylene vessels containing two litres of Elendt M7 medium. Cultures were fed daily with a suspension of mixed algae (predominantly *Scenedesmus* and *Selenastrum spp*.). Culture conditions ensured that reproduction was by parthenogenesis. Gravid adults were isolated 24 hours prior to initiation of the test. Young daphnids produced overnight were used for testing.

Preparation of test solutions
- The test substance (purity > 95%) was dissolved in 20% Tween 80 : acetone (20:80 v/v) to give an initial stock solution of 100 mg/mL. The test solutions were prepared by serial dilutions of this stock and the solvent control contained 100 µL solvent per liter.

Study design
- Test type: static
- Water media type: Freshwater
- Total exposure duration: 48 hours

Test system
- Seven test concentrations plus one control and one solvent control (100 µL auxiliary solvent per litre) each consisting of four replicates (20 animals per test group). Nominal test concentrations: 0.156, 0.313, 0.625, 1.25, 2.5, 5.0 and 10 mg 2-phenylhexanenitrile/L.
- Five first instar Daphnia were placed at random in each glass jar containing 250 mL of prepared test medium, diluent media only or diluent media plus 100 µL auxiliary solvent per litre, as appropriate, to give a loading of 50 mL test solution per organism. The jars were loosely covered with aluminium foil to minimise evaporation losses.
- Medium renewal: Daphnia were exposed to the test or control conditions for a period of 48 hours without renewal of test media.

Test water
- Elendt M7 reconstituted water was prepared using analytical grade reagents and reverse osmosis purified water.
- Elendt M7 medium:
1. Trace elements (mg/L)
H3BO3: 0.71
MnCI2.4H2O: 0.090
LiCl: 0.077
RbCl: 0.018
SrCI2·6H2O: 0.038
NaBr: 0.0040
Na2MoO4·2H2O: 0.016
CuCl2·2H2O: 0.0042
ZnCl2:0.013
CoCl2·6H2O: 0.010

KI: 0.0033
Na2SeO3: 0.0022
NH4VO3: 0.00058
Fe-EDTA solution: 1.7

2. Macro nutrients (mg/L)
CaCI2·2H2O: 294
MgSO4·7H2O: 123
KCl: 5.80
NaHCO3: 64.8
Na2SiO3.5H2O: 6.83
NaNO3: 0.274
KH2PO4: 0.143
K2HPO4: 0.184

3. Vitamins (mg/L)
Thiamine hydrochloride: 0.075
Cyanocobalamine (B12): 0.0010
Biotine: 0.00075

The above analytical grade reagents are dissolved in reverse osmosis water.

Other test conditions
- Cultures were maintained at 20 ± 2°C under a photoperiod of 16 hours light: 8 hours dark and without supplementary aeration or feeding during the 48 hour exposure period. The temperature in each vessel was measured daily and the pH and dissolved oxygen levels recorded at the start and at the end of the study.

Effect parameters measured
- Daphnia were considered to be immobilised if they were unable to swim for approximately 15 seconds after gentle agitation.

Identification and quantification of test substance
- High Performance Liquid Chromatography (HPLC) with UV detection. The analytical method was proven satisfactory during the validation phase of the study with regard to accuracy and precision with mean recoveries of 97.4%; coefficient of variation 5.1 %. The method was specific for 2-phenylhexanenitrile, there being no peak present at the characteristic retention time of 2-phenylhexanenitrile in control sample chromatography. The limit of detection for the study was calculated to be 0.007 mg 2-phenylhexanenitrile/L. Procedural recovery samples analysed concurrently with the test samples were used for the assessment of method performance. The mean procedural recovery value during this phase was 103 %; coefficient of variation 2.0%.

Results

- Mean measured test concentrations: 0.193, 0.364, 0.695, 1.26, 2.5, 4.8 and 9.3 mg 2-phenylhexanenitrile/L. Measured concentrations ranged from 98 - 127 % of nominal at 0 hours and 86 - 121 % of nominal at 48 hours. All results are expressed in terms of mean measured concentration. No immobilization was observed at 0.695 mg 2-phenylhexanenitrile/L, while at 4.8 mg/L and higher it was 100% (see Table 3 for details). The 24 and 48h-EC50s (immobilisation) were 2.7 mg/L (95% confidence limits: 2.3 - 3.1 mg/L) and 1.6 mg/L (95% confidence limits: 1.3 - 1.9 mg/L), respectively.

Table 3 - Cumulative immobilisation data for *Daphnia magna* exposed for 48 hours to 2-phenylhexanenitrile

Nominal concentration (mg 2-phenylhexanenitrile/L)	Mean measured concentration (mg 2-phenylhexanenitrile/L)	Cumulative immobilized Daphnia magna (initial population: 5 per replicate)											
		24 hours						48 hours					
		R1	R2	R3	R4	**Total**	**%**	R1	R2	R3	R4	**Total**	**%**
Control	Control	0	0	0	0	**0**	**0**	0	0	0	0	**0**	**0**
Solvent control	Solvent control	0	0	0	0	**0**	**0**	0	0	0	0	**0**	**0**
0.156	0.193	0	0	0	0	**0**	**0**	0	0	0	0	**0**	**0**
0.313	0.364	0	0	0	0	**0**	**0**	0	0	0	0	**0**	**0**
0.625	0.695	0	0	0	0	**0**	**0**	0	0	0	0	**0**	**0**
1.25	1.26	0	0	0	0	**0**	**0**	3	2	2	3	**10**	**50**
2.5	2.5	3	4	0	0	**7**	**35**	4	5	3	3	**15**	**75**
5	4.8	5	5	5	5	**20**	**100**	5	5	5	5	**20**	**100**
10	9.3	5	5	5	5	**20**	**100**	5	5	5	5	**20**	**100**

- In both controls no immobilisation was observed.
- Environmental parameters remained within acceptable limits throughout the duration of the study: pH (mean): 7.5 - 7.6; temperature (mean): 21 °C and dissolved oxygen (mean): 7.5 - 7.6 mg O2/L.
- Statistical analysis: EC50 values and 95% confidence limits were calculated according to the method of Thompson and Weil (Thompson, W.R. & Weil, C.S., 1952, Biometrics 8; 51 - 54).

Conclusion

- The 48h-EC50 (immobilisation) value for 2-phenylhexanenitrile with *Daphnia magna* was 1.6 mg/L.

4.3.3 Algal growth inhibition tests

Title: Salicynalva (2-phenylhexanenitrile) algal growth inhibition

Author: Bell, G; Thirkettle, KM and Smith, B

Year: 1996

Reliability: Klimisch 1 (reliable without restriction).

Test type

A study was performed to assess the inhibitory effect of 2-phenylhexanenitrile on the growth of the unicellular green alga *Selenastrum capricornutum* (new name: *Pseudokirchneriella subcapitata*) according to EEC Methods for Determination of Ecotoxicity Annex to Directive 92/69/EEC (O.J. No. L383A, 29.12.92) Part C, Method 3 "Algal inhibition test" and the OECD Guideline for Testing of Chemicals No. 201 "Alga, Growth Inhibition Test" and in compliance with GLP.

Test substance

- The test material used in the study is equivalent to the substance identified in the CLH dossier.
- Name of test material (as cited in study report): Salicynalva

- Chemical name: 2-Phenylhexanenitrile
- Physical state: Light yellow liquid
- Analytical purity: > 95%
- Lot/batch No.: 6162H
- Expiration date of the lot/batch: 1 October 1995
- Storage condition of test material: In darkness at room temperature

Materials and methods

Test organism
- Species: *Selenastrum capricornutum* (new name: *Pseudokirchneriella subcapitata*)
- Source: Culture Centre of Algae & Protozoa c/o Freshwater Biological Association, Cumbria, UK.

Pre-culture
- Sterile nutrient medium was inoculated from a master culture and incubated under continuous illumination (= 7000 lux) and stirring (orbital shaker) at 23 ± 2°C to give an algal suspension in log phase growth characterised by a cell density of 5.21+E06 cells per mL. The suspension was diluted using sterile nutrient medium to a cell density of 4.2+E04 cells per mL prior to use.

Preparation of test solutions
- The test substance (purity >95%) was dissolved in the auxiliary solvent Tween : acetone (20:80 v/v) to give an initial stock solution of 100 mg/mL. The test solutions were prepared by serial dilutions of this stock and the solvent control contained 100 µL solvent per liter.

Study design
- Test type: static
- Water media type: Freshwater
- Total exposure duration: 72 hours

Test system
- Six test concentrations plus one untreated control and one solvent control (100 µl auxiliary solvent per litre), each in triplicate. Nominal test concentrations: 0.22, 0.46, 1.0, 2.2, 4.6 and 10 mg 2-phenylhexanenitrile/L.

The test concentrations were verified by chemical analysis at 0 and 72 hours. Geometric means of the measured concentrations at 0 and 72 hours were used in the calculations of the effect concentrations. The mean measured concentrations were: 0.10, 0.26, 0.64, 1.8, 3.5 and 5.1 mg 2-phenylhexanenitrile/L.

Test medium
- Sterile nutrient medium as recommended in Official Journal No. L383A Part C.3

Four stock solutions (i.e. 1 - macro-nutrients; 2- Fe-EDTA; 3 - trace elements and 4 - NaHCO3 solutions) were prepared using reverse osmosis purified water. Stock solutions are sterilised by autoclaving (solutions 1-3) or by membrane filtration (solution 4) before being stored at +4°C in the

dark. Aliquots of stock solutions 1-3 are further diluted with reverse osmosis purified water and autoclaved again to produce the working strength nutrient medium. Prior to use, an aliquot of stock solution 4 is added aseptically to the medium via a membrane filter. The pH of the medium after equilibration with air is approximately 8.

Other test conditions
- Conical flasks (250 mL) each containing 100 mL of test or control culture were sealed with nescofilm and placed at random in a Gallenkamp Illuminated Orbital Incubator. The cultures were incubated, without media renewal, for 72 hours under continuous illumination of approximately 7000 lux provided by 7 x 30 W "universal white" 1 metre fluorescent tubes. The temperature was maintained at 24 ± 1°C and gaseous exchange and suspension of the algal cells was ensured by the action of the orbital shaker oscillating at 120 cycles per minute.

Measurement of growth
- Samples were taken at 0, 24, 48 and 72 hours and the cell numbers determined by direct counting using a Coulter® MuItisizer II particle counter .

Identification and quantification of test substance
- High Performance Liquid Chromatography (HPLC) with UV detection. The analytical method was proven satisfactory during the validation phase of the study with regard to accuracy and precision with mean recoveries of 108%; coefficient of variation 2.5 %. The method was specific for 2-phenylhexanenitrile, there being no peak present at the characteristic retention time of 2-phenylhexanenitrile in control sample chromatography. The limit of detection for the study was calculated to be 0.006 mg 2-phenylhexanenitrile/L. Procedural recovery samples analysed concurrently with the test samples were used for the assessment of method performance. The mean procedural recovery value during this phase was 98.6 %; coefficient of variation 6.1%.

Results
- Mean measured test concentrations (geometric mean of the measured concentrations at 0 and 72 hours): 0.10, 0.26, 0.64, 1.8, 3.5 and 5.1 mg 2-phenylhexanenitrile/L (see Table 4 for details). Values ranged from 82 - 101 % of nominal at 0 hours, apart from the lowest exposure level which was 57% of nominal, and 30 - 65 % of nominal at 72 hours. Test concentrations decreased in the course of this study which is attributed to adsorption both to the algae and to the glassware or volatilisation, since losses were observed in all treatments including that where no algae was present, but in these flasks the losses were only ca. 40 % of that observed in the flasks containing the algae.

Table 4. Measured test concentrations of 2-phenylhexanenitrile at 0 and 72 hours.

Nominal test concentration (mg/L)	Measured test concentration			
	0 h		72 h	
	mg/L	% of nominal	mg/L	% of nominal
0.22	0.1242	56.5	0.07814	35.5

0.46	0.3763	81.8	0.1865	40.5
1	0.916	91.6	0.4414	44.1
2.2	2.232	101	1.4267	64.9
4.6	4.233	92	2.84	61.7
10	8.547	85.5	3.006	30.1

The mean growth rates, cell density and inhibition of growth of the algae per test concentration during the test are given in Table 5 and in Figures 1 and 2, respectively. The following results were found based on mean measured concentrations: EbC50 (72 h): 0.81 mg/L (95% confidence limits: 0.61 - 1.07 mg/L); ErC50 (72 h): 2.58 mg/L (95% confidence limits: 2.44 - 2.74 mg/L) and NOErC (72 h): 0.26 mg/L.

All test and control cultures were inspected microscopically at 72 hours. At the 0.64 mg/L exposure level all cultures contained turgid cells, and at test concentrations of 3.5 and 5.1 mg/L all cultures showed evidence of cell debris with only a few small cells remaining intact.

Table 5. Mean growth rates of algae per test concentration of 2-phenylhexanenitrile during the test.

Test concentration (mean measured) (mg/L)	**Growth rate (day^{-1}) (mean ± SD)**			
	0-24h	**24-48h**	**48-72h**	**0-72h**
Control	1.29 ± 0.03	1.58 ± 0.07	1.32 ± 0.05	1.40 ± 0.01
Solvent control	1.08 ± 0.04	1.68 ± 0.06	1.26 ± 0.03	1.34 ± 0.01
0.1	1.08 ± 0.04	1.73 ± 0.01	1.24 ± 0.01	1.35 ± 0.01
0.26	1.05 ± 0.00	1.77 ± 0.08	1.10 ± 0.08	1.30 ± 0.00
0.64	0.34 ± 0.01	0.93 ± 0.01	2.30 ± 0.00	1.19 ± 0.00
1.8	0.33 ± 0.02	0.37 ± 0.10	2.65 ± 0.09	1.12 ± 0.02
3.5	0.32 ± 0.01	0.19 ± 0.04	0.18 ± 0.06	0.23 ± 0.01
5.1	0.039 ± 0.01	0.81 ± 0.23	-0.73 ± 0.29	0.04 ± 0.02

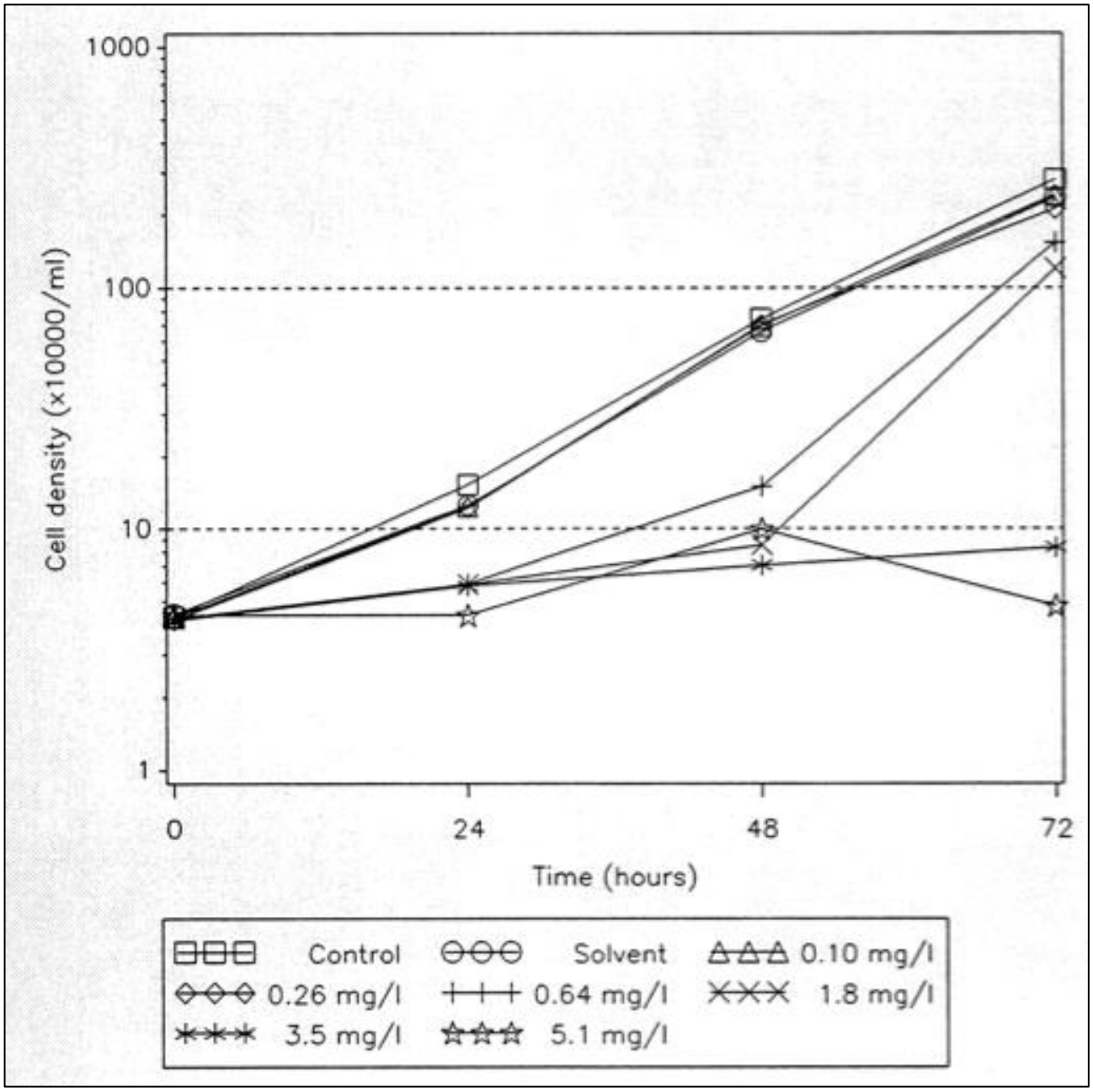

Figure 1. Mean cell density of algae per test concentration of 2-phenylhexanenitrile during the test.

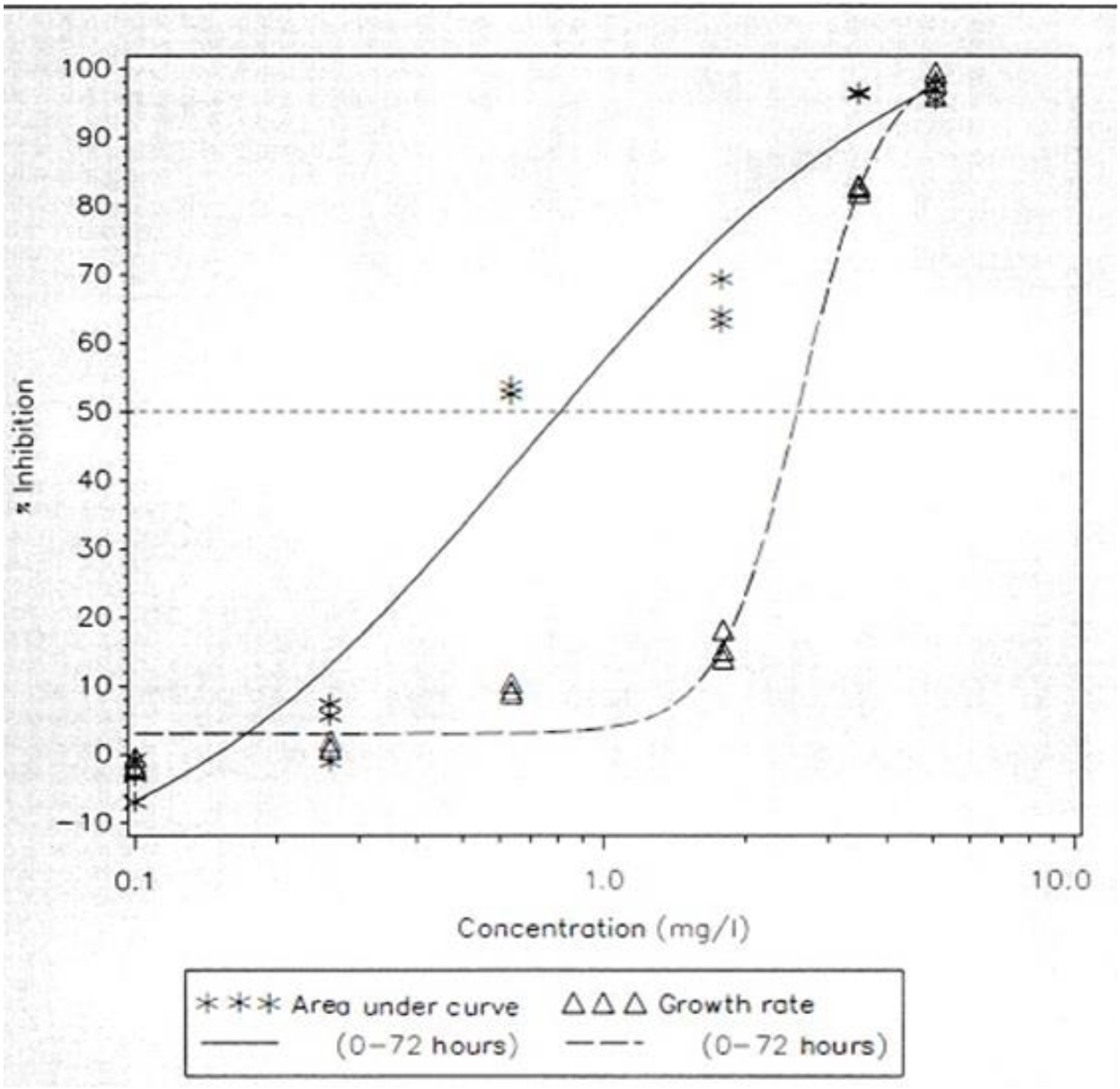

Figure 2. Inhibition of algae growth by 2-phenylhexanenitrile (0 – 72 h).

- In the control groups the biomass increased exponentially by a factor of at least 16 within the 72-hour test period.

- Environmental parameters seems to remain within acceptable limits throughout the duration of the study: pH (mean of replicates): approx. 8-9 (after equilibration with air) and temperature (mean of replicates): 24 ± 1°C.

- Statistical analysis: Percentage reductions in growth rate and the ErC50 value are calculated as for the "area under the curve" data. The ErC50 ("x "- "y "h) is the median effective concentration for inhibition of growth based on a comparison of growth rates from "x " to "y" hours. The "no-observed effect level " (NOEL) was obtained using Williams' test to compare the percentage inhibition in each treated group with that for the control/solvent control cultures (Williams' D.A., 1971/72, Biometrics 27; 103 - 117 and 28; 519 - 531).

Conclusion

- The ErC50 (72 h) of 2-phenylhexanenitrile for algae is 2.58 mg/L and the NOErC (72 h) is 0.26 mg/L.

4.3.4 Lemna sp. growth inhibition test

No data available.

4.4 Chronic toxicity

4.4.1 Fish early-life stage (FELS) toxicity test

No data available.

4.4.2 Fish short term toxicity test on embryo and sac-fry stages

No data available.

4.4.3 Aquatic Toxicity – Fish, juvenile growth test

No data available.

4.4.4 Chronic toxicity to aquatic invertebrates

No data available.

4.4.5 Chronic toxicity to algae or other aquatic plants

A 72h NOErC value of 0.26 mg/L was obtained for 2-phenylhexanenitrile in an algal growth inhibition test with *Pseudokirchneriella subcapitata* (see section 4.3.3 above).

4.4.6 Acute and/or chronic toxicity to other aquatic organisms

No data available.

4.5 ADDITIONAL HAZARDS

4.5.1 Hazardous to the ozone layer

No data available.