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TNO Report

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Determination of the effect of p-xylene (CAS#
106-42 3) on the growth of the fresh water green
alga *Selenastrum capricornutum*.
(Guidelines: OECD 201 and EU C.3)

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Summary

The toxicity of p-xylene to the fresh water green alga *Selenastrum capricornutum* was determined in a 72h growth inhibition test according to the OECD 201 and EU C.3 Guidelines, and in compliance with the OECD principles of Good Laboratory Practice.

p-Xylene is a clear liquid with a stated solubility in water of approximately 150 mg.l⁻¹. Stock solutions of the test substance were prepared by stirring an appropriate volume of p-xylene in algal medium overnight in an air tight bottle.

A standard algal growth inhibition test according to the Guidelines [1,2] was not suitable for the testing of this (expected) volatile substance, as the open test design would lead to substantial evaporative losses. Therefore, modifications according to Mayer et al. [7] were made to allow the testing in closed filled test vials.

A range finding experiment was performed to determine the concentrations for the final test. In the final test the algal cultures were incubated at a temperature of 23 °C and a light intensity of 76 – 103 μmol.s⁻¹.m⁻² for 73 hours. The algal cell density was determined after 0, 25.5, 49 and 73 hours with an electronic particle counter.

The test included analytical determination of all test substance concentrations at the start and the end of the test; the latter determination was carried out in test media containing algae.

With regard to the analysis of the test substance concentration, the following results were obtained:

- The mean measured p-xylene concentrations at the start of the test were: nd (not detectable in the controls) and 0.19, 0.47, 0.81, 1.68 and 8.01 mg.l⁻¹, respectively.
- The mean measured p-xylene concentrations at the end of the test were: nd (controls) and 0.14, 0.39, 0.56, 0.98 and 3.92 mg.l⁻¹, respectively.

The toxic effect values given, Table 1, were calculated using the geometric mean of the measured concentrations of p-xylene at the start and end of the test.

Table 1 Results of the 73-h growth inhibition test of p-xylene, calculated from the geometric mean of the measured concentrations at the start and the end of the test with *Selenastrum capricornutum* are shown in the table below.

Parameter	Dimension	Value (95% confidence limit)
NOEC	mg.l ⁻¹	0.44
NEC	mg.l ⁻¹	1.30 (1.23 – 1.36)
E _r C10	mg.l ⁻¹	1.9
E _r C50	mg.l ⁻¹	4.36 (4.06 – 4.67)
E _r C90	mg.l ⁻¹	10
E _b C10	mg.l ⁻¹	0.72 (0.44 – 0.81) ^{a)}
E _b C50	mg.l ⁻¹	2.2 (1.4 – 4.6) ^{a)}
E _b C90	mg.l ⁻¹	4.4 (1.4 – 4.6) ^{a)}

a) range between tested concentrations

NOEC = Estimated no observed effect

NEC = No Effect concentration

E_rC values = Effect concentration with regard to the growth

E_bC values = Effect concentration with regard to area under the growth curves

The statistical endpoints included in the model calculation, demonstrated a significant effect on the growth rate, and little or no effect on the growth (expressed as the area under the growth curve). The difference between the E_rC50 and the E_bC50 value therefore has no toxicological relevance.

The E_rC50 value of 4.4 mg·l⁻¹ is considered to provide the best expression of toxicity of p-xylene to algae.

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Confidentiality statement

CONFIDENTIAL. This report contains confidential and proprietary information of CEFIC Aromatics Producers Association which must not be disclosed to anyone except the employees of CEFIC Aromatics Producers Association, without the express and written approval of CEFIC Aromatics Producers Association.

Statement of GLP Compliance

I, the undersigned, hereby declare that this report constitutes a complete, true and accurate representation of the study and its results. All study activities performed by TNO Nutrition and Food Research were carried out in compliance with the current OECD Principles of Good Laboratory Practice (Organisation for Economic Co-operation and Development, Paris, ENV/MC/CHEM (98) 17).

TNO makes no GLP compliance claim for characterisation and verification of the test substance identity and properties.

Drs. A.O. Hanstveit
Study Director

Approved by:

A.F.M. Kardinaal, Ph.D
Management
Physiological Sciences Department

Quality Assurance Statement

Report title : <report title>

Report number : <report number>

Report date : <date in letters>

The study plan was inspected as follows:

Date of inspection
<date in letters>

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<date in letters>

The experimental phase of the study was inspected as follows:

Date of inspection
<date in letters>

Date of report
<date in letters>

This report was audited as follows:

Date of audit
<date in letters>

Date of report
<date in letters>

I, the undersigned, hereby declare that this report provides an accurate record of the procedures employed and the results obtained in this study; all inspections were reported to the Study Director and to laboratory management on the dates indicated.

Dr G.S. Oostenbrug
Quality Assurance Auditor
TNO Nutrition and Food Research

Date:

1 Introduction

1.1 Background

The toxicity of the product p-xylene to the fresh water green alga *Selenastrum capricornutum* was determined at the request of the sponsor.

1.2 Objective

The objective of the study was to determine the effect of p-xylene on the growth and growth rate of the algal species *Selenastrum. capricornutum* in a 72h (approx.) algal growth inhibition test according to the OECD No. 201 Guideline [1] and EU C.3 Guideline [2].

1.3 Justification of the test system

An algal growth inhibition test is specified by the relevant regulations for obtaining data for the hazard and risk assessment of new and existing chemicals, biocides and pesticides. The chosen species is the most widely used of the recommended algal species.

1.4 Guidelines

The determination was essentially conducted in accordance with the OECD 201 and EU C.3 Guidelines [1,2].

1.5 Comments on the test design

A standard algal growth inhibition test according to the Guidelines (see 1.4), is not suitable for the testing of a highly volatile substance, as the open test design would lead to substantial evaporative losses. Therefore, modifications according to Mayer et al. [7] has been made to allow the testing in closed filled test vials. A reduced initial algal density (10^3 cells·ml⁻¹), an increased NaHCO₃ concentration (300 mg·l⁻¹) were applied to avoid substantial pH changes or carbon limited growth in the closed test vials. These modifications were made to allow a substantial increase in algal biomass during the test to meet the acceptance criteria of an algal biomass increase by at least 16 times during the test of 72 hours.

1.6 Analytical monitoring

The test included analytical determination of test substance concentrations at the start and the end of the test.

1.7 Quality Standard

The study was carried out according to the OECD principles of Good Laboratory Practice [3].

1.8 Relevant dates

Study plan signed by the Study Director	:	3 June 2004
Period of range-finding test	:	2 – 5 July 2004
Period of growth inhibition test	:	6 – 9 July 2004
Period of chemical analysis	:	9 July 2004

2 Materials and methods

2.1 Test substance

The following test substance was examined:

Name	:	p-xylene
Systematic name	:	1,4-dimethylbenzene
Physical appearance	:	colourless transparent liquid
Molecular formula	:	$C_6H_4(CH_3)_2$
CAS Reg. No.	:	106-42-3
Lot no.	:	429739/1
Purity	:	$\geq 99\%$ (GC)
Impurities	:	toluene $\leq 0.5\%$ o- and m-xylene $\leq 0.5\%$
Vapour pressure	:	11.5 hPa at 25 °C
Log Pow	:	3.15
Density	:	0.86 g.cm ⁻³ at 20 °C
Solubility in water	:	156 mg.l ⁻¹
Expiration date	:	27 May 2005
Storage temperature	:	room temperature
Protection from light	:	no
TNO test substance number	:	040085

Receipt of test substance

Date	:	19 May 2004
Quantity	:	1 litre
Source	:	Sigma (Fluka)

The details above have been taken from the IUCLID file and the Fluka catalog.

2.2 Testing facilities

The study was carried out by the Physiological Sciences Department of TNO Nutrition and Food Research. The chemical analysis of the test media were carried out by the Analytical Sciences Department of TNO Nutrition and Food Research. Both laboratories are located at:

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Utrechtseweg 48
3704 HE ZEIST
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2.3 Contributing personnel

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Technician
Physiological Sciences Department

Mr. A. Schouten
Responsible for analyses

Mr. R. Engel
Technician
Analytical Sciences Department.

2.4 Test organism

The fresh-water green alga *Selenastrum capricornutum* (CCAP 278/4)¹⁾, which belongs to the order of *Chlorococcales* (class *Chlorophyceae*), was used as the test organism. The culture was supplied by the CCAP, The Freshwater Biological Association, the Ferry House, Far Sawrey, Ambleside, Cumbria LA22 OLP, England. This organism is the preferred species for regulatory testing.

A pre-culture of algae in the exponential growth phase was prepared as detailed in OECD Guideline No. 201 [1], using the medium described in 2.5.

2.5 Test medium

The medium was prepared from concentrated stock solutions in ultra pure water (Annex A). It was sterilized by micropore filtration and contained 300 mg.l⁻¹ NaHCO₃ (not 50 mg.l⁻¹ as specified in the OECD Guideline [1], this in order to improve the buffer capacity of the medium). Furthermore, the medium contained Fe-citrate, because the growth of the algae can become erratic in the absence of complexed iron.

¹⁾ The taxonomical status of this algal species has changed and is referred to as *Pseudokirchneriella subcapitata*.

3 Test methods

3.1 Preparation of test solutions

The following solutions of the test substance were made:

- For the range-finding test a stock was prepared based on the solubility in pure water (see 2.1). An amount of 109 μl p-xylene was added to 600.66 ml algal medium (this equals $181 \mu\text{l}\cdot\text{l}^{-1}$) which completely filled a bottle with a magnetic stirrer bar. With a density of $0.86 \text{ g}\cdot\text{cm}^3$, $181 \mu\text{l}\cdot\text{l}^{-1}$ p-xylene equals $156 \text{ mg}\cdot\text{l}^{-1}$. Then the bottle was tightly closed and the contents stirred during 18.5 hours. Dilutions were then prepared in medium so as to yield a nominal test substance concentration series of 0.3, 0.99, 3.0, 9.9 and $99 \text{ mg}\cdot\text{l}^{-1}$.
- For the growth inhibition test, an amount of 13 μl p-xylene was added to 1103 g algal medium which completely filled a bottle with a magnetic stirrer bar. Then the bottle was tightly closed and stirred for approximately 18 hours. The expected p-xylene concentration was $10 \text{ mg}\cdot\text{l}^{-1}$ based on a density of $0.86 \text{ g}\cdot\text{cm}^3$. From the clear stock dilutions were prepared in medium so as to yield a nominal test substance concentration series of 0.32, 1.0, 1.8, 3.2 and $10 \text{ mg}\cdot\text{l}^{-1}$.

3.2 Range-finding test and general test conditions

The range-finding test was conducted as a simplified version of OECD Guideline No. 201 [1].

Suitable numbers of 40 ml EPA vials with a Teflon® lined septum screw cap were coded.

A suspension of algae in the algal medium containing $10^5 \text{ cells}\cdot\text{ml}^{-1}$ was prepared by dilution of a pre-culture (section 2.4) containing $4.61 \times 10^5 \text{ cells}\cdot\text{ml}^{-1}$. A 0.4 ml volume of this algal suspension was added to 40 ml of the appropriate solutions of the test substance (section 3.1) in the test flasks, yielding a nominal initial cell density of $10^3 \text{ cells}\cdot\text{ml}^{-1}$. Each test vial was tightly closed after addition of the algae.

The test was set up in duplicate with four controls containing only algae. Flasks were incubated at $23 \pm 2 \text{ }^\circ\text{C}$. Algal cells were kept in suspension by rolling the flask horizontally, achieved by shaking in a Gallenkamp orbital shaker at approximately 100 rpm. The light intensity radiated by the fluorescent lamps was within the standard range of $60\text{-}120 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$. The light intensity was measured at 2 positions in the incubator with a Bottemanne Weather Instruments Photosynthetic Radiometer RA200 Q. After 3 days of incubation, algal densities ($\text{cells}\cdot\text{ml}^{-1}$) and algal biovolume ($\mu\text{m}^3\cdot\text{ml}^{-1}$) were determined with the electronic particle counter (Coulter Multisizer IIe).

3.3 Growth inhibition test

The growth inhibition test was conducted as detailed in the study plan. This study plan was developed on the basis of OECD 201 and EU C.3 Guidelines [1,2], using selected incubation conditions given in the International Standard ISO 8692 [4].

Test flasks, test solutions and algal medium were prepared as detailed in sections 3.1 and 3.2.

A suspension of algae in the algal medium containing 10^5 cells.ml⁻¹ was prepared by dilution of a pre-culture (section 2.3) containing 11.63×10^5 cells.ml⁻¹. A volume of 0.4 ml of this algal suspension was added to 40 ml of the appropriate solutions of the test substance (section 3.1) in the test flasks, yielding a nominal initial cell density of 10^3 cells.ml⁻¹. Each test vial was tightly closed after addition of the algae. At each sampling time three vials per test substance concentration will be sacrificed for measurements. Additional duplicate flasks with (t=3 days) and without (t=0 days) algae were prepared to allow chemical analysis.

The nominal test substance concentration series tested was 0.32, 1.0, 1.8, 3.2 and 9.9 mg.l⁻¹ after addition of algal suspension.

The test was carried out in triplicate with six controls containing algae only.

All flasks were incubated as described in section 3.2.

The initial density was calculated from the measured density in the algal pre-culture used for the inoculation, divided by the appropriate dilution factor.

Algal densities (cells.ml⁻¹) and algal biovolume (μm^3 .ml⁻¹) were determined after 25.5, 49 and 73 hours (see section 3.2). The mean values were used for further calculations.

The pH was measured at the start (medium without algae) and after 73 h in all cultures. The morphology of the algae was examined visually with the aid of a microscope at the start and end of the test. The light intensity at two different culture positions was measured at the start of the test.

3.4 Calculation of the EC values

The effect of a test substance on the growth of algae can be expressed by quantities denoted as the EC10, EC50 and EC90 (EC = Effect Concentration), i.e. the concentration of test substance that reduces the growth rate, the viability of the inoculum of algae by 10, 50 and 90% respectively.

In this study the EC values with respect to the growth rate and exponential growth (E_rC values), were calculated by means of a parametric model developed by Kooijman *et al.* [6] assuming an error proportional to the number of cells; a summary of the method is given in Annex B.

This calculation method is based on the assumptions made in the OECD Guideline 201. It has been used in ring tests of algal growth inhibition test Guidelines [8,9]. These ring tests have demonstrated that E_rC50 values calculated by this method are identical to those calculated by the method given in the Guidelines [9].

EC values with respect to the area under the growth curve (E_bC values) were calculated by the method given in the OECD Guideline [1]. The values were calculated by linear interpolation of a plot of the percentage reduction in growth (I_A) against the log concentration of the test substance.

3.5 Determination of the NOEC and NEC values

3.5.1 NOEC

The average specific growth rate (μ) was determined according to the OECD Guideline [1]. The NOEC (no-observed-effect concentration) was determined as the highest concentration at which no statistically significant inhibition of μ , with respect to the

control μ , was observed. Statistical significance was determined with a one tailed t-test ($\alpha=5\%$).

3.5.2 *NEC*

In addition, model calculations were carried out using the DEBtox software package according to the Dynamic Energy Budgets Theory developed by Kooijman and Bedaux [10]. Model parameters for population growth and their asymptotic standard deviation and correlation coefficients were estimated. The NEC (no-effect concentration), was calculated from the Profile Ln Likelihood function [10,11]. This method offers a completely different basis for calculating No Effect and offers complementary information to the traditional statistical NOEC or EC10/20 approach.

4 Chemical analysis

4.1 Sampling procedure

At the start of the growth inhibition test, duplicate 40 ml samples for analysis were prepared from all solutions containing nominal test substance concentrations of 0, 0.32, 1.0, 1.8, 3.2 and 10 mg·l⁻¹. Sample solutions without algae were acidified with 0.4 ml 1 M HNO₃, tightly closed and stored in a refrigerator pending analyses. The samples of stock solution were placed in 100 ml brown glass and 40 ml EPA clear vials.

At the end of the test, duplicate 40 ml samples of all test substance concentrations, in presence of algae, were taken, acidified and transferred to the analytical department.

4.2 Analytical procedure

The analytical procedure is described in Annex E.

5 Results and discussion

5.1 Range-finding test

Most test vials were opened and the content was lost, caused by a high shaking speed of the roller platform. However, for each concentration, including the blank without test substance, one vial could be retrieved to allow an estimation of the concentration-effect curve. The results of the cell counts in the various suspensions are listed in Annex C, Table C1.

The range-finding test revealed that inhibiting effects could be expected at nominal test substance concentrations $\geq 1 \text{ mg}\cdot\text{l}^{-1}$.

5.2 Growth inhibition test

5.2.1 Chemical analysis

The results of the chemical analyses are included in Annex E. In the range finding test the highest test substance concentration of $64.2 \text{ mg}\cdot\text{l}^{-1}$ in algae medium was measured in the stock solution. In the growth inhibition test a stock solution with a nominal p-xylene concentration of $10 \text{ mg}\cdot\text{l}^{-1}$ was prepared in the same way and resulted in an actual concentration of $8.0 \text{ mg}\cdot\text{l}^{-1}$. During the preparation of the stock solution, some of the p-xylene may have been lost probably due to evaporation. A summary of the results of the chemical analysis of the test substance solutions are given in Table 2. The measured concentrations were found to be 45.2 – 80.1% of the nominal concentrations at the start of the test and 30.7 – 42.3% of the nominal concentrations at the end of the test and they show a linear relationship with the nominal concentrations. As the measured concentrations were generally less than 80% of the nominal concentrations and decreased during the test, the effect values were expressed relative to exposure concentrations [5] calculated according the procedure described in annex B. No indication of transformation of the test substance was observed.

Table 2 Results of the analysis of the concentration of p-xylene in the algal test media without alga (mean values of duplicate measurements, corrected for 1% acidification)

Nominal concentration p-xylene ($\text{mg}\cdot\text{l}^{-1}$)	Concentration p-xylene ($\text{mg}\cdot\text{l}^{-1}$)				Exposure
	0 h	% of nominal	73 h	% of nominal	
0	<0.1		<0.1		0
0.32	0.19	60.3	0.14	42.3	0.14
1	0.47	46.6	0.39	38.9	0.44
1.8	0.81	45.2	0.56	30.8	0.81
3.2	1.68	52.5	0.98	30.7	1.45
10	8.01	80.1	3.92	39.2	4.65

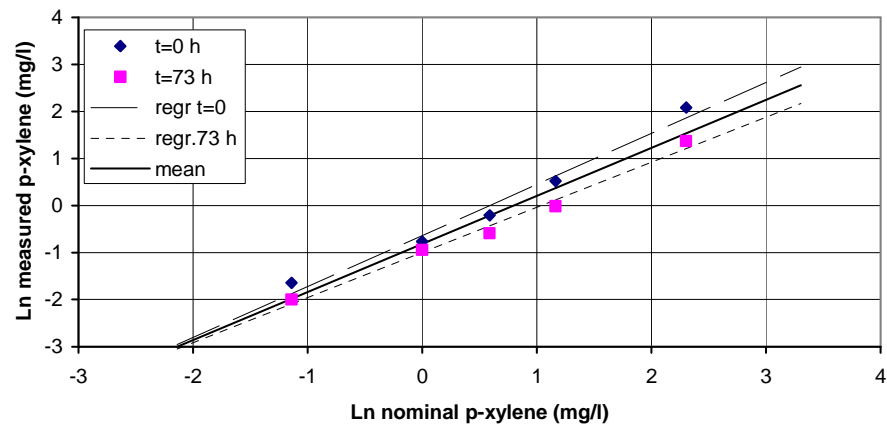


Figure 1 - Illustration of the measured p-xylene concentrations versus nominal p-xylene concentrations and the calculated geometric mean concentrations(exposure).

5.2.2 Test conditions

The temperature and the light intensity was measured to be 21.7 °C – 23.0 °C (average 22.2°C) and 76 – 103 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$, both being within the limits mentioned in the study plan.

At the start of the test the pH of the algal medium was measured to be 8.0. At the end of the test the pH of the test media varied between pH8.5 and 9.2, except for the highest test substance concentration where the algal growth was nearly completely inhibited and the pH value remained at pH 7.8 – 7.9.

5.2.3 Particle measurements

Algal density measurements (corrected for the background particle counts) in the various suspensions are shown in Annex D, Tables D1-D3. The mean values are given in Annex D, Tables D4.

5.2.4 EC values

The parametric model tested to fit the data assumes an effect on the growth rate and exponential (E_rC values). The results of the model fit are given in Annex D, Table D5. The values shown in Table D5 demonstrate that algal densities could be used for the calculation therefore the biovolumes were not used.

The growth curves for the various concentrations of the test substance are shown in Figure 2, and the concentration-effect curves in Figure 3. The data points shown in these figures represent the mean of the algal density in each of the flasks containing one concentration of test substance. The curves were obtained by parametric model calculations.

The EC50 with respect to growth rate (E_rC_{50}) was found to be 4.36 $\text{mg}\cdot\text{l}^{-1}$ with a 95% confidence interval of 4.06 through 4.67 $\text{mg}\cdot\text{l}^{-1}$.

The effect values, and the growth parameters calculated from the data, are given in Table 3.

Table 3 The model parameters calculated from the results of the growth inhibition test and the results of calculations of the area under the growth curves with p-xylene and *Selenastrum capricornutum*

Parameter	Dimension	Value (95% confidence limit)
inoculum	$10^3 \text{ cells.ml}^{-1}$	1.0 (0.91 - 1.1)
growth rate	h^{-1}	0.084 (0.082 – 0.087)
gradient	dimensionless	2.6 (1.8 – 3.4)
E_rC_{10}	mg.l^{-1}	1.9
E_rC_{50}	mg.l^{-1}	4.36 (4.06 – 4.67)
E_rC_{90}	mg.l^{-1}	10
E_bC_{10}	mg.l^{-1}	0.72 (0.44 – 0.81) ^{a)}
E_bC_{50}	mg.l^{-1}	2.2 (1.4 – 4.6) ^{a)}
E_bC_{90}	mg.l^{-1}	4.4 (1.4 – 4.6) ^{a)}

a) range between tested concentrations

The area under the growth curves was calculated using the mean values given in Annex D, Table D4 and the results are given in Annex D, Table D6.

The E_bC values shown in Table 3 have been derived from the concentration-effect curve by linear interpolation. The E_bC_{50} value is always lower than the E_rC_{50} value, the latter being a biomass independent effect. Such a difference has also been observed in international ring tests [8,9].

5.2.5 *NOEC and NEC values*

The average specific growth rate (μ) and percentage inhibition was determined according to the OECD Guideline 201 [1] and is given in Annex D, Table D7. The no-observed-effect-concentration (NOEC) of p-xylene was found to be 0.44 mg.l^{-1} .

In order to obtain a better estimate of the NOEC value, the No-Effect-Concentration (NEC) was calculated with the method given in ref. [10,11].

The NEC was found to be 1.30 mg.l^{-1} , with a 95% confidence interval of 1.23 – 1.36 mg.l^{-1} . The difference between NEC and NOEC reflect the variation in cell counts.

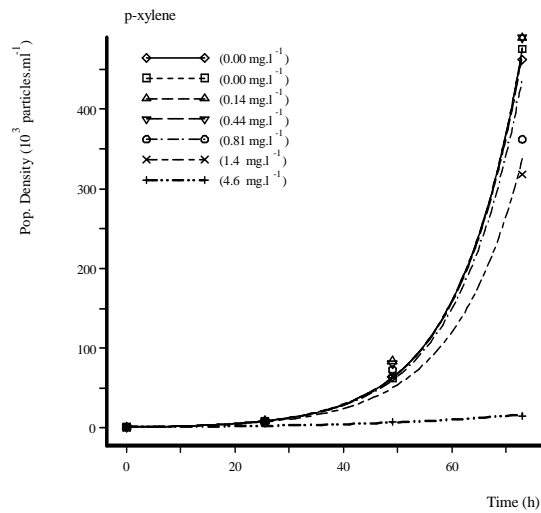


Figure 2 Growth curves of *Selenastrum capricornutum* at different concentrations of *p-xylene*.

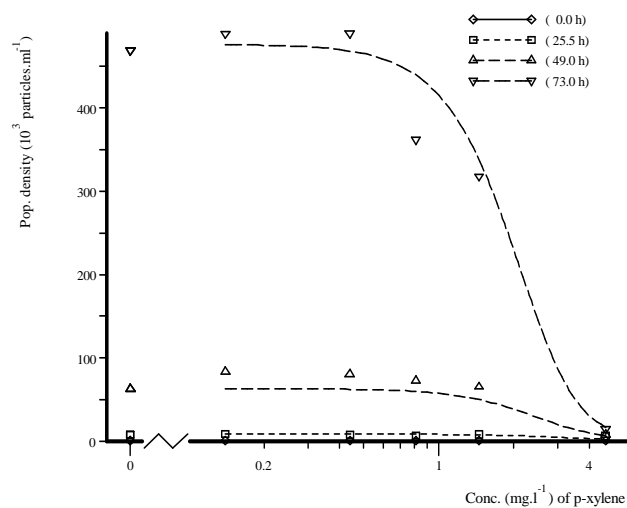


Figure 3 *p-Xylene* concentration-effect curves for *Selenastrum capricornutum*s after different incubation periods.

5.2.6 Control observations

Microscopic inspection of the morphology of algal cells in the preculture at the start of the test revealed normal cells. At the end of the test no abnormal cells were observed in the cultures containing different concentrations of *p-xylene*. No algae were found in the highest test substance concentration.

5.3 Validity criteria

The OECD Guideline No. 201 [1] recognises one validity criterion and one quality criterion, i.e. a sufficient control growth rate, and a limited increase of the test medium pH value during the test (one pH unit), respectively.

The control growth rate (0.084 h^{-1}) is higher than the minimal cell multiplication factor of 16 during a three day test given in the Guideline (corresponding to a growth rate of 0.038 h^{-1}).

The maximum increase in the pH value is 1.2 units, which is slightly outside the range specified by the Guideline. However, since the test was carried out with closed test vials, an increase in the pH of the test medium by algal growth is expected and normal. It is not expected that p-xylene is dissociated by increasing test medium pH value and the pH increase is considered to be acceptable and not influencing the quality of the study.

5.4 Conclusion

The statistical endpoints included in the model calculation, demonstrated a significant effect on the growth rate, and little or no effect on the growth (expressed as the area under the growth curve). The difference between the E_rC50 and the E_bC50 value therefore has no toxicological relevance.

The E_rC50 value of $4.4 \text{ mg}\cdot\text{l}^{-1}$ is considered to provide the best expression of toxicity of p-xylene to algae.

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7 Retention of records and samples

Remaining test substance will be retained for at least six months after submission of the final report under the sample reference 040085; thereafter, the test substance will be disposed of. Analytical samples will be discarded after submission of the final report.

Raw data, the master copy of the final report and all other information relevant to the quality and integrity of the study will be retained in the archives of TNO Nutrition and Food Research for a period of at least 15 years after reporting of the study under the study reference 5819/01. After this period the sponsor will be contacted to decide on the fate of the data.

8 Deviations from the study plan

- Only one stock solution of p-xylene was prepared because the dilutions would need very low volumes which would increase the dosing error.
- The 40 ml samples for chemical analysis were acidified with 0.4 ml 1 M HNO₃ solution.

These deviations are considered not to have affected the results of this study.

Annex A Composition of algal medium

NH ₄ Cl	15	mg.l ⁻¹
MgCl ₂ .6H ₂ O	12	mg.l ⁻¹
CaCl ₂ .2H ₂ O	18	mg.l ⁻¹
MgSO ₄ .7H ₂ O	15	mg.l ⁻¹
KH ₂ PO ₄	1.6	mg.l ⁻¹
Fe citrate.3H ₂ O	80	μg.l ⁻¹
Na ₂ EDTA.2H ₂ O	100	μg.l ⁻¹
H ₃ BO ₃	185	μg.l ⁻¹
MnCl ₂ .4H ₂ O	415	μg.l ⁻¹
ZnSO ₄ .7H ₂ O	6.3	μg.l ⁻¹
CoCl ₂ .6H ₂ O	1.5	μg.l ⁻¹
CuSO ₄ .5H ₂ O	0.015	μg.l ⁻¹
Na ₂ MoO ₄ .2H ₂ O	7	μg.l ⁻¹
NaHCO ₃	300	mg.l ⁻¹

Hardness, mg equivalent CaCO₃.l⁻¹ :

$$= 2.497 \times [\text{Ca}] \text{ in mg.l}^{-1} + 4.118 \times [\text{Mg}] \text{ in mg.l}^{-1} = 24.2$$

according to Standard Methods for the examination of water and wastewater 1998, 20th edition. APHA. AWWA. WEF

Annex B Summary of the calculation method

Exposure concentrations

Exposure concentrations used for calculations were derived from the measured concentration as follows:

- 1 Graphs of the logarithm of the nominal concentration versus the logarithm of the measured concentrations at t = 0 and 72 hours were prepared.
- 2 Straight lines were fitted through the data points of the series t = 0 and 72 hours.
- 3 The regression coefficients and intercepts are averaged. The resulting equation is used to calculate the exposure concentrations from the nominal concentrations.

The exposure concentrations (in mg.l⁻¹), shown in table B1, were calculated by:

$$\ln(\text{exposure}) = 1.02 \times \ln(\text{nominal}) - 0.82$$

Table B1. Exposure concentrations

Nominal concentration p-xylene (mg.l ⁻¹)	Exposure concentration p-xylene (mg.l ⁻¹)
0	0
0.32	0.14
1.0	0.44
1.8	0.81
3.2	1.45
10	4.65

Model

The model assumes that:

1. The number of cells in each culture increases exponentially.
2. The growth rate or the number of actively growing cells in the inoculum decreases according to a logistic function of the natural logarithm of test substance concentration.

The following equations were used:

$$N(t,c) = E_b - E(c) + E(c) \exp \{tR(c)\}$$

or when no effect is expected on the inoculum, i.e. $E(c) = E_b$:

$$N(t,c) = E_b \exp \{tR(c)\}$$

or when no effect is expected on the growth rate, i.e. $R(c) = R_b$

$$N(t,c) = E_b - E(c) + E(c) \exp \{tR_b\}$$

where

$N(t,c)$ = number of cells.ml⁻¹ at time t and concentration c of the test substance

$E(c)$ = inoculum; number of cells.ml⁻¹ in the culture containing concentration c of the test substance at t = 0

$R(c)$ = the growth rate at concentration c of the test substance

R_b and E_b = growth rate and inoculum, respectively, of the untreated cells.

In addition,

$$R(c) = R_b [1 + \exp \{R_g (\ln c - R_e)\}]^{-1}$$

and

$$E(c) = E_b [1 + \exp \{E_g (\ln c - E_e)\}]^{-1}$$

where

R_e and E_e = natural logarithm of the respective EC50 values.

R_g and E_g = the gradient of the functions for, respectively, the growth rate and the inoculum

The parameters E_b , E_e , E_g , R_b , R_e , and R_g were calculated by a weighted least square fitting of the model to the results.

Calculations were performed by APL computer programs, on a Windows XP computer.

Annex C Results of the range-finding test

Table C1 Algal density measurement (10^3 cells \cdot ml⁻¹) after a three days incubation period. Shown are single values of recovered whole flasks.

	Nominal concentration of p-xylene (mg.l ⁻¹)					
	0	0.3	1.0	3.0	9.9	99
Algal density	201	210	316	125	10	5

Annex D Results of the growth-inhibition test

Table D1 First set of algal density measurements (10^3 cells .ml⁻¹).

Time (h)	Geometric mean of measured concentrations of p-xylene (mg.l ⁻¹)						
	0	0	0.14	0.44	0.81	1.45	4.65
0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
25.5	8.6	8.9	9.1	8.9	5.2	8.4	5.0
49.0	85.7	60.0	83.4	78.5	74.3	79.3	7.4
73.0	505.9	531.1	494.9	498.8	416.8	449.7	17.7

Table D2 Second set of algal density measurements (10^3 cells .ml⁻¹).

Time (h)	Geometric mean of measured concentrations of p-xylene (mg.l ⁻¹)						
	0	0	0.14	0.44	0.81	1.45	4.65
0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
25.5	8.3	8.4	8.6	6.9	8.0	8.3	5.3
49.0	32.4	39.3	83.4	79.1	73.1	67.3	8.1
73.0	409.9	479.7	478.5	509.1	399.5	137.3	13.3

Table D3 Third set of algal density measurements (10^3 cells .ml⁻¹).

Time (h)	Geometric mean of measured concentrations of p-xylene (mg.l ⁻¹)						
	0	0	0.14	0.44	0.81	1.45	4.65
0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
25.5	7.4	7.3	7.7	7.9	7.9	8.2	8.5
49.0	73.1	87.5	84.3	83.8	71.8	49.8	7.5
73.0	470.6	416.0	494.3	462.2	269.6	367.7	13.8

Table D4 Mean values of cell algal density measurements (10^3 cells .ml⁻¹).

Time (h)	Geometric mean of measured concentrations of p-xylene (mg.l ⁻¹)						
	0	0	0.14	0.44	0.81	1.45	4.65
0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
25.5	8.1	8.2	8.5	7.9	7.0	8.3	6.3
49.0	63.7	62.3	83.7	80.5	73.1	65.4	7.7
73.0	462.1	475.6	489.2	490.0	362.0	318.2	14.9

Table D5 Modelled algal density (10^3 cells.ml⁻¹).

Time (h)	Geometric mean of measured concentrations of p-xylene (mg.l ⁻¹)						
	0	0	0.14	0.44	0.81	1.45	4.65
0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
25.5	8.7	8.7	8.7	8.7	8.5	7.7	2.7
49.0	63.1	63.1	63.0	62.4	59.8	50.1	6.7
73.0	476.6	476.6	476.2	468.6	439.7	337.9	17.0

Table D6 The area under the growth curve (A) and the percentage reduction in growth (I_A).

Parameter	Geometric mean of measured concentrations of p-xylene (mg.l ⁻¹)						
	0	0	0.14	0.44	0.81	1.45	4.65
A	7197	7328	8006	7926	6190	5516	455
I _A	0	0	-10	-9	15	24	94

Table D7 Individual growth rates and % inhibition.

Geometric mean of measured concentrations of p-xylene (mg.l ⁻¹)	Average specific growth rate (μ.d ⁻¹)	% inhibition	P(T<=t) one-tail
0	2.05	-1.3	0.2391
0	1.98	2.1	
0	2.02	-0.1	
0	2.06	-2.1	
0	2.03	-0.4	
0	1.98	1.9	
0.14	2.04	-1.0	0.2434 (NOEC)
0.14	2.03	-0.4	
0.14	2.04	-0.9	
0.44	2.04	-1.1	0.0212
0.44	2.05	-1.4	
0.44	2.02	0.2	
0.81	1.98	1.8	0.0418
0.81	1.97	2.5	
0.81	1.84	8.9	
1.45	2.01	0.6	7.25·10 ⁻¹⁰
1.45	1.62	19.9	
1.45	1.94	3.9	
4.65	0.94	53.3	
4.65	0.85	57.8	
4.65	0.86	57.3	

Annex E Results of the chemical analysis of the test substance concentration

1. Introduction

This annex describes the chemical analysis of the samples from determination of the effect of p-xylene (CAS # 106-42-3) on the growth of the fresh water green alga *Selenastrum capricornutum*. (Guidelines: OECD 201 and EU C.3).

The objective of this part of the study was to validate the analytical method for the determination of p-xylene in the test mixture, followed by determination of the concentration of p-xylene in the samples taken during the study (study 5819-01).

2. Experimental

2.1 Reference substance

On 30 June 2004 the Analytical Sciences Department received a reference sample of the test substance. The reference material was stored at ambient temperature.

2.2 Study samples

Samples (approximately 40 ml in glass bottles) were taken at the testing laboratory. After receipt on 9 July 2004, the samples were processed immediately. After analysis the samples were stored in a refrigerator (2 – 10 °C).

2.3 Analysis

2.3.1 Principle

The concentration of p-xylene in test medium was determined using High Performance Liquid Chromatography (HPLC) with UV detection. Quantification of p-xylene was achieved by comparing the peak areas in the chromatograms of the study samples with those in the chromatograms of calibration solutions.

2.3.2 Validation criteria

The method was validated, by analyzing an appropriate standard solution three times, to meet the following criteria:

- Linearity: the correlation coefficient of the calibration graph should be greater than or equal to 0.996.
- Repeatability of the concentration: the relative standard deviation should be smaller than 10 % when a test calibration solution is analyzed three times.
- Selectivity: no peak should be found in a blank test medium with a retention time of 95% - 105% of that of the test substance. If the blank sample shows a peak with a retention time in the above range and the area of this peak is >5% of the lowest concentration level, the result for the sample containing test substance will be corrected for the level found in the blank.

2.3.3 *Sample preparation*

Prior to the analysis all samples were mixed manually and centrifuged for 3 min at 10000 rpm in closed 1.5 ml vials (Eppendorf). An aliquot of the clear sample solution was transferred into an HPLC vial and analyzed as described in section 2.3.5. Sample 5819-01-001 (expected concentration 156 mg p-xylene.l⁻¹) was diluted 10 x with demineralised water and analyzed as described in section 2.3.5.

2.3.4 *Preparation of the sample for the repeatability experiment*

A validation sample containing 2.105 mg p-xylene per litre demineralised water was prepared by the Analytical Sciences Department on 9 July 2004. A 0.50 ml aliquot of a stock solution with 526.26 mg p-xylene / 100 ml HPLC mobile phase that was prepared on 30 June 2004, was diluted with 4.50 ml HPLC mobile phase solution. This solution was diluted 250 times (20 µl p-xylene solution + 5 ml demineralised water). Validation samples, including a blank (sample 5819-01-002), were analyzed as described in section and 2.3.5.

2.3.5 *Chromatography*

The validation samples, study samples and calibration solutions, prepared as described in section 2.3.6, were analysed using HPLC.

The following chromatographic conditions were used:

Column	: Phenomenex Luna 5µ C18 (2), 150/4.6 mm
Mobile phase	: acetonitrile/water = 1/1 (v/v), degassed by sonication during 15 minutes
Injection volume	: 20 µl
Flow	: 1 ml/min
Column temperature	: 30 °C
Detection	: 220 nm
Integration	: ChromQuest PC 1000

2.3.6 *Calibration*

For the two stock solutions aliquots of approximately 520 mg p-xylene, accurately weighed at 0.01 mg, were dissolved in 100 ml of mobile phase. Calibration solutions were prepared by alternately diluting the stock solutions in demineralized water to obtain concentrations between 0.26 and 20.5 mg p-xylene.l⁻¹. By using two stocks weighing errors were excluded.

The calibration solutions were analyzed as described in section 2.3.5. A calibration graph from single injections of the standards was constructed by plotting the peak area against the p-xylene concentration. The concentration of p-xylene in the samples was calculated using the calibration graph.

3 Results

3.1 Validation of the analytical method

3.1.1 Linearity

The calibration coefficient was >0.996 and therefore the calibration graph was considered to be rectilinear. A typical calibration graph is presented in Figure E1.

3.1.2 Selectivity

No peak was found in a blank test medium extract with a retention time of 95 % - 105 % of that of the test substance.

3.1.3 Repeatability

The concentrations measured and the RSD determined in the repeatability experiment with the validation samples prepared on 9 July 2004 are shown in Table E1. The RSD in the concentration of *p*-xylene in the three validation samples was 1.45 %. This value met the validation criterion.

Table E1 Repeatability of the analysis of *p*-xylene as determined from the validation samples prepared and analysed on 9 July 2004.

Concentration prepared (mg.l ⁻¹)	Concentration measured (mg.l ⁻¹)
0	< LOD ¹
2.10	1.5852
2.10	1.5662
2.10	1.5399
Mean (n=3)	1.56
RSD (n=3)	1.45 %

The measured concentration was lower than expected. The HPLC vial with the test calibration solution used for the repeatability test was analysed earlier in the sequence. Most probably *p*-xylene was lost through the perforated closure. Further investigations were not performed as no impact on the outcome of the study was anticipated. All sample solutions were injected once.

¹ The LOQ of 0.1 mg·L⁻¹ was estimated using the area of the lowest calibration standard. The LOD of 0.05 mg·L⁻¹ was estimated using the chromatographic noise.

3.2 Analysis results

The results of the study are presented in Table E2. Sample 001 (identified with the last part of the sample code) represents the stock solution used in the range finding. Samples 002 through 013 represent the concentrations at the beginning of the test. Samples 014 through 025 represent the concentrations at the end of the test.

Table E2 Concentration of p-xylene in the study samples.

Sample code	Dose level p-xylene mg.l ⁻¹	Measured concentration p-xylene mg.l ⁻¹
5819-01-001	156	64.2
5819-01-002	0	<0.1
5819-01-003	0	<0.1
5819-01-004	0.32	0.19
5819-01-005	0.32	0.19
5819-01-006	1.0	0.46
5819-01-007	1.0	0.47
5819-01-008	1.8	0.78
5819-01-009	1.8	0.83
5819-01-010	3.2	1.39
5819-01-011	3.2	1.94
5819-01-012	10	7.82
5819-01-013	10	8.04
5819-01-014	0	<0.05
5819-01-015	0.32	0.13
5819-01-016	1.0	0.46
5819-01-017	1.8	0.53
5819-01-018	3.2	0.96
5819-01-019	10	3.64
5819-01-020	0	<0.1
5819-01-021	0.32	0.14
5819-01-022	1.0	0.31
5819-01-023	1.8	0.57
5819-01-024	3.2	0.99
5819-01-025	10	4.13

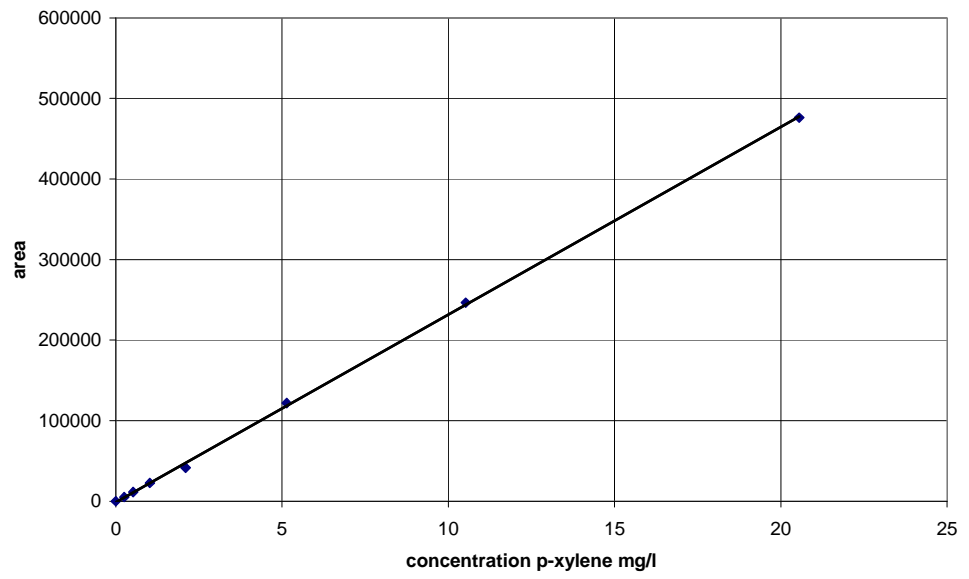


Figure E1 Calibration graph of *p*-xylene ($y = 23299x - 1247.5$ and correlation (r) 0.9998).

Annex F Endorsement of GLP compliance



voedsel en waren autoriteit

ENDORSEMENT OF COMPLIANCE

WITH THE OECD PRINCIPLES OF
GOOD LABORATORY PRACTICE

Pursuant to the Netherlands GLP Compliance Monitoring Programme and according to Directive 2004/9/EC the conformity with the OECD Principles of GLP was assessed on 7-11 June 2004 at

TNO Nutrition and Food Research
Utrechtseweg 48, P.O. Box 360
3700 AJ ZEIST

It is herewith confirmed that the afore-mentioned test facility is currently operating in compliance with the OECD Principles of Good Laboratory Practice in the following areas of expertise: Toxicity, mutagenicity, biodegradation, residues, analytical and clinical chemistry, kinetics and metabolism, and occupational toxicity.



The Hague, 19 August 2004

Dr Th. Helder

GLP Compliance Monitoring Department

Inspectorate for Health Protection and Veterinary Public Health
Food and Consumer Product Safety Authority