*TNO report*
V2360/01

# Determination of the effect of benzene on the growth of the fresh water green alga *Selenastrum capricornutum.* (OECD Guideline No. 201 and EU C.3)

TNO Nutrition and Food Research
Department of Environmental Toxicology

**Schoemakerstraat 97**
**P.O. Box 6011**
**2600 JA DELFT**
**The Netherlands**

Phone + 31 15 269 61 21
Fax + 31 15 257 26 49

Authors:
Dr P. Mayer
H. Oldersma
Drs A.O. Hanstveit

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Aromatics Producers Association
CEFIC European Chemical Industry Council
Av. E. van Nieuwenhuyse 4, bte 2
B-1160 Brussels
Belgium

Study monitor:
Mr Chr. Gilliard

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## Summary

The toxicity of benzene to the fresh water green alga *Selenastrum capricornutum* was determined in a 72h growth inhibition test according to the Guidelines OECD No. 201 and EU C.3, and in compliance with the OECD principles of Good Laboratory Practice. At the request of the sponsor, this test was conducted in closed flasks without headspace because benzene is a volatile liquid with an aqueous solubility of 1780 $mg.l^{-1}$ and an air-water distribution coefficient of 0.22 $l.l^{-1}$.

Test solutions of benzene were prepared by direct addition and subsequent serial dilution with algal medium. The test was carried out at reduced algal densities, at increased $NaHCO_3$ concentration and at a reduced algal medium pH in order to allow it to be carried out in closed flasks without headspace. Daily algal measurements were based on electronic particle counting, and daily measurements of benzene concentrations were based on liquid-liquid extraction and subsequent GC-MS analysis. At the request of the sponsor each flask was sampled only once, and was then sacrificed.

The test fulfilled the validity criterion of sufficient growth in control cultures.

The effect values were calculated using a statistic parametric model. The following parameters were calculated:

- NOEC : Estimated no-observed-effect concentration
- NEC : Calculated no-effect concentration
- ErC-values : Effect concentration with regard to the growth rate
- EbC-values : Effect concentration with regard to the area under the growth curves

Measured concentrations at the beginning of the test were between 56 to 73% of the nominal concentrations. This can be attributed to evaporative losses during the spiking and during the serial dilution of test solutions. During the test benzene concentrations remained stable between 83 and 100% of the measured concentration at the start of the test.

The following results were therefore based on the measured benzene concentrations at the start of the test.

| Parameter | Dimension | Value (95% confidence limit) |
|---|---|---|
| NOEC | $mg.l^{-1}$ | 8.0 |
| NEC | $mg.l^{-1}$ | 22.5 (20.0 – 25.6) |
| $E_rC10$ | $mg.l^{-1}$ | 34 |
| $E_rC50$ | $mg.l^{-1}$ | 100 (83 - 130) |
| $E_rC90$ | $mg.l^{-1}$ | >100 |
| $E_bC10$ | $mg.l^{-1}$ | 10 |
| $E_bC50$ | $mg.l^{-1}$ | 32 (27 – 98) [a] |
| $E_bC90$ | $mg.l^{-1}$ | >98 (extrapolated 101) |

a) range between tested concentrations

Effect values that are based on growth rate (e.g. ErC50) are the least dependent on test design, and are therefore the recommended endpoints of this study.

Effect estimates in the present study are expected to be highly reliable, due to the sufficient algal growth in the control cultures and due to the stable and analytically confirmed benzene concentration during the test.

# Contents

## Confidentiality statement

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

## GLP Compliance statement

'We, the undersigned, hereby declare that this report constitutes a true and complete representation of the procedures followed and of the results obtained in this study of TNO Nutrition and Food Research and that the study was carried out under our supervision.

The study was carried out in accordance with the OECD Principles of Good Laboratory Practice.

The chemical analysis was conducted at the TNO Institute of Environmental, Energy and Process Innovation, accredited by the Dutch Accreditation Council, but not participating in the Dutch GLP Compliance Monitoring Programme. The chemical analysis was therefore inspected by the Quality Assurance Unit of TNO Nutrition and Food Research in order to claim GLP for the entire study.

Drs A.O. Hanstveit
*Study Director*
Department of Environmental Toxicology

Date: 6 February 2001

Dr C.T. Bowmer
*Management*
*Head, Department of Environmental Toxicology*

Date: 6 February, 2001

## Contributing personnel

Dr P. Mayer
*Drafting of protocol and report*
Department of Environmental Toxicology

H. Oldersma
*Technician*
Department of Environmental Toxicology

# Quality Assurance Statement

Report title : Determination of the effect of benzene on the growth of the fresh water green alga *Selenastrum capricornutum* (OECD Guideline No. 201 and EU C.3)

Report number : V2360/01

Report date : 2 February 2001

The protocol of this study was inspected as follows:

| Date of inspection | Date of report |
|---|---|
| 16 May 2000 | 16 May 2000 |
| 18 May 2000 | 18 May 2000 |

The experimental phase of this study was inspected as follows:

| Date of inspection | Date of report |
|---|---|
| 13 June 2000 | 13 June 2000 |
| 15 June 2000 | 16 June 2000 |

This report was audited as follows:

| Date of audit | Date of report |
|---|---|
| 24 October 2000 (draft, excl. analysis) | 31 October 2000 |
| 11 January 2001 (draft, analysis) | 11 January 2001 |
| 23 January 2001 (final) | 23 January 2001 |

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.

P.B. Davis B.A.
Quality Assurance Auditor

Date : 16 February 2001

# 1 Introduction

*Background*
The toxicity of the substance benzene to the fresh water green alga *Selenastrum capricornutum* was determined at the request of the sponsor.

*Objective*
The objective of the study was to determine the NOEC, i.e. no-observed-effect-concentration, and the EC50, i.e. that concentration of benzene which results in a 50% reduction in growth rate relative to the control in a 72h (approx.) algal growth inhibition test with the algal species *Selenastrum capricornutum.*

*Justification of the test system*
An algal growth inhibition test is specified by the relevant regulations to obtaining data for the hazard and risk evaluation for chemicals in the aquatic ecosystem.

*Comments on the test design*
The determination was conducted in accordance with the Guidelines OECD No. 201 [1] and EU C.3 [2]. The test was modified according to ref. [3] in order to allow testing of the volatile test substance in a closed test system, and it included daily measurements of actual exposure concentrations.

*Quality standard*
The study was carried out in compliance with the OECD principles of Good Laboratory Practice [4].

*Relevant dates*

| | | |
|---|---|---|
| Protocol signed by the Study Director | : | 12 April 2000 |
| First amendment of the protocol signed by the Study Director | : | 5 June 2000 |
| Period of range-finding test | : | 26 - 28 April 2000 |
| Period of growth inhibition test | : | 13 - 16 June 2000 |

# 2 Materials and methods

## 2.1 Test substance

The following test substance was examined:

| | |
|---|---|
| Name | : benzene |
| CAS Reg. No. | : 71-43-2 |
| Physical appearance | : colourless liquid |
| Filling and lot code | : Fluka 365348/1 24699 |
| Purity | : >99.5%, ACS grade. |
| Solubility in water | : 1780 $mg.l^{-1}$ |
| Storage temperature | : room temperature |
| Protection from light | : yes |
| TNO test substance number | : 00-2360 |

*Receipt of test substance*

| | |
|---|---|
| Date | : 25 April 2000 |
| Quantity | : 500 ml |
| Supplier | : Fluka |

Benzene has an air water distribution coefficient of 0.22 $l \cdot l^{-1}$, and the appropriate testing of benzene requires thus a closed test design to avoid loss of test substance.

## 2.2 Testing facilities

The study was carried out by the Department of Environmental Toxicology of TNO Nutrition and Food Research. The laboratories of the Department of Environmental Toxicology are located at:

Schoemakerstraat 97
2628 VK DELFT
The Netherlands

The postal address is:

P.O. Box 6011
2600 JA DELFT
The Netherlands

The chemical analysis of the test media was carried out by the TNO Institute of Environmental, Energy and Process Innovation located at:

Laan van Westenenk 501
7334 DT APELDOORN
The Netherlands

The postal address is

P.O.Box 342
7300 AH APELDOORN
The Netherlands

## 2.3 Test organism

The fresh-water green alga *Selenastrum capricornutum* (ATCC 22662)[1)], which belongs to the order of *Chlorococcales* (class *Chlorophyceae*), was used as the test organism. This organism is the preferred species for regulatory testing.
The culture was supplied by the 'American Type Culture Collection', c/o Sales Department, 12301 Parklawn Drive, Rockville, Maryland 20852 , USA.
A preculture of algae in the exponential growth phase was prepared as detailed in OECD Guideline no. 201 [1], using the medium described in 2.4.

## 2.4 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 $mg.l^{-1}$ $NaHCO_3$ (not 50 $mg.l^{-1}$ as specified in the OECD Guideline [1]). The pH of the medium was adjusted to pH 7.0 on the day that it was used. Furthermore, the medium contained Fe-citrate, because the growth of the algae would be erratic in the absence of complexed iron.

## 2.5 Test methods

### 2.5.1 Test design

The growth inhibition test was conducted as detailed in the protocol. This protocol was developed on the basis of Guidelines OECD No. 201 [1] and EU C.3 [2], using selected incubation conditions given in the International Standard ISO

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1) The taxonomical status of this algal species is not quite settled. It is also referred to as *Pseudokirchneriella subcapitata* in culture collections.

8692 [5]. Modifications were made in order to conduct the test in "closed filled flasks" as specified by the sponsor and in accordance with a test design described by Mayer et al. [3]. In order to avoid substantial pH changes or carbon limited growth, the initial algal density was reduced ($10^3$ cells·ml$^{-1}$), the $NaHCO_3$ concentration was increased (300 mg·l$^{-1}$) and the medium pH was lowered (7.0).

A preliminary test (without GLP claim) was carried out in order to gain experience with the new test design and to find the relevant concentration range.

### 2.5.2 Preparation of test solutions and inoculation

For the definite growth inhibition test, an amount of 215.0 mg test substance was added directly to 200 ml of algal medium. Test solutions were then prepared by serial dilution with algal medium, and they were kept in closed flasks.
An algal suspension of about 1 x $10^3$ cells.ml$^{-1}$ in algal medium was prepared by dilution of a three days old pre-culture. Forty ml cylindrical glass vials with teflon (PTFE) faced silicon septum caps (EPA vials) were then completely filled by combining the algal suspension and the benzene stock solutions. The resulting algal density was about $10^3$ cells.ml$^{-1}$ and nominal benzene concentrations were 1.3, 4.3, 13, 43 and 134 mg.l$^{-1}$. Each combination of sampling point and benzene concentration was established in five fold. A single background control series without algae was added.

### 2.5.3 Incubation, test conditions and sampling

The filled vials were incubated for three days under continuous standard illumination (60-120 $\mu$mol.m$^{-2}$.s$^{-1}$) on an orbital shaker at 23 ± 2°C. The vials were allowed to "roll around" on the shaker platform in order to prevent the settling of algal cells.

Each day (0, 24, 48 and 72 hours) samples were taken with an airtight Glass/PTFE syringe through the septum of the vial. Samples for the benzene concentrations were pooled among the five replicates into 40 ml EPA vials. These were then kept cool, and they were immediately send to the analytical laboratory for further treatment. Algal densities (cells.ml$^{-1}$) and algal biovolume ($\mu$m$^3$.ml$^{-1}$) were determined daily with the Coulter Multisizer Iie electronic particle counter. Measured values were corrected for the appropriate background values.

At the end of the test, pH was determined in selected cultures. At the start and at the end of the test the morphology of the algae was examined visually with the aid of a microscope.

### 2.5.4 Calculation of the EC values

The total algal biovolume was generally used for further calculations rather than the particle number, because it is less susceptible to changes of the algal cell size. 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 yield or 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 during exponential growth ($E_rC$ values) were calculated by means of a parametric model developed by Kooijman *et al.* [6] assuming a constant error per measurement; a summary of the method is given in Annex B. This calculation method is based on the assumptions of the OECD Guideline 201. It has been used in ring tests of algal growth inhibition test Guidelines [7,8]. These ring tests have demonstrated that $E_rC50$ values calculated by this method are identical to those calculated by the method given in the Guideline [8].

EC values with respect to the area under the growth curve ($E_bC$ values) were calculated by the method given in the OECD Guideline, but they were based on algal biovolumes [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.

### 2.5.5 Determination of the NOEC and NEC values

*NOEC*
The 'no-observed-effect concentration' (NOEC) was determined based on the following criteria:

- The average inhibition based on the area under the growth curve ($I_A$ value) is less than 10%.
- The average inhibition based on final algal density is less than 10%.
- The average inhibition based on algal growth rate is less than 10%.

A treatment was considered "not to inhibit algal growth" if all three criteria are fulfilled.

*NEC*
In addition model calculations were carried out using the DEBtox software package according to the Dynamic Energy Budgets Theory developed by Kooijman and Bedeaux [10]. Model parameters for population growth and their asymptotic standard deviation and correlation coefficients were estimated. The NEC (no-effect-concentration [9,10]) was calculated from the profile ln likelihood function and was based on algal biovolume measurements.

### 2.5.6 Reference substance

The toxic response to the reference substances ($K_2Cr_2O_7$ and/or 3,5-dichlorophenol) is tested on an approximately yearly basis. The results of these reference tests demonstrate a constant quality of algal toxicity tests during an extended period of at least 15 years. The resulting EC50 values of the reference substances are also comparable to the mean values found in international ring tests [7].

## 2.6 Chemical analysis

The analytical procedure is described in Annex D.

# 3 Results and discussion

## 3.1 Chemical analysis

The results of the analytical monitoring of test substance concentrations are included in Annex D. A summary of the results are given in Table 1, which shows measured concentrations that were corrected for the 82% recovery of the analytical procedure. The exposure concentration stability is given in Table 2.

Measured concentrations at the beginning of the test were between 56 to 73% of the nominal concentrations, and this can be attributed to evaporative losses during the spiking and during the serial dilution of test solutions.

Measured benzene concentrations during the test remained stable between 83 and 100% of the measured concentration at the start of the test. Estimation of effect concentrations were therefore based on the measured benzene concentrations at the start of the test: 0, 0.73, 2.6, 8.0, 27 and 98 $mg.l^{-1}$.

*Table 1* *Measured benzene concentrations during the algal toxicity test, determined on pooled sub-samples and corrected for the analytical recovery of 82%. Measured concentrations at the start of the test are shown in bold, because they were used in all further calculations.*

| Nominal benzene concentration ($mg.l^{-1}$) | Measured benzene concentrations | | | | |
|---|---|---|---|---|---|
| | Initial ($mg.l^{-1}$) | % of nominal | 1 day ($mg.l^{-1}$) | 2 days ($mg.l^{-1}$) | 3 days ($mg.l^{-1}$) |
| 0 | **0.1** | | 0.1 | 0.1 | 0.1 |
| 1.3 | **0.7** | 56 | 0.6 | 0.6 | 0.7 |
| 4.3 | **2.6** | 60 | 2.3 | 2.2 | 2.4 |
| 13 | **8.0** | 62 | 7.1 | 6.7 | 7.3 |
| 43 | **27** | 62 | 24 | 24 | 26 |
| 134 | **98** | 73 | 92 | 87 | 93 |

*Table 2* *Exposure concentration stability during the test relative to the measured concentration at the start of the test.*

| Nominal benzene concentration ($mg.l^{-1}$) | Measured benzene concentrations (% of measured at t=0) | | | |
|---|---|---|---|---|
| | Initial | 1 day | 2 days | 3 days |
| 1.3 | 100 | 83 | 83 | 100 |
| 4.3 | 100 | 90 | 86 | 95 |
| 13 | 100 | 88 | 83 | 91 |
| 43 | 100 | 91 | 91 | 95 |
| 134 | 100 | 94 | 89 | 95 |

## 3.2 Test conditions

The temperature was registered continuously and was within the required limits. Only the cultures to be analysed after 24h had been out of temperature range during 35 minutes. The light intensity was measured at the start of the test, and it was within the limits given in the Guidelines. At the start of the test the pH of the algal medium was adjusted to pH 7.0. In the absence of algae the medium pH remained stable during the test (pH 7.0-7.3). In the presence of algae, however, the pH increased during the test with increasing algal density (pH 7.5 – 8.8).

## 3.3 Algal density and algal biomass measurements

Coulter counter measurements are presented in Annex C. Average algal densities at the different time points are presented in Annex C, Tables C1. Algal biovolume measurements are presented in Annex C, Tables C2-C6, and their averages are given in Annex C, Tables C7. The background measurements for algal biovolume are listed in Annex C, Table C8. Average algal cell sizes are given in Annex C, Table C9.

## 3.4 EC values and NOEC

### 3.4.1 EC values

Of several parametric models available, the model that assumes an effect on the growth rate and exponential growth ($E_rC$ values) appeared to fit the data. The results of the model calculations for this effect are given in Annex C, Table C10. The growth curves for the various concentrations of the test substance are shown in Figure 1, and the concentration-effect curves in Figure 2. The data points shown

in these figures represent the mean algal biovolumes of each treatment. The curves were obtained by parametric model calculations.
Additionally a semi-logarithmic plot of the growth curves is presented in Figure 3 in order to illustrate the effects on the growth rate.

The EC50 with respect to growth rate and measured initial test substance concentrations ($E_rC50$) was found to be 100 $mg.l^{-1}$, with a 95% confidence interval of 83 to 130 $mg.l^{-1}$. 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 benzene (measured concentrations) and* Selenastrum capricornutum

| Parameter | Dimension | Value (95% confidence limit) |
|---|---|---|
| Inoculum | $10^5$ $\mu m^3.ml^{-1}$ a) | 0.58 (0.33 – 0.84) |
| Growth rate | $h^{-1}$ | 0.091 (0.085 – 0.097) |
| Gradient | dimensionless | 2.0 (1.6 – 2.3) |
| $E_rC10$ | $mg.l^{-1}$ | 34 |
| $E_rC50$ | $mg.l^{-1}$ | 100 (83 – 130) |
| $E_rC90$ | $mg.l^{-1}$ | > 100 |
| $E_bC10$ | $mg.l^{-1}$ | 10 |
| $E_bC50$ | $mg.l^{-1}$ | 32 (27 – 98) b) |
| $E_bC90$ | $mg.l^{-1}$ | >98 (extrapolated 101) |

a) 1 algal cell corresponds with 73 $\mu m^3$

b) range between tested concentrations

The area under the growth curves was calculated using the mean algal biovolumes that are given in Annex C, Table C7, and the results are given in Annex C, Table C11. The $E_bC$ values shown in Table 1 have been derived from the concentration-effect curve by linear interpolation. As expected, the $E_bC50$ value was lower than the $E_rC50$ value, the latter being a biomass independent effect. Such a difference has also been observed in international ring tests [6,7].

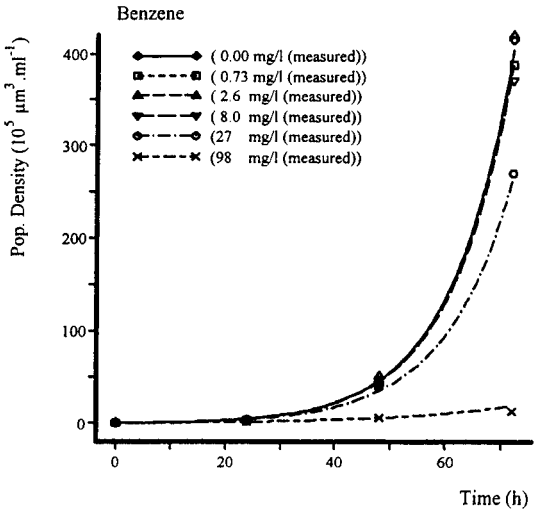


***Figure 1*** *Growth curves of* Selenastrum capricornutum *at different concentrations of benzene in a double linear plot (measurements shown in Table C7).*

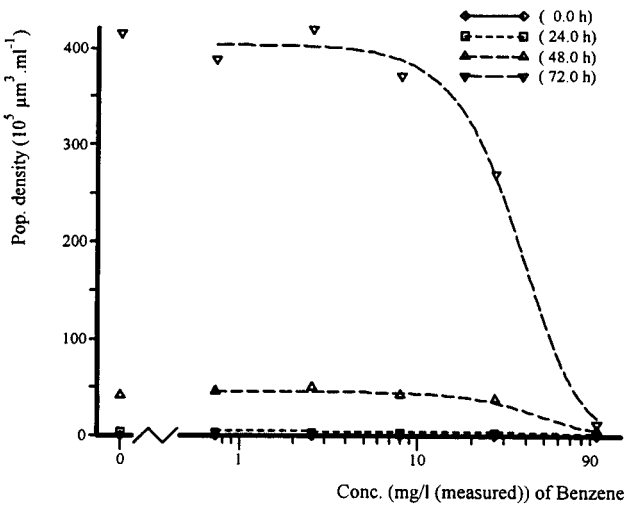


***Figure 2*** *Concentration-effect curves for* Selenastrum capricornutum *exposed to a range of benzene concentrations.*

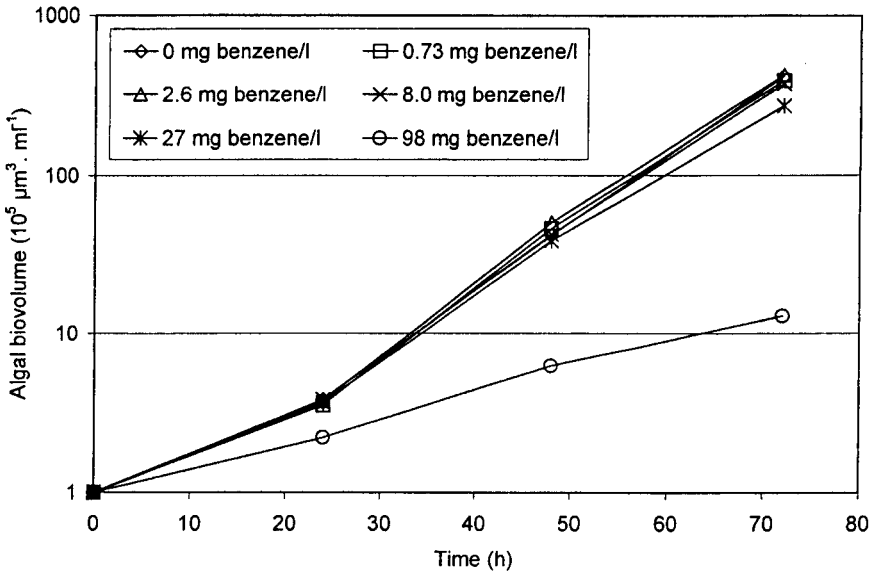


*Figure 3* *Growth curves of* Selenastrum capricornutum *at different concentrations of benzene in a semi-logarithmic plot. Measurements shown in table C7 were connected with straight lines. This representation is the most suitable to evaluate the inhibition of growth rate.*

### 3.4.2 NOEC and NEC values

By comparison of the different parameters of treated cultures with those of the controls (see Annex C), the no-observed-effect-concentration (NOEC) of benzene was found to be 8.0 mg.l$^{-1}$.

The no-effect-concentration (NEC) was calculated with the method given in reference [9,10] in order to obtain a better estimate of a non-inhibiting test substance concentration. The NEC was estimated to be 22.5 mg.l$^{-1}$, with a 95% confidence interval of 20.0 – 25.6 mg.l$^{-1}$. It was therefore concluded that a NOEC value of 8.0 mg.l$^{-1}$ constituted a reasonable approximation of this value.

### 3.4.3 Control observations

Microscopic inspection of the morphology of algal cells in the pre-culture at the start of the test revealed normal cells. At the end of the test abnormal cells were observed only in the cultures containing measured benzene concentrations of 27 and 98 mg.l$^{-1}$.

## 3.5 Validity and quality criteria

The OECD Guideline No. 201 [1] recognises one validity criterion. The algal density in the control cultures should increase by at least 16 within three days, which corresponds to a growth rate of 0.038 h$^{-1}$. The growth rate in the controls of

the present study was 0.091 $h^{-1}$, and the present test thus fulfils the validity criteria given in the guidelines [1,2].

The OECD Guideline No. 201 [1] recognises that the medium pH-value normally should not deviate by more that one unit during the test (it is not a validity criterion). This was not the case in the present study with an initial pH of 7.0 and final pH values of 7.4 to 8.8. The observed pH increase is due to the combination of a closed test design and a 3-days test duration, which lead to final cell densities that exceed the 200 000 cells/ml at which pH starts to increase in such a closed test system [3]. The closed test design was requested by the sponsor in order to keep the volatile test substance in solution. The 3 days test duration is required by the test guidelines. The pH increase was limited by reducing the initial algal density and by manipulating the algal medium composition and pH (see paragraph 2.4).

The observed pH increase is not expected to have affected the results of the present study because of two reasons. (1) The toxicity of benzene can be expected to be independent of pH within the observed range, as benzene is stable within this pH range and as it does not have a pH sensitive solution behaviour. (2) The algal population and its growth are relatively insensitive to pH within the observed range [3], and this was confirmed by high growth rates during the entire test.

## 3.6 Discussion

The system used in the present study is in most aspects equivalent to the tests described in the OECD 201 and EU C.3 guidelines [1,2]. The main difference is that the applied test design is better suited to maintain exposure concentrations of a volatile substance during the test. This was also confirmed by the chemical analysis of benzene concentrations.

The reduced initial algal density, the $NaHCO_3$ concentration and the adjusted medium pH value are not expected to have affected the results of this study. This is particularly the case for the inhibition based on growth rate, which compared to other endpoints is highly independent of the test design [11]. The effect values that are based on growth rates (e.g. $E_rC50$) are therefore the recommended endpoints of this study.

Effect estimates of the present study are expected to be highly reliable, due to the sufficient algal growth in the control cultures and due to the stable and analytically confirmed benzene concentration during the test.

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# 5 Retention of records and samples

All the data generated and all other records and information relevant to the quality and integrity of the study have been filed under the study reference 00-2360/01 in the archives of TNO Nutrition and Food Research, Utrechtseweg 48, 3704 HE Zeist, The Netherlands. These records will be retained for a period of at least 15 years after the cover date of this report.

A reference sample of the test substance will be deposited under the sample reference 00-2360-A in the sample archives of TNO Nutrition and Food Research at the same address; this sample will be stored for a period of at least two years.

# 6 Deviations from the protocol

- The batch number of the test substance was 365348/1 24699, not Sigma-Aldrich: 31,995-3 (which was a catalogue number).
- It was not possible to specify the TNO code on the test flasks; only three digits representing replicate number, time of analysis and concentration of test substance were used. However, as this closed bottle test was the only one carried out at the time. Mix-up with other studies was impossible.
- Samples for chemical analysis at t=0 were taken by adding 35 ml algal suspension to 5 ml medium or the relevant test substance stock (dilution) in airtight test vials.
- The cultures for analysis at t=24h were out of temperature range (25.6 - 25.9°C) during 35 minutes, due to a technical failure.

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

# Annex A Composition of algal medium

| | | |
|---|---|---|
| $NH_4Cl$ | 15 | $mg.l^{-1}$ |
| $MgCl_2.6H_2O$ | 12 | $mg.l^{-1}$ |
| $CaCl_2.2H_2O$ | 18 | $mg.l^{-1}$ |
| $MgSO_4.7H_2O$ | 15 | $mg.l^{-1}$ |
| $KH_2PO_4$ | 1.6 | $mg.l^{-1}$ |
| Fe-citrate.$3H_2O$ | 80 | $\mu g.l^{-1}$ |
| $Na_2EDTA.2H_2O$ | 100 | $\mu g.l^{-1}$ |
| $H_3BO_3$ | 185 | $\mu g.l^{-1}$ |
| $MnCl_2.4H_2O$ | 415 | $\mu g.l^{-1}$ |
| $ZnSO_4.7H_2O$ | 6.3 | $\mu g.l^{-1}$ |
| $CoCl_2.6H_2O$ | 1.5 | $\mu g.l^{-1}$ |
| $CuSO_4.5H_2O$ | 0.015 | $\mu g.l^{-1}$ |
| $Na_2MoO_4.2H_2O$ | 7 | $\mu g.l^{-1}$ |
| $NaHCO_3$ | 150 | $mg.l^{-1}$ |

Hardness, mg equivalent $CaCO_3.l^{-1}$:

$=2.497$ [Ca, $mg.l^{-1}$]+ 4.118[Mg, $mg.l^{-1}$] = 24.2

according to Standard Methods for the examination of water and wastewater 1985, 16th edition.APHA.AWWA.WPCF

# Annex B Summary of the calculation method of the $E_rCy$

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:

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

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 |
| Rb and Eb | = | 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 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 NT computer.

# Annex C Results of the growth-inhibition test

*Table C1* *Mean algal densities ($10^3$ cells.ml$^{-1}$, corrected for background).*

| Time | Measured benzene concentration (mg.l$^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| (h) | 0 | 0.7 | 2.6 | 8.0 | 27 | 98 |
| 0 | 1.4 | | | | | |
| 24 | 4.7 | 4.5 | 4.5 | 4.7 | 4.3 | 2.4 |
| 48 | 50.6 | 56.0 | 58.6 | 48.5 | 38.6 | 7.8 |
| 72 | 549.6 | 465.0 | 554.7 | 467.2 | 306.2 | 17.0 |

*Table C2* *First set of algal biovolumes ($10^5$ µm$^3$.ml$^{-1}$, corrected for background).*

| Time | Measured benzene concentration (mg.l$^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| (h) | 0 | 0.7 | 2.6 | 8.0 | 27 | 98 |
| 0 | 1.0 | - | - | - | - | - |
| 24 | 3.6 | 3.5 | 3.5 | 3.9 | 3.5 | 2.3 |
| 48 | 47.5 | 49.1 | 48.9 | 45.4 | 39.3 | 8.4 |
| 72 | 422.3 | 424.5 | 430.1 | 357.7 | 306.1 | 20.2 |

*Table C3* *Second set of algal biovolumes ($10^5$ µm$^3$.ml$^{-1}$, corrected for background).*

| Time | Measured benzene concentration (mg.l$^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| (h) | 0 | 0.7 | 2.6 | 8.0 | 27 | 98 |
| 0 | 0.9 | - | - | - | - | - |
| 24 | 3.8 | 3.4 | 3.6 | 3.7 | 3.4 | 2.2 |
| 48 | 45.5 | 48.3 | 56.8 | 46.7 | 40.4 | 6.2 |
| 72 | 400.3 | 404.9 | 393.5 | 393.3 | 277.5 | 13.0 |

*Table C4* *Third set of algal biovolumes ($10^5$ µm$^3$.ml$^{-1}$, corrected for background).*

| Time | Measured benzene concentration (mg.l$^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| (h) | 0 | 0.7 | 2.6 | 8.0 | 27 | 98 |
| 0 | 0.8 | - | - | - | - | - |
| 24 | 3.8 | 3.4 | 3.7 | 3.6 | 3.8 | 2.4 |
| 48 | 38.9 | 44.6 | 48.7 | 41.6 | 39.2 | 6.6 |
| 72 | 422.9 | 376.1 | 435.1 | 335.9 | 250.5 | 14.5 |

*Table C5* *Fourth set of algal biovolumes ($10^5$ $\mu m^3.ml^{-1}$, corrected for background).*

| Time (h) | Measured benzene concentration ($mg.l^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 0.7 | 2.6 | 8.0 | 27 | 98 |
| 0 | 1.3 | - | - | - | - | - |
| 24 | 3.8 | 3.7 | 3.6 | 3.8 | 3.8 | 2.2 |
| 48 | 35.5 | 42.7 | 49.0 | 41.4 | 36.3 | 4.8 |
| 72 | 409.9 | 368.5 | 437.5 | 364.5 | 245.7 | 4.5 |

*Table C6* *Fifth set of algal biovolumes ($10^5$ $\mu m^3.ml^{-1}$, corrected for background).*

| Time (h) | Measured benzene concentration ($mg.l^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 0.7 | 2.6 | 8.0 | 27 | 98 |
| 0 | 1.0 | - | - | - | - | - |
| 24 | 4.0 | 3.6 | 4.0 | 3.9 | 3.6 | 2.2 |
| 48 | 41.4 | 47.2 | 48.3 | 37.5 | 37.8 | 5.2 |
| 72 | 421.5 | 367.5 | 405.1 | 406.1 | 269.3 | 11.9 |

*Table C7* *Mean values of algal biovolumes ($10^5$ $\mu m^3.ml^{-1}$, corrected for background). The initial algal biovolume for all treatments was calculated as the mean of the control cultures.*

| Time (h) | Measured benzene concentration ($mg.l^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 0.7 | 2.6 | 8.0 | 27 | 98 |
| 0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| 24 | 3.8 | 3.5 | 3.7 | 3.8 | 3.6 | 2.2 |
| 48 | 41.8 | 46.4 | 50.4 | 42.5 | 38.6 | 6.2 |
| 72 | 415.4 | 388.3 | 420.3 | 371.5 | 269.8 | 12.9 |

*Table C8* *Total particle volumes in the background series ($10^5$ $\mu m^3.ml^{-1}$).*

| Time (h) | Measured benzene concentration ($mg.l^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 0.7 | 2.6 | 8.0 | 27 | 98 |
| 0 | 0.16 | - | - | - | - | - |
| 24 | 0.31 | 0.52 | 0.45 | 0.25 | 0.33 | 0.23 |
| 48 | 0.96 | 0.51 | 0.94 | 0.86 | 0.50 | 0.47 |
| 72 | 1.93 | 1.45 | 1.66 | 0.73 | 1.28 | 0.79 |

*Table C9* *Mean cell size ($\mu m^3.cell^{-1}$, corrected for background).*

| Time | Measured benzene concentration ($mg.l^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| (h) | 0 | 0.7 | 2.6 | 8.0 | 27 | 98 |
| 0 | 73 | - | - | - | - | - |
| 24 | 82 | 79 | 81 | 81 | 85 | 95 |
| 48 | 82 | 83 | 86 | 88 | 100 | 80 |
| 72 | 76 | 84 | 76 | 80 | 88 | 76 |

*Table C10* *Modelled total biovolumes ($10^5$ $\mu m^3.ml^{-1}$).*

| Time | Measured benzene concentration ($mg.l^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| (h) | 0 | 0.7 | 2.6 | 8.0 | 27 | 98 |
| 0 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 |
| 24 | 5.2 | 5.2 | 5.2 | 5.1 | 4.5 | 1.9 |
| 48 | 45.7 | 45.7 | 45.5 | 44.4 | 34.6 | 6.0 |
| 72 | 404.2 | 404.0 | 402.4 | 387.9 | 266.1 | 19.1 |

*Table C11* *The area under the growth curve with respect to cell numbers (A) and the inhibition based on this parameter ($I_A$).*

| Para- | Measured benzene concentration ($mg.l^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| meter | 0 | 0.7 | 2.6 | 8.0 | 27 | 98 |
| A | 6017 | 5797 | 6280 | 5508 | 4191 | 297 |
| $I_A$ | 0% | 4% | -4% | 8% | 30% | 101% |

# Annex D Chemical analysis

TNO Environment, Energy and Process Innovation

TNO-MEP
Business Park E.T.V.
Laan van Westenenk 501
PO Box 342
7300 AH Apeldoorn
The Netherlands
Telephone: +31 55 549 34 93
Fax: +31 55 549 33 90
Internet: www.mep.tno.nl

QUALIFIED BY STERLAB Reg.nr. L-026

*TNO-report*

TNO Nutrition and Food Research Institute
Department of Environmental Toxicology
Attn. Drs. P. Mayer
P.O. Box 6011
2600 JA Delft

**Subject:** **The determination of benzene in aqueous samples of a 72 h algal grow inhibition test with the green algae *Selenastrum capricornutum, IMW-00-2360-01***

| | | |
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CONTENTS:



Issued by:
Department Environmental Quality

J.A.D. van Renesse van Duivenbode
(Author)
Date: 02-02-2001

R.J.B. Peters
(Principal investigator)
Date: 02-02-2001

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## 1. Introduction

The Department of Environmental Toxicology of TNO Nutrition and Food Research (TNO Nutrition) has asked the Department of Environmental Quality of TNO Environmental Sciences, Energy Research and Process Innovation (TNO-MEP) to determine the concentration of benzene in aqueous samples from a 72 h algal growth inhibition test with the green algae *Selenastrum capricornutum*. The study number of the growth inhibition test is IMW-00-2360-01.

## 2. Samples

Description; 24 aqueous samples of about 40 ml were received in glass crimp-cap vials. No headspace was observed in the vials.
Date of delivery; June 13, 14, 15 and 16, 2000. Each day six samples were received.
Our sample code, the sample code of the commissioner and a description of the samples are given with the results in table 1 in section 4.

## 3. Methods

### 3.1 Analyses of benzene in aqueous samples

The samples are analyzed using a liquid-liquid extraction with carbon disulfide. $D_6$-benzene was added to the extraction liquid as an internal standard in a concentration of 95 mg/l. Typically, 2.0 ml of the aqueous samples was extracted with 2.0 ml of the extraction liquid in a 4 ml vial by shaking for 2 minutes. All samples were extracted on the day of delivery and the extracts were stored in the refrigerator at a temperature of about -18°C until gas chromatographic analysis. All extract were analyzed on the day after the extraction with the exception of the samples that were received on June 16, 2000, which were extracted and analyzed on the date of delivery.

The extracts were analyzed by gas chromatography with mass spectrometry (GC/MS). The conditions were as follows:

*GC-conditions*

| | | |
|---|---|---|
| gas chromatograph | : | Hewlett Packard 6890 |
| column | : | DB-5MS, 60 m x 0,25 mm (ID), film thickness 0,25 µm |
| carrier gas | : | helium |
| column flow | : | 1 ml/min |
| split flow | : | 25 ml/min |
| injection technique | : | split |
| injection volume | : | 1 µl |
| injector | : | 280°C |
| interface GC/MS | : | 280°C |
| oven GC | : | from 30°C (3 min) to 150°C (1 min) with 3°/min. |

*MS-conditions*

| | | |
|---|---|---|
| mass spectrometer | : | Hewlett Packard 5973 |
| ionisation | : | Electron Impact (EI) 70 eV |
| mode | : | selected-ion monitoring SIM |
| ions for benzene | : | 78 en 51 amu |
| ions for $D_6$-benzene | : | 84 en 54 amu |
| dwell time | : | 50 msec. |

The identification of benzene was based on the retention time in the chromatogram and correct ratio of the qualifier ion. The quantitation was based on the peak area of the target ion and a relative response factor obtained from external standards that were analyzed with the sample extracts. With each series of extracts two external standards with concentrations of 8 and 80 mg/l were analyzed.

### 3.2. Performance characteristics of the method

The method of analyses was validated before the analyses were performed. The validation resulted in a standard operating procedure which was used for the analysis of the samples. Some important validation parameters are listed below.

#### 3.2.1 Measuring range

The measuring range was determined as the linearity of the instrumental analysis. This was determined by the analysis of a series of standards ranging from 0.8 to 2130 mg/l. The instrumental analyses was linear in this range.

#### 3.2.2 Limit of detection and quantitation.

The limits of detection and quantitation of the analytical method were determined as 3 and 10 times the signal/noise ratio in the chromatogram. For benzene the limit of detection is 0.06 mg/l and the limit of quantitation 0.19 mg/l.

#### 3.2.3 Repeatability

The repeatability of the analytical method is determined as the standard deviation in the results of six analysis of an aqueous sample with a benzene concentration of 85.2 mg/l. The standard deviation of the complete method was smaller than 2%.

#### 3.2.4 Recovery

The recovery of benzene was determined by the analysis of six aqueous sample with a benzene concentration of 97 mg/l. The recovery was found to be 82% ± 1%. The analysis results in section 4 are not corrected for this recovery.

## 4. Results

The results of the analysis of the test samples are given in table 1.

**Table 1. Results of the benzene analysis of aqueous samples.**

| Sample code TNO-MEP 52000- | Sample code TNO Nutrition IMW-00- | Date of delivery | Nominal test concentration (mg/l) | Time after incubation (hrs.) | Benzene concentration (mg/l) |
|---|---|---|---|---|---|
| 205-04 | 2360-01/3 | 13-jun-00 | 0 | 0 | 0.1 |
| 205-05 | 2360-01/4 | 13-jun-00 | 1.3 | 0 | 0.6 |
| 205-06 | 2360-01/5 | 13-jun-00 | 4.3 | 0 | 2.1 |
| 205-07 | 2360-01/6 | 13-jun-00 | 13 | 0 | 6.6 |
| 205-08 | 2360-01/7 | 13-jun-00 | 43 | 0 | 22 |
| 205-09 | 2360-01/8 | 13-jun-00 | 134 | 0 | 80 |
| 205-10 | 2360-01/9 | 14-jun-00 | 0 | 24 | 0.1 |
| 205-11 | 2360-01/10 | 14-jun-00 | 1.3 | 24 | 0.5 |
| 205-12 | 2360-01/11 | 14-jun-00 | 4.3 | 24 | 1.9 |
| 205-13 | 2360-01/12 | 14-jun-00 | 13 | 24 | 5.8 |
| 205-14 | 2360-01/13 | 14-jun-00 | 43 | 24 | 20 |
| 205-15 | 2360-01/14 | 14-jun-00 | 134 | 24 | 75 |
| 205-16 | 2360-01/15 | 15-jun-00 | 0 | 48 | 0.1 |
| 205-17 | 2360-01/16 | 15-jun-00 | 1.3 | 48 | 0.5 |
| 205-18 | 2360-01/17 | 15-jun-00 | 4.3 | 48 | 1.8 |
| 205-19 | 2360-01/18 | 15-jun-00 | 13 | 48 | 5.5 |
| 205-20 | 2360-01/19 | 15-jun-00 | 43 | 48 | 20 |
| 205-21 | 2360-01/20 | 15-jun-00 | 134 | 48 | 71 |
| 205-22 | 2360-01/21 | 16-jun-00 | 0 | 72 | 0.1 |
| 205-23 | 2360-01/22 | 16-jun-00 | 1.3 | 72 | 0.6 |
| 205-24 | 2360-01/23 | 16-jun-00 | 4.3 | 72 | 2.0 |
| 205-25 | 2360-01/24 | 16-jun-00 | 13 | 72 | 6.0 |
| 205-26 | 2360-01/25 | 16-jun-00 | 43 | 72 | 21 |
| 205-27 | 2360-01/26 | 16-jun-00 | 134 | 72 | 76 |