## Section A6.6.4 Genotoxicity in vivo
**Annex Point IIA6.6.4** **In vivo mammalian bone marrow assay**

### 1 REFERENCE

Kawachi T, Komatsu T, Kada T, Ishidate M, Sasaki M, Sugiyama T, Tazima Y, 1980, Results of recent studies on the relevance of various short-term screening tests in Japan; The predictive value of short-term screening tests in carcinogenicity evaluation; Williams et al. (eds.), Elsevier/North-Holland Biomedical Press, 253-267 (A6.6.4/01)
Anonymous, Benzoates, Review Report, OECD-SIDS, 2001 (A6.6.4/02)

### 2 RESULTS

General remark: Since the sodium salt of benzoic acid instantaneously dissociates to the benzoic acid, the studies with sodium benzoate are also representative for benzoic acid and potassium benzoate.

**Kawachi T, Komatsu T, Kada T, Ishidate M, Sasaki M, Sugiyama T, Tazima Y, 1980 (6.6.4/01)**

General remark: Since the sodium salt of benzoic acid instantaneously dissociates to the benzoic acid, the studies with sodium benzoate are also representative for benzoic acid and potassium benzoate.

A cytogenic assay in male rats given single or multiple gavage doses of 50, 500, or 5,000 mg/kg **sodium benzoate** showed no significant increase in chromosomal aberrations in the bone marrow.

| Type of test | Species | Results | Remarks | Reference |
|---|---|---|---|---|
| Bone marrow assay<br>50, 500 or 5000 mg/kg sodium benzoate | Rat | Negative | No further information available | Kawachi T, Komatsu T, Kada T, Ishidate M, Sasaki M, Sugiyama T, Tazima Y, 1980 (6.6.4/01) |

**OECD-SIDS, 2001 (A6.6.4/02)**

A host mediated assay using male rats given multiple gavage doses of 50, 500, or 5,000 mg/kg **sodium benzoate** showed no elevation of mutant frequencies in *Salmonella typhimurium* G46; no elevation of mutant frequencies in *Salmonella typhimurium* TA 1530; no increase in recombinant frequencies in *Saccharomyces cerevesiae* D3.
(FDA PB 245453, 1974, cited in Anonymous, OECD-SIDS, 2001 (A6.6.4/02))
A host mediated assay using male rats given a single gavage dose of 50, 500, or 5,000 mg/kg **sodium benzoate** showed an elevation of mutant frequencies in *Salmonella typhimurium* TA 1530
in the intermediate dose level; the other doses were negative.
(FDA PB 245453, 1974, cited in Anonymous, OECD-SIDS, 2001 (A6.6.4/02))

A dominant lethal assay using male rats given single or multiple gavage doses of 50, 500, or 5,000 mg/kg **sodium benzoate** was non-mutagenic.
(FDA PB 245453, 1974, cited in Anonymous, OECD-SIDS, 2001 (A6.6.4/02))
**Remark:** IPCS CICAD 26 (2000) mentioned this dominant lethal assay as a positive result, however evaluation of the raw data in the original report (by experts of the industry consortium and a recent independent review by Prof. R. Kroes) gives no support for this. In addition the authors of the study clearly conclude negative. FDA also evaluated this study as negative. In

**Section A6.6.4** **Genotoxicity in vivo**

**Annex Point IIA6.6.4** **In vivo mammalian bone marrow assay**

addition sodium benzoate doesn't contain a structural alert for genotoxicity.

| Type of test | Species | Results | Remarks | Reference |
|---|---|---|---|---|
| Host-Mediated Assay<br>50, 500 or 5000 mg/kg sodium benzoate | Male rats | Negative | Elevated mutant frequency with TA 1530 in the intermediate single gavage dosing only (clear negative after multiple gavage dosing) | cited in Anonymous, OECD-SIDS, 2001 (A6.6.4/02) |
| Dominant lethal assay<br>50, 500 or 5000 mg/kg sodium benzoate | Male random bred rats | Negative | IPCS CICAD 26 (2000) mentioned this test as positive result; however evaluation of the raw data in the original report (by experts of the industry consortium and a recent independent review by Prof. R. Kroes) gives no support for this. In addition the authors of the study clearly conclude negative. FDA also evaluated this study as negative. In addition sodium benzoate doesn't contain a structural alert for genotoxicity. | cited in Anonymous, OECD-SIDS, 2001 (A6.6.4/02) |

**3 CONCLUSION**

Benzoic acid and sodium benzoate have no mutagenic properties

**Evaluation by Competent Authorities**

Use separate "evaluation boxes" to provide transparency as to the comments and views submitted

**EVALUATION BY RAPPORTEUR MEMBER STATE**

## Section A6.6.4 Genotoxicity in vivo
**Annex Point IIA6.6.4** **In vivo mammalian bone marrow assay**

| | |
|---|---|
| **Date** | 2008/01/07 |
| **Material and methods** | N/A |
| **Results and discussion** | The submitted part of the publication of Kawachi et al. does neither contain specification of the doses applied (50, 500, 5000 mg/kg bw) nor of the number of dosing (single or multiple or both).<br>The reference for the cited literature from the OECD SIDS dossier is:<br>Host mediated assay: Fabrizio DBA (1974). Mutagenic evaluation of compound FDA 71-27 Sodium Benzoate. Litton Bionetics, Inc. Sodium benzoate (single dose or 5 daily doses) was given orally to **mice (ICR strain, 8-10 males/group)**, not to male rats.<br>Dominat lethal assay: Fabrizio DBA (1974). Mutagenic evaluation of compound FDA 71-27 Sodium Benzoate. Litton Bionetics, Inc. Sodium benzoate (single dose or 5 daily doses) was given orally (gavage) to 5 M rats/group. |
| **Conclusion** | Sodium benzoate was not mutagenic in vivo tests. |
| | **COMMENTS FROM OTHER MEMBER STATE** *(specify)* |
| **Date** | *Give date of comments submitted* |
| **Evaluation of applicant's justification** | *Discuss if deviating from view of rapporteur member state* |
| **Conclusion** | *Discuss if deviating from view of rapporteur member state* |
| **Remarks** | |

## Section A6.7 Carcinogenicity / Oral / Rat

**Annex Point IIA6.7**

Official use only

| | | |
|---|---|---|
| | | **1 REFERENCE** |
| **1.1** | **Reference** | Sodemoto Y, Enomoto M, 1980, Report of carcinogenesis bioassay of sodium benzoate in rats: Absence of carcinogenicity of sodium benzoate in rats<br>Journal of Environmental Pathology and Toxicology, 4, 87-95 |
| **1.2** | **Data protection** | No |
| 1.2.1 | Data owner | Not applicable |
| 1.2.2 | Companies with letter of access | - |
| 1.2.3 | Criteria for data protection | No data protection / published data |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** |
| **2.1** | **Guideline study** | No. Study conducted according to prevailing standards and subsequently accepted for international registration. Results considered to be valid |
| **2.2** | **GLP** | No. GLP was not compulsory at the time the study was performed. |
| **2.3** | **Deviations** | Not applicable |
| | | **3 MATERIALS AND METHODS** |
| **3.1** | **Test material** | Sodium benzoate |
| 3.1.1 | Lot/Batch number | No lot/batch number available |
| 3.1.2 | Specification | Delivered from Drs. K. Suzuki and F. Nakadate, National Institute of Hygienic Science, Tokyo, Japan |
| 3.1.2.1 | Description | Pellets at concentrations of 1 and 2% in the basal diet |
| 3.1.2.2 | Purity | Pellets at concentrations of 1 and 2% in the basal diet |
| 3.1.2.3 | Stability | The test substance was considered to be stable for the duration of the study. |
| **3.2** | **Test Animals** | Non-entry field |
| 3.2.1 | Species | Rat |
| 3.2.2 | Strain | Fischer 344 |
| 3.2.3 | Source | Japan CLEA Co. Ltd., Japan |
| 3.2.4 | Sex | Male and female |
| 3.2.5 | Age/weight at study initiation | Age: 4-5 weeks<br>Weight: 110-150 g |
| 3.2.6 | Number of animals per group | 50 males and 52 females/group |
| 3.2.6.1 | at interim sacrifice | Scheduled sacrifice of several animals from each group for morphological examination in the middle of the experimental period, no number given for the intermediate sacrifice |
| 3.2.6.2 | at terminal sacrifice | all surviving animals |
| 3.2.7 | Control animals | Yes, 25 males and 43 females |

## Section A6.7 Carcinogenicity / Oral / Rat

**Annex Point IIA6.7**

| | | | |
|---|---|---|---|
| **3.3** | **Administration/ Exposure** | Oral | |
| 3.3.1 | Duration of treatment | 25 month | |
| 3.3.2 | Interim sacrifice(s) | After 18 month | |
| 3.3.3 | Final sacrifice | after 25 month | |
| 3.3.4 | Freqency of exposure | Daily | |
| 3.3.5 | Postexposure period | No data given | |
| | | **Oral** | |
| 3.3.6 | Type | In food | |
| 3.3.7 | Concentration | In food 0-1-2% (ca 0-700-1400 mg/kg bw/d)<br>Preliminary 6 week toxicity study (0-0.5-1-2-4-8% in the diet)<br>Food consumption per day ad libitum | |
| 3.3.8 | Vehicle | Moistened with food | |
| 3.3.9 | Concentration in vehicle | 1-2% | |
| 3.3.10 | Total volume applied | No data given | |
| 3.3.11 | Controls | Vehicle, plain diet or other | |
| **3.4** | **Examinations** | | |
| 3.4.1 | Body weight | Yes, weekly | |
| 3.4.2 | Food consumption | Yes, daily | |
| 3.4.3 | Water consumption | No | |
| 3.4.4 | Clinical signs | Yes in experimental period | |
| 3.4.5 | Macroscopic investigations | Tumours | |
| 3.4.6 | Ophthalmoscopic examination | No | |
| 3.4.7 | Haematology | No | |
| | | Number of animals: | all animals, 10 animals/sex/group or other |
| | | Time points: | After 3, 6, 12, 18, 24 months of treatment, end of study or other |
| | | Parameters: | Haematocrit, haemoglobin concentration, erythrocyte count, total and differential leukocyte count, platelet count, clotting time, prothrombin time, thromboplastin time |
| | | Other: | - |
| 3.4.8 | Clinical Chemistry | No | |

## Section A6.7 Carcinogenicity / Oral / Rat

**Annex Point IIA6.7**

| | | | |
|---|---|---|---|
| | | Number of animals: | All animals, 10 animals/sex/group or other |
| | | Time points: | After 3, 6, 12, 18, 24 months of treatment, end of study or other |
| | | Parameters: | Sodium, potassium, glucose, total cholesterol, urea, blood urea nitrogen, total bilirubin, creatinine, total protein and albumin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma glutamyl transpeptidase, sorbitol dehydrogenase, methaemoglobin, lipids, hormone (specify hormones), acid/base balance, cholinesterase inhibition. |
| | | Other | - |
| 3.4.9 | Urinalysis | No | |
| | | Number of animals: | All animals, 10 animals/sex/group or other |
| | | Time points: | After 3, 6, 12, 18, 24 months of treatment, end of study or other |
| | | Parameters: | Appearance, volume, osmolality, specific gravity, pH, protein, glucose, blood |
| | | Other | - |
| 3.4.10 | Pathology | Yes | |
| 3.4.10.1 | Organ Weights | No | |
| | | from: | all surviving animals, at interim sacrifice, at terminal sacrifice |
| | | Organs: | Liver, kidneys, adrenals, testes, epididymides, uterus, ovaries, thymus, spleen, brain, heart |
| | | | Other |
| 3.4.11 | Histopathology | Yes | |
| | | from: | all dose groups |
| | | from: | all surviving animals at terminal sacrifice and at interim sacrifice |
| | | Organs: | Various organs, no further data given |
| | | | Results are found in : pituitary, mammary gland, testis, maxilla, uterus |
| | | | Other |
| 3.4.12 | Other examinations | No other examinations | |
| **3.5** | **Statistics** | Results of the statistical tests for dose related trends were not significant ($p < 0.05$). | |
| **3.6** | **Further remarks** | | |

**4 RESULTS AND DISCUSSION**

## Section A6.7 Carcinogenicity / Oral / Rat

**Annex Point IIA6.7**

**4.1 Body weight**

No effects

Body weight – male rats

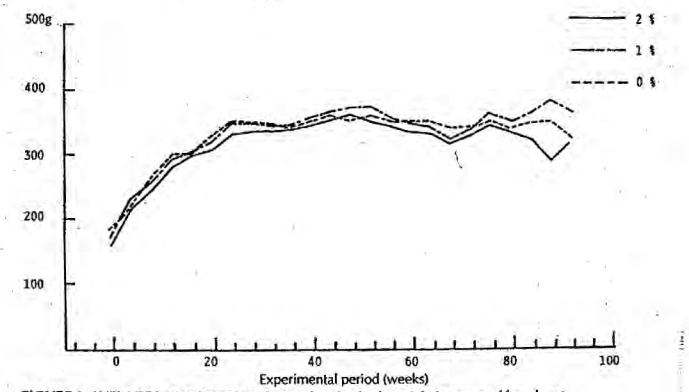


FIGURE 4. AVERAGE BODY WEIGHT. Curves showing body weight increase of female rats.

Body weight – female rats

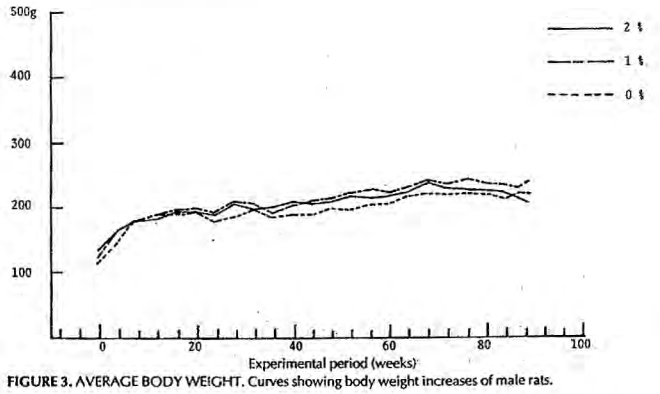


FIGURE 3. AVERAGE BODY WEIGHT. Curves showing body weight increases of male rats.

In the figures 3 and 4 of the publication, showing curves of the body weight change, males and females obviously were exchanged by mistake.

**4.2 Food consumption**

No effects

Consumed diet (average values)

| | % sodium benzoate in food | | |
|---|---|---|---|
| | **0** | **1** | **2** |
| Male | 13.8 ± 0.9 | 14.1 ± 1.0 | 14.0 ± 0.9 |
| Female | 9.68 ± 0.3 | 10.2 ± 1.2 | 10.1 ± 0.5 |

**4.3 Water consumption**

No data

**4.4 Clinical signs**

No treatment related effects

**4.5 Macroscopic investigations**

No effects

**4.6 Ophthalmoscopic examination**

No data

## Section A6.7 Carcinogenicity / Oral / Rat

**Annex Point IIA6.7**

| | | |
|---|---|---|
| **4.7** | **Haematology** | No data |
| **4.8** | **Clinical Chemistry** | No data |
| **4.9** | **Urinalysis** | No data |
| **4.10** | **Pathology** | During the first 16 month of the experimental period, the average mortality rate was 14.5%. Except for myeloproliferative disorder developed in one female control rat, all the dead animals showed pneumonia with abscess. Around 100 rats including those of the control groups dies after 16 month of hemorrhagic pneumonia with edema which was probably induced by mixed infection of sialodacryoadenitis and mycoplasma. No differences in mortality rates between treated and control groups were observed throughout the experimental course. |
| **4.11** | **Organ Weights** | No data |

## Section A6.7 Carcinogenicity / Oral / Rat

**Annex Point IIA6.7**

**4.12 Histopathology** Incidence of tumour / malignant tumour (%)

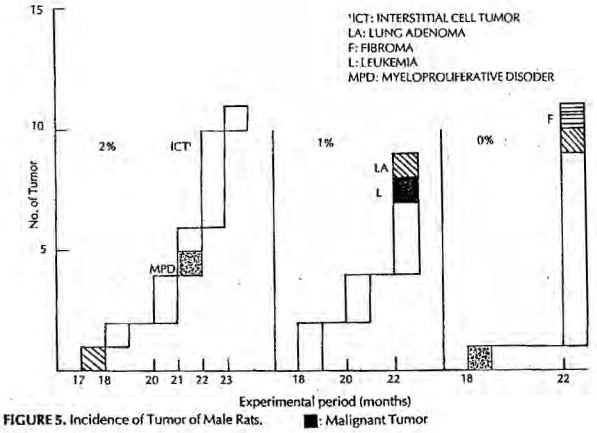


FIGURE 5. Incidence of Tumor of Male Rats. ■: Malignant Tumor

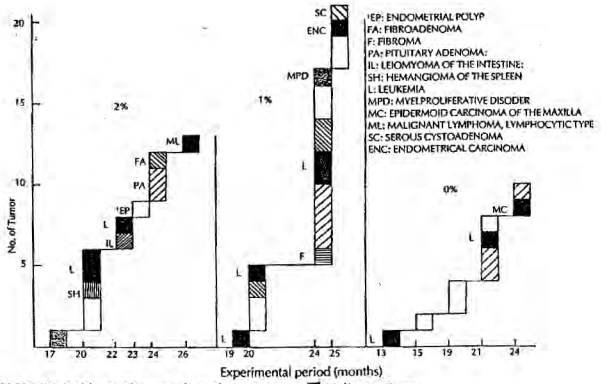


FIGURE 6. Incidence of Tumor of Female Rats. ■:Malignant Tumor

Tumors appearing in treated rats were similar in type and number to those of controls.

Benign tumors included chromophobe adenoma of the pituary body, endometrial polyp, fibroadenoma of the mammary glands and interstitial cell tumour of the testis.

One male rat of the 1% group, four female rats of the control group developed malignant tumours including leukaemia, malignant lymphoma, epidermoid carcinoma of the maxilla, after 17 – 24 month of feeding. These tumours corresponded with the spontaneous tumours of Fisher rats, as reported from observation throughout their natural life spans (Sass et al., 1975; Coleman et al., 1977 and Haley, 1978).

**4.13 Other examinations** -

**4.14 Time to tumours** -

**4.15 Other** -

## Section A6.7 Carcinogenicity / Oral / Rat

**Annex Point IIA6.7**

### 5 APPLICANT'S SUMMARY AND CONCLUSION

**5.1 Materials and methods**

Sodium benzoate was tested for its carcinogenicity in a feeding study in rats. 50 males and 52 females per group were feed for 18 month or 25 month.

Clinical signs, body weight change, food consumption, survival rate, pathohistology (autopsy and histology of various organs) and tumour rates were determined.

**5.2 Results and discussion**

No carcinogenic effect in Fischer rats from sodium benzoate up to 2% in the diet (ca 1400 mg/kg bw/d) was evident in this study.

No hypersensitivity occurring in the preliminary short-term study, no other lesions were reported.

During the first 16 month of the experimental period, the average mortality rate was 14.5%. Except for myeloproliferative disorder developed in one female control rat, all the dead animals showed pneumonia with abscess. Around 100 rats including those of the control groups died after 16 month of hemorrhagic pneumonia with edema which was probably induced by mixed infection of sialodacryoadenitis and mycoplasma. No differences in mortality rates between treated and control groups were observed throughout the experimental course. Nor were there any significant differences in growth and food intake between groups of rats.

Tumors appearing in treated rats were similar in type and number to those of controls.

One male rat of the 1% group, four female rats of the control group developed malignant tumours including leukaemia, malignant lymphoma, epidermoid carcinoma of the maxilla, after 17 – 24 month of feeding. These tumours corresponded with the spontaneous tumours of Fisher rats, as reported from observation throughout their natural life spans (Sass et al., 1975; Coleman et al., 1977 and Haley, 1978).

**5.3 Conclusion**

5.3.1 Reliability 2

5.3.2 Deficiencies There is a lack of information on the intermediate scheduled sacrifice. Overall, there is enough information and survival rates up to 16 months are sufficient for the conclusion that sodium benzoate up to 2% in the diet (ca 1400 mg/kg bw/d) has no carcinogenic effect in Fischer rats.

## Section A6.7 Carcinogenicity / Oral / Rat

**Annex Point IIA6.7**

| Evaluation by Competent Authorities | |
|---|---|
| | Use separate "evaluation boxes" to provide transparency as to the comments and views submitted |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | 2008/01/09 |
| **Materials and Methods** | Major corrections required as follows:<br>3.3.2 Interim sacrifice: Yes. No. of animals and time of sacrifice not specified.<br>3.3.3 Final sacrifice: between mo 18 and 25 at unspecified schedule<br>3.3.8 Vehicle: not specified<br>3.3.9 Concentration in vehicle: n/a<br>3.3.11 Controls: Plain diet<br>3.4.7 Haematology: not studied<br>3.4.8 Clinical chemistry: not studied<br>3.4.9 Urinanalysis: not studied<br>3.4.10.1 Organ weights: not studied<br>3.4.11 Histopathology: Unspecified organs ("various") of all animals by HE staining of unspecified numbers of paraffin embedded sections.<br>3.4.12 Other examinations: Gross morphologic examination (autopsy)<br>3.5 Statistics: t-test |
| **Results and discussion** | Applicant's version acceptable with changes as follows:<br>4.4 Clinical signs: not specified in the report<br>4.5 Macroscopic investigations: not specified in the report<br>4.12 Histopathology / 5.2 Results and Discussion: The number of tumors remained considerably lower in all groups compared to historical control data. Sass et al. (1975) reported the following incidences of spontaneous neoplasms in F344 rats. Leukemia: 25 %, mammary: 23/41 % (M/F), pituitary: 24/36 % (M/F), testicular interstitial: 85 % (M). Here, these types of tumors were reported at the following rates for the individual treatment groups. Leukemia: 0-8 %, mammary: 0-6 %, pituitary: 0-8 %, testicular interstitial: 14-32 % (M). |
| **Conclusion** | NOAEL: 20000 ppm (280/202 mg/kg bw/d (M/F)) |
| **Reliability** | 2 (see remarks) |
| **Acceptability** | Acceptable with limitations. |

## Section A6.7 Carcinogenicity / Oral / Rat

**Annex Point IIA6.7**

**Remarks**

Does not meet current relevant testing guidelines such as OECD 451 or EC method B.32. Based on the low no. of detected tumors, the reliability study is regarded as restricted. Data reporting was inappropriate and occasionally incorrect.

Three additional study summaries have been provided in Table A6_7-2. The methods used by Jagota and Dani (1985) and Spustova and Oravec (1989) were regarded as not suitable for risk assessment.

| Exposure, Species | NOAEL / LOAEL (mg/kg bw /d) | Main effects | Reference |
|---|---|---|---|
| Oral, 2.5 yr, Mouse, Sodium benzoate | 3000 / > 3000 (20000 / > 20000 ppm) | No changes in mortality and tumor incidence | Toth (1984) |
| Rat liver microsomes in vitro | n/a | Not suitable for risk assessment | Jagota and Dani (1985) |
| Cytotoxicity | n/a | Not suitable for risk assessment | Spustova and Oravec (1989) |

**COMMENTS FROM ...**

**Date** *Give date of comments submitted*

**Materials and Methods** *Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.*
*Discuss if deviating from view of rapporteur member state*

**Results and discussion** *Discuss if deviating from view of rapporteur member state*

**Conclusion** *Discuss if deviating from view of rapporteur member state*

**Reliability** *Discuss if deviating from view of rapporteur member state*

**Acceptability** *Discuss if deviating from view of rapporteur member state*

**Remarks**

**Table A6_7-1. Table for Clinical Chemistry, Haematology and Urinalysis (modify if necessary)**

**No data given**

**Table A6_7-2. Results of Carcinogenicity study**

| Parameter | control data | | | | low dose | | high dose | | dose-response + / | |
|---|---|---|---|---|---|---|---|---|---|---|
| | historical | | study | | | | | | | |
| | m | f | m | f | m | f | m | f | m | f |
| | | | | | | | | | | |
| Number of animals examined | - | - | 25 | 43 | 50 | 52 | 50 | 52 | n.a. | n.a. |
| Mortality | - | - | 11 | 39 | 42 | 12 | 35 | 19 | - | - |
| clinical signs | - | - | - | - | - | - | - | - | n.a. | n.a. |
| food consumption | - | - | - | - | - | - | - | - | n.a. | n.a. |
| Overall tumour incidence: | - | - | 9 | 8 | 7 | 16 | 11 | 11 | - | - |
| No. of animals with malignant neoplasms | - | - | 0 | 3 | 1 | 5 | 0 | 4 | - | - |
| leukaemia, malignant lymphoma, epidermoic carcinoma of thmaxilla | - | - | 0 | 3 | 1 | 5 | 0 | 4 | - | - |

**Table A6_7-2. Additional Data**

**Reference:** Toth; B., 1984 (A6.7/02)
**Duration:** Lifelong; sodium benzoate
**Dose:** 0-2% (average uptake: 0-124 mg/d for males, 0-119 mg/d for females; no data given for body weights)
Preliminary 6 week toxicity study (0.5-1-2-4-8% in the drinking water)
**Application:** Oral (in the drinking water)
**Species/Strain:** Mouse, Albino Swiss
**Animal number:** 50/sex
**Initial body weight:** Not reported, (age: 39 days)
**Control group:** 99/sex
**Parameters:** Clinical signs, body weight change, water consumption, survival rate, complete pathohistology (including the brain), and tumour rates
**Findings:** No adverse effect on survival rates or on the other parameters
**Conclusion:** No carcinogenic effect in Albino Swiss mice from sodium benzoate at 2% in the drinking water was evident in this study
NOEL for other lesions also at 2% in the drinking water (ca 120 mg/mouse/d).

**Reference:** Jagota, S.K. & Dani, H.M. 1985 (A6.7/03)

Microsomal degranulation test

A method for the detection of carcinogens indicated by degranulation of microsomes (loss of their ribosomes) is tested. Benzoic acid (no concentration given) was used as a known non-carcinogen. Eight known carcinogens caused a high percentage of microsomal degranulation, whereas benzoic acid together with 6 other known non-carcinogens failed to cause this effect. The authors recommended the technique as a quick, inexpensive and accurate screening for the prediction of environmental carcinogens.

**Reference:** Spustová, V. & Oravec, C. 1989 (A6.7/04)

*In vitro* test with mouse ascites tumour strains with benzoate

Ascites mouse carcinoma test with hippurate

In an *in vitro* test with several mouse ascites tumour strains, an anti-tumour effect of benzoate and its main metabolite hippurate was found. The concentration range was 0.5 to 5.0 mmolar.

The growth of ascites tumors implanted into mice four days before administration of hippurate (1000 mg/kg bw ip, twice daily, 4 days) could be reduced. The authors suggested to investigate the possible clinically relevant anti-tumour effect of hippurate further.

| **Route** | **Species Strain Sex no/group** | **Dose levels frequency of application** | **Results / Tumours** | **Reference** |
|---|---|---|---|---|
| Oral lifelong | Mouse Albino Swiss 50 males and 50 females per group | Sodium benzoate 0-2% (average uptake: 0-124 mg/d for males, 0-119 mg/d for females | No carcinogenic effect in Albino Swiss mice from sodium benzoate at 2% in the drinking water was evident in this study NOEL for other lesions also at 2% in the drinking water (ca 120 mg/mouse/d) No tumours | Toth; B., 1984 (A6.7/02) |
| Microsomal degranulation test | A method for the detection of carcinogens indicated by degranulation of microsomes (loss of their ribosomes) is tested. Benzoic acid (no concentration given) was used as a known non-carcinogen. Eight known carcinogens caused a high percentage of microsomal degranulation, whereas benzoic acid together with 6 other known non-carcinogens failed to cause this effect. | | | Jagota SK, Dani HM 1985 (A6.7/03) |
| *In vitro* test with mouse ascites tumour strains with benzoate | In an *in vitro* test with several mouse ascites tumour strains, an anti-tumour effect of benzoate and its main metabolite hippurate was found. The concentration range was 0.5 to 5.0 mmolar. | | | Spustová V, Oravec C, 1989 (A6.7/04) |
| Ascites mouse carcinoma test with hippurate | The growth of ascites tumors implanted into mice four days before administration of hippurate (1000 mg/kg bw ip, twice daily, 4 days) could be reduced. The authors suggested to investigate the possible clinically relevant anti-tumour effect of hippurate further | | | Spustová V, Oravec C, 1989 (A6.7/04) |

**Section A6.8.1/01** **Teratogenicity Study**
**Annex Point IIA6.8.1** **Rat / oral**

Official use only

| | | |
|---|---|---|
| | | **1 REFERENCE** |
| **1.1** | **Reference** | Onodera H, Ogiu T, Matsuoka C, Furuta K, Takeuchi M, Oono Y, Kubota T, Miyahara M, Maekawa A, Odashima S, 1978, Studies on effects of sodium benzoate on fetuses and offspring of Wistar rats<br>Eis. Shik. Hok., 96, 1978, 47-54 |
| **1.2** | **Data protection** | No |
| 1.2.1 | Data owner | Not applicable |
| 1.2.2 | Companies with letter of access | - |
| 1.2.3 | Criteria for data protection | No data protection / published data |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** |
| **2.1** | **Guideline study** | No. Study conducted according to prevailing standards and subsequently accepted for international registration. Results considered to be valid |
| **2.2** | **GLP** | No. GLP was not compulsory at the time the study was performed. |
| **2.3** | **Deviations** | Not applicable |
| | | **3 MATERIALS AND METHODS** |
| **3.1** | **Test material** | Sodium benzoate |
| 3.1.1 | Lot/Batch number | No lot/batch number available |
| 3.1.2 | Specification | No data given |
| 3.1.2.1 | Description | White crystalline solid substance |
| 3.1.2.2 | Purity | No data given |
| 3.1.2.3 | Stability | No data given |
| **3.2** | **Test Animals** | Non-entry field |
| 3.2.1 | Species | Rat |
| 3.2.2 | Strain | Wistar |
| 3.2.3 | Source | Nihon Rat K.K. (presently known as Hokudo Co., Ltd.) |
| 3.2.4 | Sex | Male and female |
| 3.2.5 | Age/weight at study initiation | Male 8 - 9 weeks old approx. 260 - 270 g<br>Female 7 - 10 weeks old approx. 260 - 270 g<br>after 4 weeks of rearing they were used for mating |
| 3.2.6 | Number of animals per group | 5 groups of 27 - 30 rats |
| 3.2.7 | Control animals | Yes |
| 3.2.8 | Mating period | 2 male rats cohabited with 5 nulliparous female rats during night time |
| **3.3** | **Administration/ Exposure** | Oral |

**Section A6.8.1/01** **Teratogenicity Study**
**Annex Point IIA6.8.1** **Rat / oral**

| | | |
|---|---|---|
| 3.3.1 | Duration of exposure | Rat: during the entire gestation period |
| 3.3.2 | Post exposure period | Off spring rats: 3 weeks and 8 weeks |
| | | **Oral** |
| 3.3.3 | Type | In feed |
| 3.3.4 | Concentration | 0, 1, 2, 4 and 8% in feed<br>Food consumption was measured every day.<br>Water consumption per day ad libitum |
| 3.3.5 | Vehicle | Solid feed (CE-2), manufactured by Nihon Kuna |
| 3.3.6 | Concentration in vehicle | 0, 1, 2, 4 and 8% in feed |
| 3.3.7 | Total volume applied | Total amount of sodium benzoate (g/kg) |

| Sodium benzoate (%) | Total amount of food consumption per rat | Sodium benzoate (g/kg) | |
|---|---|---|---|
| | | total amount | approx. per day |
| 0 | 413.3 | 0 | 0 |
| 1 | 415.5 | 14.0 | 0.71 |
| 2 | 394.5 | 26.2 | 1.33 |
| 4 | 242.0 | 37.5 | 1.90 |
| 8 | 54.1 | 19.3 | 0.98 |

| | | |
|---|---|---|
| 3.3.8 | Controls | Solid feed only |
| **3.4** | **Examinations** | On the 20th gestation day (one day before the expected delivery date) 22-25 rats from each group were sacrificed. The numbers of viable fetuses, dead fetuses, restored embryos, retention of the placenta, implants, as well as the weight of fetuses, weight of placenta, and weight of ovaries were recorded while the presence of visceral abnormalities in pregnant rats as well as external anomalies in fetuses were sought.<br>5 pregnant rats from each group underwent natural delivery, and the number of born offspring rats and survival rate were recorded at the time of birth.<br>After three weeks, approximately half of the young rats were sacrificed and after looking for visceral abnormalities, skeletal preparations were created for all of these cases in the same manner used for the fetuses.<br>The remaining half of weaned young rats were reared until they were 8 weeks old under observation. During this time, their weight and food consumption were measured once a week. |
| 3.4.1 | Body weight | Yes, **see table A6_8_1_01/1** and **A6_8_1_01/2** |
| 3.4.2 | Food consumption | Yes, daily |
| 3.4.3 | Clinical signs | Yes, **see table A6_8_1_01/3** and **A6_8_1_01/4** |
| 3.4.4 | Examination of uterine content | Gravid uterine weight - Yes, no further data given<br>Number of corpora lutea - No |

**Section A6.8.1/01** **Teratogenicity Study**
**Annex Point IIA6.8.1** **Rat / oral**

| | | | |
|---|---|---|---|
| | | Number of implants – Yes, **see table A6_8_1_01/1** and **A6_8_1_01/2** | |
| | | Number of dead fetuses and resorbed embryos– Yes, **see table A6_8_1_01/1** and **A6_8_1_01/2** | |
| 3.4.5 | Examination of foetuses | No entry field | |
| 3.4.5.1 | General | Sex, no. of dead foetuses, foetal weight, litter size, organ weight | |
| 3.4.5.2 | Skeleton | Yes, **see table A6_8_1_01/3** and **A6_8_1_01/4** | |
| 3.4.5.3 | Soft tissue | Yes, **see table A6_8_1_01/3** and **A6_8_1_01/4** | |
| 3.4.5.4 | Organ weight | Yes, **see table A6_8_1_01/5** | |
| **3.5** | **Further remarks** | | |
| | | **4 RESULTS AND DISCUSSION.** | |
| **4.1** | **Maternal toxic Effects** | At 4% and 8% decreased body weight gain and food consumption | |
| **4.2** | **Teratogenic / embryotoxic effects** | Adverse effects only at maternally toxic concentrations in the diet. At 4% and 8% decreased weight of fetuses (gestation day 20), shortly after birth 100% mortality of the offspring; increased incidences of abnormalities/malformations of organs (eye, kidney, brain) and skeletal system | |
| **4.3** | **Other effects** | No other effects observed | X |
| | | **5 APPLICANT'S SUMMARY AND CONCLUSION** | |
| **5.1** | **Materials and methods** | Sodium benzoate was assessed for its embryotoxic effects.<br>Because benzoic acid has poor solubility, sodium benzoate was used instead for this study. When using sodium benzoate, the dosage had to be increased by 1.2 times in order to obtain the same efficacy as that of benzoic acid | |
| **5.2** | **Results and discussion** | At 4% and 8% decreased body weight gain and food consumption of the mothers, decreased weight of fetuses (gestation day 20), shortly after birth 100% mortality of the offspring; increased incidences of abnormalities/malformations of organs (eye, kidney, brain) and skeletal system<br>At 1% and 2% no statistically significant difference in organ or skeletal abnormalities between control and these 2 experimental groups.<br>Number of fetuses with abnormalities at 0%, 1%, 2%, 4% and 8% were 0/41, 1/33, 1/41, 12/36 and 11/26, respectively; at 1%, the affected fetus showed bilateral anophthalmia, at 2%, the affected fetus showed unilateral pyelectasis; at 4% and 8% high incidences of eye and kidney abnormalities were evident. **See table A6_8_1_01/3** | |
| **5.3** | **Conclusion** | | |
| 5.3.1 | LO(A)EL maternal toxic effects | 1.90 g sodium benzoate/kg bw day / total 37.5 g/kg bw (approximately 1.60 g benzoic acid/kg bw day) | X |
| 5.3.2 | NO(A)EL maternal toxic effects | 1.33 g sodium benzoate/kg bw day / total 26.2 g/kg bw (approximately 1.11 g benzoic acid/kg bw day) | X |
| 5.3.3 | LO(A)EL embryotoxic / teratogenic effects | 1.90 g sodium benzoate /kg bw day / total 37.5 g/kg bw<br>Adverse effects only at maternally toxic concentrations in the diet | X |

| Section A6.8.1/01 | Teratogenicity Study | |
|---|---|---|
| Annex Point IIA6.8.1 | Rat / oral | |
| 5.3.4 NOEL embryotoxic / teratogenic effects | 1.33 g sodium benzoate /kg bw day / total 26.2 g/kg bw<br>Adverse effects only at maternally toxic concentrations in the diet | X |
| 5.3.5 Reliability | 2 | |
| 5.3.6 Deficiencies | Not applicable | |

| | Evaluation by Competent Authorities |
|---|---|
| | Use separate "evaluation boxes" to provide transparency as to the comments and views submitted |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | 2008/09/15 |
| **Materials and Methods** | Applicant's version acceptable. |
| **Results and discussion** | Applicant's version adopted with addition:<br>4.3 Other effects: Survival rate of the offspring at week 8 was 95.5 % in the 2 % dose group compared to 100 % in controls and the 1 % dose group. |
| **Conclusion** | Based on decreased survival and loss of bw:<br>maternal LOAEL: 4 % sodium benzoate in food (~ 1900 mg/kg bw/d)<br>maternal NOAEL: 2 % sodium benzoate in food (~ 1330 mg/kg bw/d)<br><br>Based on decreased number of offspring and increased rate of abnormalities:<br>developmental LOAEL: 4 % sodium benzoate in food (~ 1900 mg/kg bw/d)<br>developmental NOAEL: 2 % sodium benzoate in food (~ 1330 mg/kg bw/d)<br><br>Based on slightly decreased postnatal survival:<br>offspring LOAEL: 2 % sodium benzoate in food (~ 1330 mg/kg bw/d)<br>offspring NOAEL: 1 % sodium benzoate in food (~ 710 mg/kg bw/d) |
| **Reliability** | 2 |
| **Acceptability** | Acceptable. |
| **Remarks** | Substance tested: sodium benzoate. Doses of 710, 1330 and 1900 mg/kg sodium benzoate correspond to approx. 600, 1130 and 1610 mg/kg benzoic acid. |
| | **COMMENTS FROM ...** |
| **Date** | *Give date of comments submitted* |
| **Materials and Methods** | *Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.*<br>*Discuss if deviating from view of rapporteur member state* |
| **Results and discussion** | *Discuss if deviating from view of rapporteur member state* |
| **Conclusion** | *Discuss if deviating from view of rapporteur member state* |
| **Reliability** | *Discuss if deviating from view of rapporteur member state* |
| **Acceptability** | *Discuss if deviating from view of rapporteur member state* |
| **Remarks** | |

**Section A6.8.1/01** **Teratogenicity Study**
**Annex Point IIA6.8.1** **Rat / oral**

## Table A6_8_1_01/1 Table for Teratogenic effects (separate data for all dosage groups)

### Maternal effects

Modify if necessary and give historical data if available

| | Sodium benzoate | | | | | |
|---|---|---|---|---|---|---|
| **Parameter** | **Control** | **1 % 710 mg/ kg/day** | **2 % 1330 mg/ kg/day** | **4 % 1900 mg/ kg/day** | **8 % 980 mg/ kg/day** | **dose-response + / -** |
| **Number of dams examined** | **15** | **15** | **16** | **18** | **12** | - |
| **Clinical findings during application of test substance** | | | | | | |
| Areas of hairless | - | - | - | - | - | - |
| Scab formation | - | - | - | - | - | - |
| **Mortality of dams** *state %* | **0%** | **0%** | **0%** | **11%** | **17%** | + |
| **Abortions** | | - | - | - | - | - |
| **Body weight on day 0** | **265 g** | **265 g** | **260 g** | **270 g** | **265 g** | - |
| **Body weight on day 7** | **290 g** | **285 g** | **280 g** | **255 g** | **230 g** | + |
| **Body weight on day 14** | **325 g** | **310 g** | **305 g** | **260 g** | **215 g** | + |
| **Body weight on day 20** | **370 g** | **365 g** | **360 g** | **270 g** | **205 g** | + |
| **Body weight gain (mean day 0 – 21)** | **105 g** | **100 g** | **100 g** | **0 g** | **- 60 g** | + |
| **Food consumption, mean (g)** | | | | | | |
| **Food consumption (mean, day 1-7)** | **18** | **18** | **16** | **7** | **3** | + |
| **Food consumption (mean, day 7-14)** | **21** | **21** | **20** | **14** | **3** | + |
| **Food consumption (mean, day 14-17)** | **25** | **23** | **23** | **18** | **4** | + |
| **Food consumption (mean, day 17-20)** | **20** | **23** | **23** | **16** | **4** | + |
| **Water consumption** *if test substance is applied with drinking water* | - | - | - | - | - | - |
| **Necropsy findings in dams dead before end of test** | – | – | – | – | – | – |

**Section A6.8.1/01** **Teratogenicity Study**

**Annex Point IIA6.8.1** **Rat / oral**

**Table A6_8_1_01/2 Table for Teratogenic effects (separate data for all dosage groups)**

**Litter response (Caesarean section data)**

| | Sodium benzoate | | | | | |
|---|---|---|---|---|---|---|
| **Parameter** | **Control** | **1 % 710 mg/ kg/day** | **2 % 1330 mg/ kg/day** | **4 % 1900 mg/ kg/day** | **8 % 980 mg/ kg/day** | **dose-response + / -** |
| **Implants 20th gestation day** state total/number of dams | **194 / 15** | **179 / 15** | **194 / 16** | **213 / 18** | **141 / 12** | + |
| **Number of live fetuses** | **181** | **159** | **183** | **151** | **117** | + |
| **Number of dead fetuses** | **13** | **20** | **11** | **62** | **24** | + |
| **Total number of litters** | **15** | **15** | **16** | **18** | **12** | - |
| **Fetuses / litter** | **12.9** | **11.9** | **12.1** | **11.8** | **11.8** | - |
| **Live fetuses / litter** *state ratio* | **12.1** | **10.6** | **11.4** | **8.4** | **9.8** | + |
| **Dead fetuses / litter** *state ratio* | **0.8** | **1.3** | **0.7** | **3.4** | **2** | + |
| **Fetus weight (mean)** *[g]* | **3.70** | **3.71** | **3.63** | **2.62** | **2.22** | + |
| **Fetal sex ratio** *[state ratio m/f]* | **88/93** | **85/74** | **99/84** | **84/57** | **58/59** | - |
| | | | | | | |
| **Implants / natural delivery** state total/number of dams | **64 / 5** | **61 / 5** | **55 / 4** | **52 / 4** | **49 / 5** | - |
| **Number of live fetuses** | **48** | **49** | **45** | **26** | **4** | + |
| **Number of dead fetuses** | **16** | **12** | **10** | **26** | **45** | + |
| **Total number of litters** | **5** | **5** | **4** | **4** | **5** | - |
| **Fetuses / litter** | **12.8** | **12.2** | **13.8** | **13** | **9.8** | + |
| **Live fetuses / litter** *state ratio* | **9.6** | **9.8** | **11.3** | **6.5** | **0.8** | + |
| **Dead fetuses / litter** *state ratio* | **3.2** | **2.4** | **2.5** | **6.5** | **9.0** | + |
| **Rate of perinatal death** | **0%** | **0%** | **0%** | **100%** | **100%** | + |
| **Survival rate at 8 weeks** | **100%** | **100%** | **95.5%** | **0%** | **0%** | + |
| fetus weight (mean) **/ 3 weeks old male** [g] | **39.4** | **39.5** | **39.4** | - | - | - |
| fetus weight (mean) **/ 3 weeks old female** [g] | **40.0** | **39.5** | **37.3** | - | - | - |
| fetus weight (mean) **/ 8 weeks old male** [g] | **242.8** | **245.5** | **254.5** | - | - | - |
| fetus weight (mean) **/ 8 weeks old female** [g] | **190.0** | **187.8** | **184.0** | - | - | - |

**Section A6.8.1/01** **Teratogenicity Study**

**Annex Point IIA6.8.1** **Rat / oral**

**Table A6_8_1_01/3 Table for Teratogenic effects (separate data for all dosage groups)**

**Examination of the fetuses**

| | Sodium benzoate | | | | | |
|---|---|---|---|---|---|---|
| **Parameter** | **Control** | **1 % 710 mg/ kg/day** | **2 % 1330 mg/ kg/day** | **4 % 1900 mg/ kg/day** | **8 % 980 mg/ kg/day** | **dose-response + / -** |
| **External malformations 20th gestation day** [%] | **0** | **0** | **0** | **0** | **0** | **-** |
| **External anomalies** [%] | **0** | **0** | **0** | **11.3** | **0.9** | **+** |
| **Internal anomalies** [%] | **0** | **3** | **2.5** | **33.3** | **42.3** | **+** |
| Bilateral anophthalmia (number) | **0** | **1** | **0** | **0** | **0** | **-** |
| Unilateral pyelectasis (number) | **0** | **0** | **1** | **0** | **0** | **-** |
| Unilateral microphthalmia (number) | **0** | **0** | **0** | **5** | **6** | **+** |
| Bilateral microphthalmia (number) | **0** | **0** | **0** | **1** | **0** | **-** |
| Unilateral anophthalmia (number) | **0** | **0** | **0** | **2** | **1** | **+** |
| Hydrocephalus | **0** | **0** | **0** | **3** | **3** | **+** |
| Bilateral pyelectasis (number) | **0** | **0** | **0** | **2** | **2** | **+** |
| Unilateral renal hydroplasia (number) | **0** | **0** | **0** | **1** | **0** | **-** |
| Cerebral hydroplasia (number) | **0** | **0** | **0** | **0** | **1** | **-** |
| **Skeletal malformations (cleft palate)** [%] | **0** | **0** | **0** | **0** | **0** | **-** |
| **Skeletal anomalies** [%] | | | | | | |
| Ossification state (Number of ossified sacral and caudal vertebrae) | **8.61** | **8.48** | **8.47** | **5.53** | **4.89** | **+** |
| **Skeletal variants [number/examinated rats]** | | | | | | |
| Lumbar ribs | **32/140** | **29/126** | **45/142** | **49/115** | **15/91** | **+** |
| Cervical ribs | **0/140** | **0/126** | **0/142** | **5/115** | **4/91** | **+** |
| Varied sternebrae | **50/140** | **47/126** | **51/142** | **112/115** | **91/91** | **+** |
| Fusion of the thoracic arches | **1/140** | **0/126** | **1/142** | **0/115** | **0/91** | **-** |
| Spincule | **1/140** | **0/126** | **0/142** | **0/115** | **0/91** | **-** |
| Small pubis | **1/140** | **0/126** | **0/142** | **0/115** | **0/91** | **-** |
| Shortening of the cervical arches | **0/140** | **0/126** | **1/142** | **0/115** | **0/91** | **-** |
| Waved rib | **0/140** | **0/126** | **1/142** | **4/115** | **0/91** | **+** |
| Fusion of the cervical arches | **0/140** | **0/126** | **0/142** | **5/115** | **1/91** | **+** |
| Lack of cervical centrum | **0/140** | **0/126** | **0/142** | **1/115** | **0/91** | **-** |
| Deformed centrums | **0/140** | **0/126** | **0/142** | **3/115** | **1/91** | **+** |
| Fusion of ribs | **0/140** | **0/126** | **0/142** | **1/115** | **0/91** | **-** |
| Shortening of rib | **0/140** | **0/126** | **0/142** | **0/115** | **1/91** | **-** |
| Lack of rib | **0/140** | **0/126** | **0/142** | **0/115** | **1/91** | **-** |

## Table A6_8_1_01/4 Table for Teratogenic effects (separate data for all dosage groups)

**Examination of the offsprings / 8 weeks old**

| | Sodium benzoate | | | | | |
|---|---|---|---|---|---|---|
| **Parameter** | **Control** | **1 % 710 mg/ kg/day** | **2 % 1330 mg/ kg/day** | **4 % 1900 mg/ kg/day** | **8 % 980 mg/ kg/day** | **dose-response + / -** |
| **External malformations** [%] | **0** | **0** | **0** | - | - | - |
| **External anomalies** [%] | **0** | **0** | **0** | - | - | - |
| **Internal anomalies** [%] | **0** | **0** | **0** | - | - | - |
| **Skeletal variants [number/examinated rats]** | | | | | | |
| **Lumbar rib** | **3/48** | **1/49** | **0/42** | - | - | - |
| **Cervical rib** | **8/48** | **21/49** | **17/42** | - | - | - |
| **Varied sternebrae** | **0/48** | **2/49** | **0/42** | - | - | - |
| **Detachment of dorsal nodules of cervical vertebrae** | **0/48** | **0/49** | **1/42** | - | - | - |
| **Pathological findings [number/examinated rats]** | | | | - | - | - |
| **Inhibited growth** | **0/48** | **1/49** | **0/42** | - | - | - |
| **Delay of opening eyes** | **0/48** | **0/49** | **4/42** | - | - | - |
| **Urinary bladder stones** | **0/48** | **0/49** | **1/42** | - | - | - |

**Table A6_8_1_01/5. Table for Teratogenic effects (separate data for all dosage groups)**
**Average organ weight / 8 weeks old offsprings**

| Parameter | Sodium benzoate | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | | 1 % 710 mg/kg/day | | 2 % 1330 mg/kg/day | | Dose-response + / - |
| | M | F | M | F | M | F | |
| Thymus [g] | 0.714 | 0.717 | 0.866 | 0.590 | 0.801 | 0.604 | - |
| Spleen [g] | 0.616 | 0.509 | 0.594 | 0.465 | 0.730 | 0.548 | - |
| Heart [g] | 0.939 | 0.772 | 0.958 | 0.731 | 1.034 | 0.760 | - |
| Lung [g] | 1.597 | 1.264 | 1.519 | 1.181 | 1.311 | 1.175 | - |
| Liver [g] | 14.22 | 11.37 | 14.95 | 11.06 | 15.43 | 10.93 | - |
| Adrenal / right [g] | 0.018 | 0.024 | 0.022 | 0.028 | 0.027 | 0.028 | - |
| Adrenal / left [g] | 0.021 | 0.024 | 0.026 | 0.029 | 0.026 | 0.028 | - |
| Testis / right [g] | 0.991 | - | 1.023 | - | 1.008 | - | - |
| Testis / left [g] | 1.011 | - | 1.072 | - | 1.011 | - | - |
| Ovary / right [g] | - | 0.028 | - | 0.032 | - | 0.034 | - |
| Ovary / left [g] | - | 0.030 | - | 0.029 | - | 0.031 | - |
| Kidney / right [g] | 1.197 | 0.852 | 1.187 | 0.873 | 1.251 | 0.873 | - |
| Kidney / left [g] | 1.233 | 0.834 | 1.231 | 0.870 | 1.253 | 0.862 | - |

## Section A6.8.1/02 Teratogenicity Study
## Annex Point IIA6.8.1 Rat / oral

| | | | Official use only |
|---|---|---|---|
| | | **1 REFERENCE** | |
| **1.1** | **Reference** | Kimmel CA, Wilson JG, Schumacher HJ, 1971, Studies on metabolism and identification of the causative agent in aspirin teratogenesis in rats Teratology, 4, 15-24 | |
| **1.2** | **Data protection** | No | |
| 1.2.1 | Data owner | Not applicable | |
| 1.2.2 | Companies with letter of access | - | |
| 1.2.3 | Criteria for data protection | No data protection / published data | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **Guideline study** | No. Study conducted according to prevailing standards and subsequently accepted for international registration. Results considered to be valid | |
| **2.2** | **GLP** | No. GLP was not compulsory at the time the study was performed. | |
| **2.3** | **Deviations** | Not applicable | |
| | | **3 MATERIALS AND METHODS** | |
| **3.1** | **Test material** | Benzoic acid / As given in section 2 | |
| 3.1.1 | Lot/Batch number | No lot/batch number available | |
| 3.1.2 | Specification | As given in section 2 | |
| 3.1.2.1 | Description | White crystalline solid substance | X |
| 3.1.2.2 | Purity | >99% | X |
| 3.1.2.3 | Stability | The test substance was considered to be stable for the duration of the study. | |
| **3.2** | **Test Animals** | Non-entry field | |
| 3.2.1 | Species | Rat | |
| 3.2.2 | Strain | Wistar | |
| 3.2.3 | Source | Albino Farms | |
| 3.2.4 | Sex | Female | |
| 3.2.5 | Age/weight at study initiation | 200 – 250 g | |
| 3.2.6 | Number of animals per group | 7 - 10 | X |
| 3.2.7 | Control animals | Yes, 6 | |
| 3.2.8 | Mating period | Not applicable | |
| **3.3** | **Administration/ Exposure** | Oral | |
| 3.3.1 | Duration of exposure | | X |

**Section A6.8.1/02** **Teratogenicity Study**
**Annex Point IIA6.8.1** **Rat / oral**

| | | | |
|---|---|---|---|
| | | rat/mouse: day 9 after day 0 of gestation | |
| 3.3.2 | Postexposure period | 20 days | X |
| | | **Oral** | |
| 3.3.3 | Type | Gavage | |
| 3.3.4 | Concentration | Gavage<br>1. 510 mg/kg bw<br>2. 510 mg/kg bw and 2 hours later 250 or 500 mg/kg bw acetylsalicylic acid (aspirin), a known teratogen<br>For Determination of free salicylate in maternal serum and embryos:<br>3. 510 mg/kg bw and 2 hours later 500 mg/kg bw sodium salicylate ( (teratogenic effects similar to those of aspirin<br>Food and water consumption per day ad libitum | X |
| 3.3.5 | Vehicle | Benzoic acid: Moistened with carboxy-methylcellulose<br>Acetylsalicylic acid Moistened with carboxy-methylcellulose<br>Sodium salicylate Moistened with water | X |
| 3.3.6 | Concentration in vehicle | Benzoic acid: 10.2%<br>Acetylsalicylic acid 5% or 10%<br>Sodium salicylate 10% | |
| 3.3.7 | Total volume applied | 5 ml | |
| 3.3.8 | Controls | Vehicle only | |
| **3.4** | **Examinations** | | |
| 3.4.1 | Body weight | Yes, no further data | |
| 3.4.2 | Food consumption | No | |
| 3.4.3 | Clinical signs | Yes, no further data | |
| 3.4.4 | Examination of uterine content | Gravid uterine weight - No<br>Number of corpora lutea - No<br>Number of implantations – Yes, Implantation and resorption rate<br>Or other | |
| 3.4.5 | Examination of foetuses | No entry field | |
| 3.4.5.1 | General | Litter size, no. of dead foetuses, foetal weight | |
| 3.4.5.2 | Skelet | Yes | |
| 3.4.5.3 | Soft tissue | Yes | |
| **3.5** | **Further remarks** | The concentration of acetylsalicylic acid in maternal serum and in fetuses with and without pre-treatment by benzoic acid was determined. | |

**Section A6.8.1/02** **Teratogenicity Study**
**Annex Point IIA6.8.1** **Rat / oral**

| | | | |
|---|---|---|---|
| | | **4 RESULTS AND DISCUSSION.** | |
| **4.1** | **Maternal toxic Effects** | No effects | |
| **4.2** | **Teratogenic / embryotoxic effects** | No effects | X |
| **4.3** | **Other effects** | Benzoic acid significantly increased the salicylate concentration in both at 6 and 12 hours after salicylate treatment ($p < 0.05$ for serum, $p < 0.02$ for embryos).<br>Benzoic acid greatly increased the time for total salicylate excretion from the body and thereby increased the duration of embryo exposure. | |
| | | **5 APPLICANT'S SUMMARY AND CONCLUSION** | |
| **5.1** | **Materials and methods** | Benzoic acid was assessed for its teratogenic / embryotoxic effects.<br>Gavage on day 9 of gestation: 510 mg/kg bw or 510 mg/kg bw and 2 hours later 250 or 500 mg/kg bw acetylsalicylic acid (aspirin), a known teratogen.<br>For determination of free salicylate in maternal serum and embryos gavage of 510 mg/kg bw benzoic acid and 2 hours later 500 mg/kg bw sodium salicylate (teratogenic effects similar to those of aspirin). | |
| **5.2** | **Results and discussion** | No teratogenic effect after gavage of 510 mg/kg bw benzoic acid on day 9 of gestation.<br>After application of benzoic acid alone the survival and malformation rates (100% and 3%) were comparable with the rates in the vehicle control group (90% and 4%); historical control data: 96% and 1%.<br>The teratogenic effect of acetylsalicylic acid after 250 and 500 mg/kg bw was enhanced by pre-treatment with benzoic acid from rates of 21% and 76% after acetylsalicylic acid alone to rates of 43% and 91%.<br>The authors concluded, that benzoic acid prolonged the excretion time of acetylsalicylic acid, resulting in a longer exposition time for the fetuses. | |
| **5.3** | **Conclusion** | | |
| 5.3.1 | LO(A)EL maternal toxic effects | No data given | X |
| 5.3.2 | NO(A)EL maternal toxic effects | No data given | X |
| 5.3.3 | LO(A)EL embryotoxic / teratogenic effects | No data given | X |
| 5.3.4 | NOEL embryotoxic / teratogenic effects | 510 mg/kg bw | X |
| 5.3.5 | Reliability | 2 | X |
| 5.3.6 | Deficiencies | Not applicable | X |

**Section A6.8.1/02** **Teratogenicity Study**

**Annex Point IIA6.8.1** **Rat / oral**

| | **Evaluation by Competent Authorities** |
|---|---|
| | Use separate "evaluation boxes" to provide transparency as to the comments and views submitted |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | 2008/09/16 |
| **Materials and Methods** | Acceptable only with corrections as follows:<br>3.1.2.1 Description: not specified<br>3.1.2.2 Purity: not stated<br>3.2.6 Number of animals: 7<br>3.3.1 Duration of Exposure: Single exposure on day 9 of gestation<br>3.3.2 Postexposure Period: 11 days<br>3.3.4 Concentration: 510 mg/kg bw<br>3.3.5 Vehicle: 0.2 % carboxymethylcellulose (suspension) |
| **Results and discussion** | CA-Table 1 added.<br>4.2 Teratogenic/embryotoxic effects: No increase in the percentage of dead or resorbed fetuses and survivors with abnormalities at comparable number of implantations per animal. See Annex 1, CA-Table 1 (corrected tables A6_8-2 and A6_8-3)<br>Otherwise, the applicant's version is adopted. |
| **Conclusion** | No indications for strong embryotoxicity or teratogenicity when administered to rats at a single dose of 510 mg/kg on day 9 of gestation.<br>Maternal toxicity:<br>LOAEL: > 510 mg/kg bw (single dose, d 9)<br>NOAEL: 510 mg/kg bw (single dose, d 9)<br>Developmental toxicity:<br>LOAEL: > 510 mg/kg bw (single dose, d 9)<br>NOAEL: 510 mg/kg bw (single dose, d 9)<br>Benzoic acid may increase the (developmental) toxicity of other substances when given at high dosage. |
| **Reliability** | 3 (see remarks) |
| **Acceptability** | not acceptable (see remarks) |
| **Remarks** | The study was not designed to evaluate the teratogenicity of benzoic acid. Given the fast elimination of benzoic acid, single exposure on day 9 would be inappropriate. The number of exposed animals (7) is insufficient. There was only one dose group. No individual data on the type of abnormalities or malformations was provided.<br><br>Six additional study summaries have been provided in Table A6_8-4. Of these, Verret et al. (1980) is not relevant for human risk assessment. An original study report was only submitted for Crane and Lachane (1985). This was evaluated and relevant conclusions are summarised in CA-Table 2. |
| | **COMMENTS FROM ...** |
| **Date** | *Give date of comments submitted* |

## Section A6.8.1/02 Teratogenicity Study
## Annex Point IIA6.8.1 Rat / oral

| | |
|---|---|
| **Materials and Methods** | *Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.*<br>*Discuss if deviating from view of rapporteur member state* |
| **Results and discussion** | *Discuss if deviating from view of rapporteur member state* |
| **Conclusion** | *Discuss if deviating from view of rapporteur member state* |
| **Reliability** | *Discuss if deviating from view of rapporteur member state* |
| **Acceptability** | *Discuss if deviating from view of rapporteur member state* |
| **Remarks** | |

### Table A6_8_1_02/1. Table for Teratogenic effects (separate data for all dosage groups) Maternal effects

No data given

### Table A6_8_1_02/2. Table for Teratogenic effects (separate data for all dosage groups)

| Parameter | control data | | Gavage (mg/kg/bw) | | | | | dose-response + / - |
|---|---|---|---|---|---|---|---|---|
| | historical | study | Benzoic acid 510 | Aspirin 250 | Benzoic acid 510 + 250 Aspirin | Aspirin 500 | Benzoic acid 510 + 500 Aspirin | |
| **Total number of dams** | n.a. | **6** | **7** | **5** | **10** | **7** | **10** | n.a. |
| **Implantations** | - | 57 | 88 | 58 | 117 | 69 | 82 | n.a. |
| **Resorptions** | - | 6 | 0 | 2 | 12 | 30 | 42 | n.a. |
| **Total number of fetuses** | - | 63 | 88 | 60 | 129 | 89 | 124 | n.a. |
| **Implantation loss (%)** | 4 | 10 | 0 | 3 | 9 | 34 | 34 | n.a. |

### Table A6_8_1_02/3. Table for Teratogenic effects (separate data for all dosage groups) Examination of the fetuses

| Parameter | control data | | Gavage (mg/kg/bw) | | | | | dose-response + / - |
|---|---|---|---|---|---|---|---|---|
| | historical | study | Benzoic acid 510 | Aspirin 250 | Benzoic acid 510 + 250 Aspirin | Aspirin 500 | Benzoic acid 510 + 500 Aspirin | |
| **Malformation rates (%)** | **1** | **4** | **3** | **21** | **43** | **76** | **91** | n.a. |

### Table A6_8-4. Additional data Teratogenicity

**Rat**

**Reference:** FDR Lab., 1972 (cited in BUA, 1995, A6.8.1/03)
**Duration:** Day 6-15 of gestation; observation until day 20 of gestation; sodium benzoate
**Dose:** 0-1.75-8-38-175 mg/kg bw/d
**Application:** Oral (by gavage)
**Species/Strain:** Rat, Wistar, females
**Animal number:** 20 females

**Initial body weight:** No data given
**Control group:** Yes
**Parameters:** Maternal toxicity (no more data given), nidation rate, survival rate, abnormalities of soft and skeletal tissues
**Findings:** No adverse effect
**Conclusion:** The NOEL is at 175 mg/kg bw/d sodium benzoate (highest tested dose).

**Reference:** Crane SC, Lachance PA, 1985 (A6.8.1/04)
**Duration:** During the whole gestation and lactation period, pubs after weaning up to day 45 of age; sodium benzoate
**Dose:** 0-0.1-0.5-1% (ca 0-50-250-500 mg/kg bw/d)
**Application:** Oral (in the diet)
**Species/Strain:** Rat, Wistar
**Animal number:** 10 females/group
**Initial body weight:** 200-250 g
**Control group:** 10 females
**Parameters:** Body weight change, food consumption; spontaneous locomotor activity (at day 6, 9, 12, 15, 18, 21 of age and thereafter continuously up to termination); brain levels of serotonin, dopamine and norepinephrine (at day 9, 15, 21 of age and at termination), brain weight
**Findings:** No adverse effect
**Conclusion:** The NOEL is at 1% sodium benzoate in the diet (500 mg/kg bw/d).

**Rabbit**

**Reference:** FDR Lab., 1972 (cited in BUA, 1995, A6.8.1/03)
**Duration:** Day 6-18 of gestation; observation until day 29 of gestation; sodium benzoate
**Dose:** 0-2.5-12-54-250 mg/kg bw/d
**Application:** Oral (by gavage)
**Species/Strain:** Rabbit, Dutch-belted, females
**Animal number:** 14-32 female
**Initial body weight:** No data given
**Control group:** Yes
**Parameters:** Maternal toxicity (no more data given), nidation rate, survival rate, abnormalities of soft and skeletal tissues
**Findings:** No adverse effect
**Conclusion:** The NOEL is at 250 mg/kg bw/d sodium benzoate (highest tested dose).

**Mouse**

**Reference:** FDR Lab., 1972 (cited in BUA, 1995, A6.8.1/03)
**Duration:** Day 6-15 of gestation; observation until day 17 of gestation; sodium benzoate
**Dose:** 0-1.75-8-38-175 mg/kg bw/d
**Application:** Oral (by gavage)
**Species/Strain:** Mouse, CD-1, females
**Animal number:** 25-31 female
**Initial body weight:** No data given
**Control group:** Yes
**Parameters:** Maternal toxicity (no more data given), nidation rate, survival rate, abnormalities of soft and skeletal tissues
**Findings:** No adverse effect
**Conclusion:** The NOEL is at 175 mg/kg bw/d sodium benzoate (highest tested dose).

**Hamster**

**Reference:** FDR Lab., 1972 (cited in BUA, 1995, A6.8.1/03)
**Duration:** Day 6-10 of gestation; observation until day 14 of gestation; sodium benzoate
**Dose:** 0-3-14-65-300 mg/kg bw/d
**Application:** Oral (by gavage)
**Species/Strain:** Golden hamster, outbred, females
**Animal number:** 22 females
**Initial body weight:** No data given
**Control group:** Yes

**Parameters:** Maternal toxicity (no more data given), nidation rate, survival rate, abnormalities of soft and skeletal tissues
**Findings:** No adverse effect
**Conclusion:** The NOEL is at 300 mg/kg bw/d sodium benzoate (highest tested dose).

**Teratogenicity screening test, Hen**
**Reference:** Verrett, M.J. et al., 1980 (A6.8.1/05)
**Duration:** Single injection; sodium benzoate
**Dose:** Up to 5 mg/egg (only the highest of 5 doses is given)
**Application:** Injection into the eggs; four test conditions: injection via the air cell or via the yolk at preincubation (hour 0) or 96 hours after
**Species/Strain:** Hen, Leghorn
**Animal number:** Ca 25 chicken embryos/test condition (= ca 100/dose)
**Control group:** Ca 100/vehicle or untreated control
**Parameters:** Concerning acute toxicity and teratogenicity
**Findings:** LD50 (injection via air cell at 96 hours): 4.74 mg/egg
No teratogenic effect with sodium benzoate.
Ten of the simultaneously tested 79 compounds produced teratogenic effects
**Conclusion:** No teratogenic effect up to a dose of 5 mg sodium benzoate/egg (highest tested dose).
Remark: The authors propose this test for screening large numbers of compounds.

**Summary**

Taken together all teratogenic investigations, at a dose of ca 500 mg/kg bw/d neither maternaltoxic nor adverse effects on fetuses and offspring were evident. This is the same magnitude as the overall NOEL in short-term and chronic toxicity studies (basis for ADI).

| Test substance | Species | Result (mg/kg bw) | Reference |
|---|---|---|---|
| Benzoic acid | Rat | NOEL: 510 | Kimmel CA, Wilson JG, Schumacher HJ, 1971 (A6.8.1/02) |
| Sodium benzoate | Rat | NOEL: 175 (highest tested dose) | FDR Lab., 1972, cited in Anonymous, BUA Report, 1995 (A6.8.1/03) |
| Sodium benzoate | Rat | NOEL: ca. 500 | Crane SC, Lachance PA, 1985 (A6.8.1/04) |
| Sodium benzoate | Rabbit | NOEL: 250 (highest tested dose) | FDR Lab., 1972, cited in Anonymous, BUA Report, 1995 (A6.8.1/03) |
| Sodium benzoate | Mouse | NOEL: 175 (highest tested dose) | FDR Lab., 1972, cited in Anonymous, BUA Report, 1995 (A6.8.1/03) |
| Sodium benzoate | Golden hamster | NOEL: 300 (highest tested dose) | FDR Lab., 1972, cited in Anonymous, BUA Report, 1995 (A6.8.1/03) |

**Teratogenicity screening test**

| | | | |
|---|---|---|---|
| Sodium benzoate | Hen | NOEL: 5 mg per egg (highest tested dose) | Verrett MJ, Scott WF, Reynaldo EF, Alterman EK, Thomas CA,1980 (A6.8.1/05) |

**Annex 1. Evaluation by Rapporteur Member State**

**CA-Tables**

**CA-Table 1. Teratogenic effects (corrected tables A6_8-2 and A6_8-3)**

| Parameter | Historical control | Vehicle group | Benzoic acid (510 mg/kg bw, d 9) | treatment related effect |
|---|---|---|---|---|
| **Total no. of dams** | n.a. | 6 | 7 | - |
| **Average no. of implantations per dam** | n.a. | 10.5 | 12.5 | - |
| **Dead and resorbed fetuses (%)** | 4 | 10 | 0 | - |
| **Abnormalities (%)** | 1 | 4 | 3 | - |

**CA-Table 2. Additional studies**

| Duration | Species, Strain, No/group | Dose levels | Critical effects 1) dams 2) fetuses | NOAEL | Reference |
|---|---|---|---|---|---|
| from d 5 of gestation,during lactation to d 45,<br><br>Sodium benzoate | Rat, Wistar, 10 F (8 pups per litter group) | 0-1000-5000-10000 ppm<br><br>(~0-50-250-500 mg/kg bw/d) | 1 and 2) no effects on mortality, bw and food consumption | Maternal: 500 mg/kg bw/d<br>Developmental: 500 mg/kg bw/d | Crane and Lachane (1985) |

## Section A6.8.2
**Annex Point IIA6.8.2**

## Multigeneration Reproduction Toxicity Study
## Four generation study / Rat / oral

| | | | Official use only |
|---|---|---|---|
| | | **1 REFERENCE** | |
| **1.1** | **Reference** | Kieckebusch W, Lang K, 1960, Die Verträglichkeit der Benzoesäure im chronischen Fütterungsversuch<br>Arzneim. Forsch., 10, 1001-1003 (published) | |
| **1.2** | **Data protection** | No | |
| 1.2.1 | Data owner | Not applicable | |
| 1.2.2 | Companies with letter of access | - | |
| 1.2.3 | Criteria for data protection | No data protection / published data | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **Guideline study** | No. Study conducted according to prevailing standards and subsequently accepted for international registration. Results considered to be valid | |
| **2.2** | **GLP** | No. GLP was not compulsory at the time the study was performed. | |
| **2.3** | **Deviations** | Not applicable | |
| | | **3 MATERIALS AND METHODS** | |
| **3.1** | **Test material** | Benzoic acid / As given in section 2 | |
| 3.1.1 | Lot/Batch number | No lot/batch number available | |
| 3.1.2 | Specification | As given in section 2 | |
| 3.1.2.1 | Description | White crystalline solid substance | |
| 3.1.2.2 | Purity | >99% | |
| 3.1.2.3 | Stability | The test substance was considered to be stable for the duration of the study. | |
| **3.2** | **Test Animals** | Non-entry field | |
| 3.2.1 | Species | Rat | |
| 3.2.2 | Strain | Strain from Farbwerke Bayer AG, Elberfeld | |
| 3.2.3 | Source | Farbwerke Bayer AG, Elberfeld, Germany | |
| 3.2.4 | Sex | Male and female | |
| 3.2.5 | Age/weight at study initiation | Mean weight: 40 - 50 g | |
| 3.2.6 | Number of animals per group | 20 male and 20 female | |
| 3.2.7 | Mating | See table 6_8_2-1 below | |
| 3.2.8 | Duration of mating | 14 days | |
| 3.2.9 | Deviations from standard protocol | not applicable | |
| 3.2.10 | Control animals | Yes | |
| **3.3** | **Administration/ Exposure** | Oral | |
| 3.3.1 | Animal assignment to dosage groups | See table 6_8_2-1 below | |

| Section A6.8.2<br>Annex Point IIA6.8.2 | | Multigeneration Reproduction Toxicity Study<br>Four generation study / Rat / oral | |
|---|---|---|---|
| 3.3.2 | Duration of exposure before mating | 11 - 12 weeks | |
| 3.3.3 | Duration of exposure in general P, F1, F2 males, females | P, F1 (Generation 1 and 2):<br>F2 (Generation 3):<br>F3 (Generation 4): | lifelong<br>16 weeks (sacrifice)<br>until breeding |
| | | **Oral** | |
| 3.3.4 | Type | In food | |
| 3.3.5 | Concentration | Food: 0, 275 or 550 mg/kg bw/d<br>First 8 weeks: paired feed-Technic, then food and water consumption ad libitum | |
| 3.3.6 | Vehicle | Moistened with water, aqueous solution, corn oil or other | |
| 3.3.7 | Concentration in vehicle | 0%<br>0.5% = 0.225mmol/100g<br>1% = 0.450 mmol/100g | |
| 3.3.8 | Total volume applied | No data given | |
| 3.3.9 | Controls | Plain diet | |
| **3.4** | **Examinations** | | |
| 3.4.1 | Clinical signs | Yes | |
| 3.4.2 | Body weight | Yes | |
| 3.4.3 | Food/water consumption | No data given | |
| 3.4.4 | Oestrus cycle | No data given | |
| 3.4.5 | Sperm parameters | Testis weight | |
| 3.4.6 | Offspring | Number and sex of pups | |
| 3.4.7 | Organ weights F2 | Testes<br>Brain<br>Heart<br>Liver<br>Kidneys<br>Spleen | |
| 3.4.8 | Histopathology F2 | Animals of third generation after 16 weeks<br>Brain<br>Heart<br>Liver<br>Spleen<br>Kidneys<br>Testes | |
| 3.4.9 | Histopathology F1 not selected for mating, F2 | No data given | |
| **3.5** | **Further remarks** | | |
| | | **4 RESULTS AND DISCUSSION.** | |
| **4.1** | **Effects** | Non-entry field | |

## Section A6.8.2
**Annex Point IIA6.8.2**

## Multigeneration Reproduction Toxicity Study
## Four generation study / Rat / oral

4.1.1 Parent males and females

Body weight gain

| | | | Benzoic acid | | |
|---|---|---|---|---|---|
| | Control | | 0,5% | | 1% |
| | m | f | m | f | m |
| P (Generation 1) after | | | | | |
| 4 weeks | 70 ± 5.0 | 67 ± 4.1 | 69 ± 2.7 | 64 ± 2.4 | 75 ± 1.4 |
| 8 weeks | 159 ± 6.6 | 124 ± 5.2 | 160 ± 5.0 | 135 ± 5.4 | 147 ± 2.8 |
| 12 weeks | 219 ± 6.3 | - | 221 ± 5.6 | - | 237 ± 6.0 |

No effects on growth

Organ weight, see table A6_8_2-2

4.1.2 F1 males and females

Body weight gain

| | | | Benzoic acid | | |
|---|---|---|---|---|---|
| | Control | | 0,5% | | 1% |
| | m | f | m | f | m |
| F1 (Generation 2) after | | | | | |
| 4 weeks | 69 ± 2.3 | 57 ± 3.3 | 71 ± 2.2 | 60 ± 6.7 | 73 ± 3.3 |
| 8 weeks | 167 ± 2.0 | 122 ± 3.6 | 161 ± 2.6 | 131 ± 3.5 | 158 ± 4.6 |
| 12 weeks | 243 ± 5.5 | - | 238 ± 3.3 | - | 231 ± 5.6 |

No effects on growth

Organ weight, see table A6_8_2-2

4.1.3 F2 males and females

Body weight gain

| | | | Benzoic acid | | |
|---|---|---|---|---|---|
| | Control | | 0.5% | | 1% |
| | m | f | m | f | m |
| F2 (Generation 3) after | | | | | |
| 4 weeks | 73 ± 3.0 | 60 ± 3.5 | 68 ± 3.8 | 71 ± 2.4 | 72 ± 3.5 |
| 8 weeks | 157 ± 4.5 | 134 ± 4.1 | 158 ± 5.3 | 137 ± 3.9 | 168 ± 2.0 |
| 12 weeks | 240 ± 4.6 | - | 226 ± 5.8 | - | 233 ± 3.8 |

No effects on growth

Organ weight, see table A6_8_2-2

**Section A6.8.2** **Multigeneration Reproduction Toxicity Study**
**Annex Point IIA6.8.2** **Four generation study / Rat / oral**

4.1.4 F3 males and females

Body weight gain

| | | | Benzoic acid | | |
|---|---|---|---|---|---|
| | Control | | 0,5% | | 1% |
| | m | f | m | f | m |
| F3 (Generation 4) after | | | | | |
| 4 weeks | 93 ± 1.7 | 76 ± 4.8 | 84 ± 2.9 | 70 ± 3.1 | 73 ± 5.3 |
| 8 weeks | 189 ± 5.3 | 137 ± 4.3 | 168 ± 3.7 | 143 ± 2.4 | 159 ± 6.2 |
| 12 weeks | 255 ± 4.6 | - | 245 ± 4.4 | - | 240 ± 4.4 |

No effects on growth

Organ weight, see table A6_8_2-2

**4.2 Other**

Influence of benzoic acid on lifetime of rats

| | **% benzoic acid in food** | | |
|---|---|---|---|
| | **0** | **0.5** | **1.0** |
| **Number of animals** | **78** | **79** | **80** |
| **Mean age (days)** | **785 ± 20** | **899 ± 28** | **827 ± 25** |
| Age < 600 days | 13 | 8 | 13 |
| Age 601 - 800 days | 23 | 18 | 11 |
| Age 801 - 1000 days | 32 | 27 | 42 |
| Age > 1000 days | 10 | 26 | 14 |

No treatment related mortality.

Feeding of 0.5% benzoic acid in food led to prolongation of lifetime. The oldest rat has been 1346 days old and taken 200 g benzoic acid (1% in food) during its life.

## 5 APPLICANT'S SUMMARY AND CONCLUSION

**5.1 Materials and methods**

Benzoic acid was tested for its chronic toxicity.

3 groups of 20 male and 20 female rats received a daily dose of 0, 0.5 or 1% benzoic acid (0, 275 or 550 mg/kg bw) in food.

P, F1 (Generation 1 and 2): lifelong
F2 (Generation 3): 16 weeks (sacrifice)
F3 (Generation 4): until breeding

Rats of third generation were scarified after 16 weeks and brain, heart, liver, spleen, kidney and testes were examined.

**5.2 Results and discussion**

No treatment related mortality. Feeding of 0.5% benzoic acid in food led to prolongation of lifetime.

No treatment related changes in brain, heart, liver, spleen, kidney and testes.

The NOEL is 1% benzoic acid in the diet (550 mg/kg bw/d)

**5.3 Conclusion**

5.3.1 LO(A)EL

*Give critical effect and dose/concentration*

| Section A6.8.2<br>Annex Point IIA6.8.2 | Multigeneration Reproduction Toxicity Study<br>Four generation study / Rat / oral |
|---|---|
| 5.3.1.1 Parent males | No data given |
| 5.3.1.2 Parent females | No data given |
| 5.3.1.3 F1 males | No data given |
| 5.3.1.4 F1 females | No data given |
| 5.3.1.5 F2 males | No data given |
| 5.3.1.6 F2 females | No data given |
| 5.3.2 NO(A)EL | Non-entry field |
| 5.3.2.1 Parent males | 1% benzoic acid in the diet (550 mg/kg bw/d) |
| 5.3.2.2 Parent females | 1% benzoic acid in the diet (550 mg/kg bw/d) |
| 5.3.2.3 F1 males | 1% benzoic acid in the diet (550 mg/kg bw/d) |
| 5.3.2.4 F1 females | 1% benzoic acid in the diet (550 mg/kg bw/d) |
| 5.3.2.5 F2 males | 1% benzoic acid in the diet (550 mg/kg bw/d) |
| 5.3.2.6 F2 females | 1% benzoic acid in the diet (550 mg/kg bw/d) |
| 5.3.3 Reliability | 2 |
| 5.3.4 Deficiencies | Not applicable |

| Evaluation by Competent Authorities | |
|---|---|
| | Use separate "evaluation boxes" to provide transparency as to the comments and views submitted |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | 2008/01/10 |
| **Materials and Methods** | Acceptable with corrections as follows:<br>3.1.2.1 Description: no data provided<br>3.1.2.2 Purity: no data provided<br>3.3.5 Concentration: According to data provided in the study as well as default assumptions, 0, 5000 and 10000 ppm benzoic acid in the food would correspond to approximate average doses of 0, 250 and 500 mg/kg bw/d.<br>3.3.6 Vehicle: None<br>3.3.7 Concentration in vehicle: n/a<br>5.1 The first sentence must read: "… was tested for its reproductive toxicity." |
| **Results and discussion** | Parental and Offspring:<br>No increase in mortality or changes in bw and bw gain, no clinical signs of toxicity, no changes in organ weight (brain, heart, liver, spleen, kidneys, testes) or histopathology in male and female rats fed 0.5 and 1.0 % benzoic acid in the diet.<br>Reproductive:<br>No significant treatment related reduction in no. of pregnancies, no. of offspring, offspring survival or offspring bw development, no significant change in testes weight at 0.5 and 1.0 % benzoic acid in the diet compared to controls. |
| **Conclusion** | Parental/reproductive/offspring NOAEL: 10000 ppm (~500 mg/kg bw/d)<br>Parental/reproductive/offspring LOAEL: > 10000 ppm (> 500 mg/kg bw/d) |
| **Reliability** | 2 (see remarks) |
| **Acceptability** | Acceptable |
| **Remarks** | Does not meet current EU and OECD standards with regard to experimental design and reporting. |
| | **COMMENTS FROM ...** |

## Section A6.8.2 Multigeneration Reproduction Toxicity Study
**Annex Point IIA6.8.2** **Four generation study / Rat / oral**

| | |
|---|---|
| **Date** | *Give date of comments submitted* |
| **Materials and Methods** | *Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.*<br>*Discuss if deviating from view of rapporteur member state* |
| **Results and discussion** | *Discuss if deviating from view of rapporteur member state* |
| **Conclusion** | *Discuss if deviating from view of rapporteur member state* |
| **Reliability** | *Discuss if deviating from view of rapporteur member state* |
| **Acceptability** | *Discuss if deviating from view of rapporteur member state* |
| **Remarks** | |

**Table A6_8_2-1. Table for animal assignment for mating (modify as appropriate)**

| | | Number of animals | | |
|---|---|---|---|---|
| | | **Controls** | **Low Dose 0.5% (275 mg/kg/day)** | **High Dose 1% (550 mg/kg/day)** |
| **Parents** | **m** | 20 | 20 | 20 |
| | **f** | 20 | 20 | 20 |
| **$F_1$** | **m** | 20 | 20 | 20 |
| | **f** | 20 | 20 | 20 |
| **$F_2$** | **m** | 20 | 19 | 20 |
| | **f** | 20 | 19 | 20 |
| **$F_3$** | **m** | 20 | 19 | 20 |
| | **f** | 20 | 19 | 20 |
| **Total** | **m** | 80 | 78 | 80 |
| | **f** | 80 | 78 | 80 |

**Table A6_8_2-2. Table for reproductive toxicity study (modify if appropriate)**

**Organ weights (g) as % of body weight / rats of third generation after 16 weeks**

| | | | Benzoic acid | | | |
|---|---|---|---|---|---|---|
| | **Control** | | **0.5%** | | **1%** | |
| | **m** | **f** | **m** | **f** | **m** | **f** |
| Body weight | 236 ± 5 | 262 ± 5 | 329 ± 5 | 276 ± 5 | 316 ± 3 | 248 ± 6 |
| Brain | 0.57 ± 0.01 | 0.69 ± 0.02 | 0.58 ± 0.01 | 0.68 ± 0.01 | 0.61 ± 0.003 | 0.75 ± 0.02 |
| Heart | 0.34 ± 0.02 | 0.35 ± 0.01 | 0.35 ± 0.01 | 0.39 ± 0.01 | 0.33 ± 0.01 | 0.35 ± 0.01 |
| Liver | 3.49 ± 0.1 | 5.32 ± 0.3 | 3.34 ± 0.1 | 5.19 ± 0.2 | 3.49 ± 0.1 | 5.40 ± 0.2 |
| Spleen | 0.21 ± 0.00 | 0.25 ± 0.04 | 0.21 ± 0.04 | 0.23 ± 0.01 | 0.22 ± 0.01 | 0.25 ± 0.01 |
| Kidneys | 0.72 ± 0.02 | 0.80 ± 0.03 | 0.70 ± 0.04 | 0.79 ± 0.01 | 0.74 ± 0.02 | 0.78 ± 0.02 |
| Testes | 0.85 ± 0.03 | - | 0.83 ± 0.04 | - | 0.92 ± 0.01 | - |

No treatment related changes.

The higher liver weights of female rats related in higher liver weight in case of lactation. The differences in liver weight related in different litters.

## Table A6_8_2-2. Table for reproductive toxicity study (modify if appropriate)

**Data on reproduction**

| | % benzoic acid in food | | |
|---|---|---|---|
| | **0** | **0.5** | **1.0** |
| **Total number of females** | **80** | **78** | **80** |
| **Number of infertile females (%)** | 14 | 10 | 4 |
| **Number of females with delayed sexual maturity** | 7.5 | 17 | 9 |
| **Number of pups per litter** | 9.0 ± 0.35 | 9.5 ± 0.26 | 9.6 ± 0.29 |
| **Total number of pups** | 625 | 688 | 741 |
| **Raised pups (% of total number of pups)** | 74 | 66 | 65 |
| | | | |
| **Pairing in week 48** | | | |
| **Total number of females** | **37** | **38** | **38** |
| **Number of pups per litter** | 6.9 | 7.7 | 7.5 |
| **Total number of pups** | 173 | 139 | 171 |
| **Raised pups (% of total number of pups)** | 72 | 61 | 73 |

## Section A6.9 Neurotoxicity

**Annex Point IIA6.9**

Official use only

| | | |
|---|---|---|
| | | **1 REFERENCE** |
| **1.1** | **Reference** | Mattson JL, Albee RR, Gorzinski StJ, 1989. Similarities of toluene and o-cresol neuroexcitation in rats<br>Neurotoxicology and Teratology, 11, 1989, 71-75 |
| **1.2** | **Data protection** | No |
| 1.2.1 | Data owner | Not applicable |
| 1.2.2 | Companies with letter of access | - |
| 1.2.3 | Criteria for data protection | No data protection / published data |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** |
| **2.1** | **Guideline study** | No. Study subsequently accepted for international registration. Results considered to be valid |
| **2.2** | **GLP** | No. GLP was not compulsory at the time the study was performed. |
| **2.3** | **Deviations** | Not applicable |
| | | **3 MATERIALS AND METHODS** |
| **3.1** | **Test material** | Benzoic acid, as given in section 2 |
| 3.1.1 | Lot/Batch number | Lot number 648406, Baker Chemicals |
| 3.1.2 | Specification | As given in section 2 |
| 3.1.2.1 | Description | White crystalline solid substance |
| 3.1.2.2 | Purity | 99.9% |
| 3.1.2.3 | Stability | The test substance was considered to be stable for the duration of the study. |
| **3.2** | **Reference Substance (positive control)** | Toluene |

## Section A6.9 Neurotoxicity

**Annex Point IIA6.9**

| | | |
|---|---|---|
| **3.3** | **Test Animals** | Non-entry field |
| 3.3.1 | Species | Rat |
| 3.3.2 | Strain | Fischer 344 |
| 3.3.3 | Source | No data given |
| 3.3.4 | Sex | Male |
| 3.3.5 | Rearing conditions | Benzoic acid, o-cresol and hippuric acid Exposure:<br>The rats were placed in a restrainer and intravenously infused with benzoic acid. The vehicle was phosphate buffered saline. Benzoic acid (2% solution) was administered iv to two rats at approximately 2 mg/min for a total dose of 108 mg. SEPs were regularly monitored for the appearance of excitation.<br>Toluene Exposure:<br>The rats were placed in a restrainer and a beaker that contained gauze saturated with toluene was placed over the rat's nose. Exposure continued until the rats were in light anaesthesia (late Stage n or early Stage III, Plane I). Anaesthesia was maintained for several minutes in some animals and for several hours in others. SEPs were monitored every few minutes several hours in others. SEPs were monitored every few minutes during exposure. EEGs were occasionally recorded from the somatosensory cortex (nasal reference). Exposure was decreased if the SEP began to flattern, and was increased if the SEP began to lose the excitatory oscillations. Three rats were exposed one time only, and four were exposed for up to 7 hr on four different days (over a two week period). Four rats also were given iv toluene by jugular catheter at various rates to determine the rate of infusion necessary to induce SEP excitation. |
| 3.3.6 | Age/weight at study initiation | Weight: 225-275 g |
| 3.3.7 | Number of animals per group | Benzoic acid: to two rats<br>Toluene: Four rats were given iv toluene Three rats were also exposed one time only to toluene, and four were exposed for up to 7 hr on four different days (over a two week period). |
| 3.3.8 | Control animals | No |
| **3.4** | **Administration** | iv |
| 3.4.1 | Exposure | Single dose |
| 3.4.2 | Dose Levels | Benzoic acid: 2% solution, approximately 2 mg/min for a total dose of 108 mg.<br>Toluene: 5 mg |
| 3.4.3 | Vehicle | Phosphate buffered saline |
| 3.4.4 | Concentration in vehicle | 2% |
| 3.4.5 | Total volume applied | No data given |
| 3.4.6 | Postexposure period | No |
| 3.4.7 | Anticholinergic substances used | No |
| 3.4.8 | Controls | No controls |

## Section A6.9 Neurotoxicity

**Annex Point IIA6.9**

| | | |
|---|---|---|
| **3.5** | **Examinations** | |
| 3.5.1 | Body Weight | No data given |
| 3.5.2 | Signs of Toxicity | Somatosensory evoked potential (SEP):<br>SEPs were recorded from the somatosensory cortex, with a nasal reference. Ventrolateral caudal nerves were stimulated at the base of the tail and a response was recorded at the somatosensory cortex. The stimulating electrodes were small needles set into the bottom of a plastic tray that fit the tail (7). A 3 mA, 50 μsec electric pulse was presented at 1,1 pulse/sec. The amplifier bandpass settings were1-\500 Hz. Simultaneous recordings were made with sweep durations of 35 and 150 msec each. The final SEP was an average of 200 sweeps for each duration. |
| 3.5.3 | Observation schedule | Benzoic acid: SEPs were collected at 15 min intervals.<br>Toluene: SEPs were collected after 15 to 60 min of exposure. |
| 3.5.4 | Clinical Chemistry | No<br>Number of animals: -<br>Time points: -<br>Parameters: - |
| 3.5.5 | Pathology | No<br>Organs: - |
| 3.5.6 | Histopathology | No<br>Organs: -<br>- |
| **3.6** | **Further remarks** | |

**4 RESULTS AND DISCUSSION**

| | | |
|---|---|---|
| **4.1** | **Body Weight** | No data given |
| **4.2** | **Clinical signs of toxicity** | **Somatosensory evoked potential**<br>Benzoic acid and hippuric acid:<br>Rats given iv benzoic acid showed no evidence of SEP excitation<br>Toluene:<br>Intravenous toluene caused SEP excitation. A rapid IV bolus of 5 mg toluene would cause excitation within a minute, and steady infusion at roughly 40 mg/min would cause excitation after 5 to 10 minutes. It seemed, therefore, that many combinations of dose, dose interval, and dose duration might cause toluene neuroexcitation.<br>O-cresol:<br>readily induced SEP excitation. |
| **4.3** | **Clinical Chemistry** | No data given |
| **4.4** | **Pathology** | No data given |
| **4.5** | **Histopathology** | No data given |

**5 APPLICANT'S SUMMARY AND CONCLUSION**

## Section A6.9 Neurotoxicity

**Annex Point IIA6.9**

| | | |
|---|---|---|
| **5.1** | **Materials and methods** | The neuroexcitation potential of benzoic acid as a metabolite of toluene was examined. Benzoic acid (2% solution) was intravenously administered to rats at a rate of ca 2 mg/min, resulting in a total dose of 108 mg. Somatosensory evoked potentials (SEP) were recorded from the somatosensory cortex with nasal reference. The results were compared to tests with toluene, o-cresol and hippuric acid as the main metabolite of benzoic acid. |
| **5.2** | **Results and discussion** | Neither benzoic acid nor hippuric acid did cause neuroexcitation or changes in EEG.<br>Neurotoxicity studies including testing of delayed neurotoxicity according to current guidelines are not available. There are no results in other studies indicating any necessity of neurotoxicity testing. |
| **5.3** | **Conclusion** | |
| 5.3.1 | LOAEL | No data given |
| 5.3.2 | NOAEL | 108 mg / iv |
| 5.3.3 | Reliability | 2 |
| 5.3.4 | Deficiencies | Not applicable |

| | **Evaluation by Competent Authorities** |
|---|---|
| | Use separate "evaluation boxes" to provide transparency as to the comments and views submitted |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | 2012/02/13 |
| **Materials and Methods** | Acceptable with additions:<br>3.3.7 Number of animals per group: SEP – 2 animals, SEP oscillations – 6 animals<br>3.3.8 Control animals: internal controls by derivation of potential prior to infusion<br>3.4.1 Exposure: i.v. infusion over 60 min<br>3.5.2 / 5.1: In addition, SEP oscillations in the frequency band between 30 and 120 Hz were assessed in 6 animals prior to and during benzoic acid infusion. |
| **Results and discussion** | Benzoic acid administered by i.v. infusion over 60 min at an approximate dose of 500 mg/kg bw did not affect somatosensory evoked potential (SEP) and SEP osciallations in male rats. |
| **Conclusion** | Neuroexcitation (somatosensory evoked potential):<br>LOAEL: > 500 mg/kg bw/d (M)<br>NOAEL: ~500 mg/kg bw/d (M) |
| **Reliability** | 3 (see remarks) |
| **Acceptability** | with restrictions |
| **Remarks** | The number of animals in the benzoic acid group is too low to provide reliable results |
| | **COMMENTS FROM ...** |
| **Date** | *Give date of comments submitted* |

Gelöscht: 2008/01/11

Gelöscht: 2

Gelöscht: Acceptable

Gelöscht: None

## Section A6.9 Neurotoxicity

**Annex Point IIA6.9**

| | |
|---|---|
| **Materials and Methods** | *Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.*<br>*Discuss if deviating from view of rapporteur member state* |
| **Results and discussion** | *Discuss if deviating from view of rapporteur member state* |
| **Conclusion** | *Discuss if deviating from view of rapporteur member state* |
| **Reliability** | *Discuss if deviating from view of rapporteur member state* |
| **Acceptability** | *Discuss if deviating from view of rapporteur member state* |
| **Remarks** | |

| | | |
|---|---|---|
| **Section A6.12**<br>**Annex Point IIA6.12** | **Human Case Report**<br>**6.12.1 Medical surveillance data an manufacturing plant personnel** | Official use only |
| | **1 REFERENCE** | |
| | Anonymous, 2001, OECD-SIDS, 2001 (A6.12.1) | |
| | **2 RESULTS** | |
| | THE OECD-SIDS (SIDS Initial Assessment Report for 13th SIAM (Bern, 7th - 9th November 2001) states:<br>In the several past decades of production, no cases of health complaints (sensitisation inclusive) have occurred.<br>Also from companies that use the substances no health complaints (sensitisation inclusive) have ever been reported. | |
| | **Evaluation by Competent Authorities** | |
| | *Use separate "evaluation boxes" to provide transparency as to the comments and views submitted* | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | 2008/04/10 | |
| **Evaluation of applicant's justification** | Applicant's version is adopted. | |
| **Conclusion** | Applicant's version is acceptable. The OECD-SIDS is cited correctly. | |
| **Remarks** | | |
| | **COMMENTS FROM OTHER MEMBER STATE** *(specify)* | |
| **Date** | *Give date of comments submitted* | |
| **Evaluation of applicant's justification** | *Discuss if deviating from view of rapporteur member state* | |
| **Conclusion** | *Discuss if deviating from view of rapporteur member state* | |
| **Remarks** | | |

**Section A6.12** **Human Case Report** Official use only

**Annex Point IIA6.12** **6.12.2 Direct observations, e.g. clinical cases and poisoning incidents**

## 1 REFERENCE

Bignani G, 1924, Ricerche sulla sintesi ippurica nell organismo umano, Biochim. Ter. Sper., 11, 383-393 (A6.12.2/02)

Gerlach V, 1909, Physiologische Wirkungen der Benzoesäure und des benzoesauren Natron. VII. Zusammenfassung der Resultate, Gerlach V, (ed.); Verlag von Heinrich Staadt, Wiesbaden, Germany (A6.12.2/03)

Swanson WW, 1925, The effect of sodium benzoate ingestion upon the composition of the blood and urine with especial reference to the possible synthesis of glycine in the body, J. Biol. Chem., 62, 565-673 (A6.12.2/04)

Wiley HM, Bigelow WD, 1908, Influence of benzoic acid and benzoates on digestion and health. Bulletin 84, Pt. IV, Bureau of Chemistry, U. S. Department of Agriculture, cited in Anonymous, GRAS, 1972, (A6.12.2/01)

## 2 RESULTS

**Experiments with human volunteers**

| Kind of study Doses | Number of humans | Results | References |
|---|---|---|---|
| Acute<br>Single dose of 21 to 42 g benzoic acid | 5 | Excreted nearly completely within few hours | Bignani G, 1924 (A6.12.2/02) |
| Acute<br>Single dose of 10 g benzoic acid within 3.5 hour<br><br>0.5 or 1 g benzoic acid or sodium benzoate | 1 (self-experiment) | No observed effect on respiration, body temperature and pulse<br><br>No effect on total acidity of gastric juice, free HCl and digestion | Gerlach V, 1909 (A6.12.2/03) |
| Acute<br>Single dose of 3 to 10 g sodium benzoate administered | 5 male | Decrease of urea and uric acid in urine and an increase of uric acid (but not of urea) in blood and plasma. No symptoms were reported | Swanson WW, 1925 (A6.12.2/04) |
| Subacute<br>Daily administration of 1 g benzoic acid or 1.5 g sodium benzoate for 6 days<br><br>Daily intake of 0.5 g, 1 g, 2 g benzoic acid or 1.5 g or 2.0 g sodium benzoate for 44 days | No data given | No effect on total protein and on the utilization of nitrogen and lipid components of the food.<br><br>No symptoms and no influence on body weight, body temperature, respiration and pulse. | Gerlach V, 1909 (A6.12.2/03) |

| | | | |
|---|---|---|---|
| Daily intake of 1 g benzoic acid over a period of about 90 days | | Did not cause unfavourable effects | |
| Subacute Total administration of 35 g for 20 days (1 g for 5 days, 1.5 g for 5 days, 2 g for 5 days and 2.5 g for 5 days) | 12 | Benzoic acid produced marked symptoms as discomfort, malaise, nausea, headache, weakness, burning and irritation of the oesophagus and indigestion. Only 3 volunteers took the intended entire dose of 35 g. | Wiley HM, Bigelow WD, 1908, cited in Anonymous, GRAS, 1972 (A6.12.2/01) |

**3 CONCLUSION**

Benzoic acid is not acute toxic to humans. Signs of toxicity after continued intake of several grams per day are unspecific and partly attributed to the acidic nature of the test article (discomfort, malaise, nausea, headache, weakness, burning and irritation of the oesophagus and indigestion).

Additional data are available and reported for medical use (e.g. Quick test, single oral 6 g to test the liver function, (A6.12.3/05)).

## Evaluation by Competent Authorities

*Use separate "evaluation boxes" to provide transparency as to the comments and views submitted*

**EVALUATION BY RAPPORTEUR MEMBER STATE**

| | |
|---|---|
| **Date** | 2008/01/10 |
| **Evaluation** | Applicant's version is acceptable. |
| **Conclusion** | Applicant's version is acceptable. |
| **Remarks** | None |

**COMMENTS FROM OTHER MEMBER STATE** *(specify)*

| | |
|---|---|
| **Date** | *Give date of comments submitted* |
| **Evaluation of applicant's justification** | *Discuss if deviating from view of rapporteur member state* |
| **Conclusion** | *Discuss if deviating from view of rapporteur member state* |
| **Remarks** | |

**Section A6.12** **Human Case Report** Official use only

**Annex Point IIA6.12** **6.12.3 Health records**

**1 References**

Batshaw ML, Painter MJ, Sproul GT, Schafer IA, Thomas GH, Brusilow S, 1981, Therapy of urea cycle enzymopathies: Three case studies, The Johns Hopkins Medical Journal, 148, 34-40 (A6.12.3/01)

Brusilow SW, Maestri NE, 1996, Urea cycle disorders: Diagnosis, pathophysiology and therapy, Adv Pediatr, 43, 127-170 (A6.12.3/02)

Feillet F, Leonard JV, 1998, Alternative pathway therapy for urea cycle disorders, J. Inher. Metab. Dis., 21, 1, 101-111 (A6.12.3/03)

Green TP, Marchessault RP, Freese DK, 1983, Disposition of sodium benzoate in Existborn infants with hyperammonemia, The Journal of Pediatrics, 102, 785-790 (A6.12.3/04)

Quick AJ, 1931, The conjugation of benzoic acid in man, The Journal of Biological Chemistry, XCII, 1, 65-85 (A6.12.3/05)

Senator H, 1879, Über die Wirkung der Benzoesäure bei der rheumatischen Polyarthritis, Zeitschr. f. klin. Medicin, 1, 2, 243-264 (A6.12.3/06)

Takeda E, Kuroda Y, Toshima K, Watanabe T, Naito E, Miyao M, 1983, Effect of long-term administration of sodium benzoate to a patient with partial ornithine carbamoyl transferase deficiency, Clinical Pediatrics, 22, 3, 1983, 206-208 (A6.12.3/07)

Waldo JF, Masson JM, Lu W, Tollstrup J, 1948, The effect of benzoic acid and caronamide on blood penicillin levels and on renal function, Amer. J. Med. Sci., 117, 563-568 (A6.12.3/08)

**2 Results**

**Experiences with medical use of benzoic acid/benzoates**

| Kind of study Doses | Number of humans | Results | References |
|---|---|---|---|
| Patient with partial ornithine transcarbamoylase deficiency (hyperammonemia) 360 mg sodium benzoate per day for 13 month | 1 boy (8 month) | Effective in reducing the frequency and severity of hyperammonemia in this patient | Batshaw ML, Painter MJ, Sproul GT, Schafer IA, Thomas GH, Brusilow S, 1981 (A6.12.3/01) |
| Protection against hyperammonemia, or genetic effects in the urea cycle Sodium benzoate IV or oral. | 6 | Therapy with drugs or haemodialysis will usually correct the hyperammonemia and may prevent or minimise brain damage. | Brusilow SW, Maestri NE, 1996 (A6.12.3/02) |
| Urea cycle disorders Sodium benzoate and diet | - | Sodium benzoate could be used to increase waste nitrogen excretion. Combined with diet: remarkably | Feillet F, Leonard JV, 1998 (A6.12.3/03) |

| | | | |
|---|---|---|---|
| | | effective. | |
| Protection against hyperammonemia<br>Sodium benzoate<br>125 mg/kg IV | 4<br>Newborn | There was a considerable interpatient variability in benzoate metabolism, parameters as glycine, benzoate, hippurate and/or ammonia levels in plasma and urine have to be carefully monitored to avoid any toxic effect of benzoate. | Green TP, Marchessault RP, Freese DK, 1983 (A6.12.3/04) |
| Test on liver function (making use of the conversion of benzoic acid to hippuric acid)<br>Oral 6 g benzoic acid | 3 | The most important factor in the excretion of hippuric acid is the rate of synthesis of glycine in the body. | Quick AJ, 1931 (A6.12.3/05) |
| Treatment of rheumatic arthritis<br>benzoic acid<br>4 to 6 g per day<br>10 to 25 g per day | 46 patient with acute symptoms | Effective against rheumatic arthritis. Unfavourable effects were not reported. | Senator H, 1879 (A6.12.3/06) |
| Patient with partial ornithine carbamoyl transferase deficiency (hyperammonemia)<br>200 mg sodium benzoate per day for 13 month | 1 girl (8 years) | Effective in reducing the frequency and severity of hyperammonemic attacks in this patient. | Takeda E, Kuroda Y, Toshima K, Watanabe T, Naito E, Miyao M, 1983 (A6.12.3/07) |
| Increase of the penicillin level in blood<br>12 g benzoic acid (divided into 8 administrations per day) for five days (in one case for 14 days) | 11 | No influence on the blood urea nitrogen levels and on the endogenous creatinine clearance, i.e. no renal impairment became evident under these conditions. About one third of the patients complained of gastric burning and anorexia | Waldo JF, Masson JM, Lu W, Tollstrup J, 1948 (A6.12.3/08) |

The data are examples for medical use. Generally, the first reported use for a specific indication is given. It is virtually impossible to list all data and reports on oral medical use, and it is unnecessary for the purpose of this dossier as well, because the concentrations used for biocidal purposes are much lower than for medical uses.

| Evaluation by Competent Authorities | |
|---|---|
| | *Use separate "evaluation boxes" to provide transparency as to the comments and views submitted* |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | 2008/01/10 |
| **Evaluation** | Applicant's version is acceptable. |
| **Conclusion** | Applicant's version is acceptable. |
| **Remarks** | Since 1979 benzoic acid is used for the acute and long-term treatment of ornithine transcarbamylase deficiency (OTCD), a X-linked chromosomal disorder with a high mortality rate. The recommended maintenance dose for long-term treatment starting in neonates is 250 mg/kg bw/d sodium benzoate (oral, or i.v.) while doses for the treatment of acute hyperammonemia in neonates can be as high as 4380 mg/kg bw/d i.v. (Enns GM, Berry SA, Berry GT, Rhead WJ, Brusilow SW, Harmosh A. New Engl J Med 356(22):2282-2292 (2007)). |
| | **COMMENTS FROM OTHER MEMBER STATE** *(specify)* |
| **Date** | *Give date of comments submitted* |
| **Evaluation of applicant's justification** | *Discuss if deviating from view of rapporteur member state* |
| **Conclusion** | *Discuss if deviating from view of rapporteur member state* |
| **Remarks** | |

| | | |
|---|---|---|
| **Section A6.12**<br>**Annex Point IIA6.12** | **Human Case Report**<br>**6.12.4 Epidemiological studies on the general population** | Official use only |
| | **1 REFERENCE** | |
| | Wibbertmann A et al., 2000, Concise international chemical assessment document No 26 Benzoic Acid and Sodium Benzoate<br>IPCS (International Programme on chemical safety), 1. Draft, prepared by Wibbertmann, A. (A6.12.4) | |
| | **2 RESULTS** | |
| | The food category that contributed most to benzoate intake was soft drinks (Australia/New Zealand, Finland, France, UK and US), Soya sauce (China and Japan).<br><br>Estimates of the mean intake of benzoates with food performed for consumers were in a range of 0.18 mg/kg bw/d (Japan) to 14 mg/kg bw/d (China) (12.6 to 980 mg per 70 kg body weight).<br><br>In this range is the average value of 312 mg benzoic acid (sum of acid and salts) for the human daily intake per capita in USA.<br><br>Intake in Europe is expected to be in the same range. Additional exposure of the general population from the use of benzoic acid as disinfectant in veterinary hygiene can be excluded.<br><br>Studies (also epidemiological studies) in connection with the known pseudoallergic reactions (non-immune immediate contact reactions, NIICRs) of benzoic acid/benzoates: see A6.12.6 | |
| | **3 CONCLUSION**<br>From the use of benzoic acid as disinfectant, any additional exposure of the general population can be excluded or is negligible compared to the mean intake by daily food. | |
| | **Evaluation by Competent Authorities** | |
| | *Use separate "evaluation boxes" to provide transparency as to the comments and views submitted* | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | 2008/01/10 | |
| **Evaluation** | Applicant's version is acceptable. | |
| **Conclusion** | Applicant's version is acceptable. | |
| **Remarks** | None | |
| | **COMMENTS FROM OTHER MEMBER STATE** *(specify)* | |
| **Date** | *Give date of comments submitted* | |
| **Evaluation of applicant's justification** | *Discuss if deviating from view of rapporteur member state* | |
| **Conclusion** | *Discuss if deviating from view of rapporteur member state* | |

**Remarks**

**Section A6.12**
**Annex Point IIA6.12**

# Human Case Report

**6.12.6 Sensitisation/allergenicity observations**

Official use only

## 1 REFERENCE

Due to the large volume of Benzoic acid and its salts being marketed as food additives, there is a large body of information on studies, and in particular on allergenicity observations. In this summary only the most important reports are cited as example. X


Baer RL, Serri F A, Weissenbach-Vial C, 1955, Studies on allergic sensitization to certain topical therapeutic agents, AMA Arch. Dermatol. Syphil., 71, 19-23 (A6.12.6/01)

Basketter DA, Wilhelm K-P, 1996, Studies on non-immune immediate contact reactions in an unselected population, Contact Dermatitis, 35, 1996, 237-240 (A6.12.6/02)

Brasch J, Henseler T, Frosch P, 1993, Patch test reaction to a preliminary preservative series, Dermatosen, 41, 2, 71-76 (A6.12.6/03)

Broeckx W, Blondeel A, Dooms-Goossens A, Achten G, 1987, Cosmetic intolerance, Contact Dermatitis, 16, 189-194 (A6.12.6/04)

Genton C, Frei PC, Pecoud A, 1985, Value of oral provocation tests to aspirin and food additives in the routine investigation of asthma and chronic urticaria, J. Allergy Clin. Immunol., 76, 40-45 (A6.12.6/05)

Lahti A, 1980, Non-immunologic contact urticaria, Acta Derm Venereol, 60, 91, 49 pages (A6.12.6/06)

Lahti A et al., 1993, Alcohol vehicles in tests for non-immunologic immediate contact reactions, Contact Dermatitis, 29, 22-25 (A6.12.6/0107)

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Ros A, Juhlin L, Michaelsson G, 1976, A follow-up study of patients with recurrent urticaria and hypersensitivity to aspirin, benzoates and azo dyes, British Journal of Dermatology, 95, 19-24 (A6.12.6/13)

Schaubschläger WW, Becker WM, Schade U, Zabel P, Schlaak M, 1980, Release of mediators from human gastric mucosa and blood in adverse reactions to benzoate, Int. Arch. Allergy Appl. Immunol., 96, 1991, 97-101 (A6.12.6/14)

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Ylipieti S, Lahti A, 1989, Effect of the vehicle on non-immunologic immediate contact reactions, Contact Dermatitis, 21, 105-106 (A6.12.6/16)

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## 2 RESULTS

### Allergenicity

**Non-immune immediate contact reactions (NIICR's)**

Benzoic acid does not cause allergic reactions.
Benzoic acid and its salts are known as pseudoallergic substances which may provoke syndromes like urticaria, rhinitis or asthma.
The pseudoallergic reactions mimic signs and symptoms of allergic disorders but without underlying immunologic mechanisms. Therefore these are coined non-immune immediate contact reactions (NIICRs) including non-immune contact urticaria (NICU), in contrast to immunological contact urticaria which is mediated at least partly by specific IgE antibodies.

Investigations of Lahti, 1980 (A6.12.6/06) indicated that NIICRs due to benzoates are not mediated by histamine. The reactions may be mediated by other vasoactive substances. Benzoates can also have a direct influence on dermal vessels.

The release of histamine and prostaglandin from mucosa was significant increased by sodium benzoate. The mucosa of the control persons did not react. Furthermore there was a significant difference in prostaglandin release between patients with positive oral provocation and the control persons. (Schaubschläger WW, Becker WM, Schade U, Zabel P, Schlaak M, 1991(A6.12.6/14))

Ultraviolet irradiation appears to reduce (Larmi, 1989 (A6.12.6/08)) and infra-red and laser irritation (1064 nm) to increase (Larmi E, Lahti A, Hannuksela M, 1989a (A6.12.6/09)) the NIICRs induced by benzoic acid.

**Testing in patients with a known or suspected sensitivity to pseudoallergic (or allergic) substances.**

Intensive testing has been carried out to find the causative substance in people for whom sensitivity to pseudoallergic (or allergic) substances is known or at least suspected.

**Skin tests**

Patch tests

| Test substance | Solvent | Result | Reference |
|---|---|---|---|
| Benzoic acid (5%) | Petrolatum | 5 of 113 patients reacts with erythema | Baer RL, Serri F A, Weissenbach-Vial C et al., 1955 (A6.12.6/01) |
| Sodium benzoate (10%) | Petrolatum | 7 of 2045 humans reacts with irritation (reaction index – 0.66) | Brasch J, Henseler T, Frosch P, 1993 (A6.12.6/03) |
| Benzoic acid | Cosmetics | 34 of 5202 patients (0.7%) showed sensitivity | Broeckx W, Blondeel A, Dooms-Goossens A, Achten G, 1987 (A6.12.6/04) |
| Benzoic acid | 5% in Vaseline | 10 of 465 patients (2.1%) showed positive results | Meynadier JM et al., 1982 (A6.12.6/12) |

Lahti, 1980 (A6.12.6/06)

Chamber test with benzoic acid 5%

| | | Positive | |
|---|---|---|---|
| **Patients** | | **Patients** | **%** |
| 36 | atopics | 10 | 28 |
| 23 | urticaria | 9 | 39 |
| 26 | non-atopic dermatitis | 14 | 54 |
| 25 | comparison series | 10 | 40 |
| Total 110 patients | | 43 | 39 |

Patch test with benzoic acid 5%

| **Patients** | **Open** | | | | **Closed** | | | |
|---|---|---|---|---|---|---|---|---|
| | **Redness and oedema** | | **Redness** | | **Redness and oedema** | | **Redness** | |
| | **No.** | **%** | **No.** | **%** | **No.** | **%** | **No.** | **%** |
| 51 atopic | 30 | 59 | 8 | 16 | 16 | 31 | 17 | 33 |
| 55 non-atopic | 43 | 78 | 8 | 15 | 33 | 62 | 15 | 27 |

**Oral provocation tests**

| Test substance | Result | Reference |
|---|---|---|
| Sodium benzoate 50 mg and 500 mg | 11% of the patients showed exacerbations (usually in the higher dose) | Warin RP, Smith RJ, 1982 (A6.12.6/15) |
| Sodium benzoate 10, 50, 250 and 500 mg | 6 of 33 humans reacts with urticaria | Genton C, Frei PC, Pecoud A, 1985 (A6.12.6/05) |
| Sodium benzoate 20 mg | Positive response Control: 0 of 29 patients: 4 of 29 | Schaubschläger WW, Becker WM, Schade U, Zabel P, Schlaak M, 1991 (A6.12.6/14) |

Lahti, 1980 (A6.12.6/06)
Oral challenge test with 200 mg benzoic acid

| **Patients** | **Objective symptoms** | | **Only subjective symptoms** | | **Total** | |
|---|---|---|---|---|---|---|
| | **No.** | **%** | **No.** | **%** | **No.** | **%** |
| 51 atopic | 8 | 16 | 7 | 14 | 15 | 29 |
| 55 non-atopic | 2 | 4 | 4 | 7 | 6 | 11 |

Oral challenge test with 500 mg sodium benzoate

| **Patients** | **Objective symptoms** | | **Only subjective symptoms** | | **Total** | |
|---|---|---|---|---|---|---|
| | **No.** | **%** | **No.** | **%** | **No.** | **%** |
| 51 atopic | 3 | 6 | 14 | 28 | 17 | 33 |
| 55 non-atopic | 3 | 6 | 6 | 11 | 9 | 16 |

**Test reactions depending different parameters**

The number of patients reacting and the strength of reaction depend on different parameters.

**The used vehicle**

Lahti A, 1980 (A6.12.6/06)
Frequency of urticarial reactions 0.25% in water and 1% in petrolatum was the same
0.1% showed the same frequency of reactions in petrolatum and water, but more oedema reactions were seen when water was the vehicle.

Lahti A et al., 1993 (A6.12.6/07)
16 healthy medical students
Strongest reactions: propylene glycol vehicles. Increasing the concentration of propylene glycol in isopropanol and ethanol from 25% to 50% weakened the reactions to 125 mM benzoic acid ($p<0.05$).
The vehicles without benzoic acid did not cause any reactions.

Ros A, Juhlin L, Michaelsson G, 1976 A6.12.6/13
Cross reactivity in NIICRs for sodium benzoate and salicylates has been described
75 patients total with recurrent urticaria and angio-oedema > 4 month and with positive provocation test to aspirin, azo dyes and/or benzoates were tested.

| | |
|---|---|
| Aspirin only | 12 positive provocations |
| Azo dyes only | 7 positive provocations |
| Benzoates only | 2 positive provocations |
| Benzoates + Aspirin | 11 positive provocations |
| Benzoates + Aspirin + azo dyes | 24 positive provocations |
| Aspirin + azo dyes | 12 positive provocations |

Ylipieti S, Lahti A, 1989 (A6.12.6/16)
11 healthy medical students and 3 patients with urticaria were treated with 10 mL doses of 1000, 500, 250, 100 and 50mM benzoic acid in different solvents. In Petrolatum and 2-propyl alcohol/ water (50/50) no difference in the strength of reactions was seen. But reactions were stronger than in synthetic lanolin and ethyl alcohol.

**The part of the body where the substance is applied**
Larmi E, Lahti A, Hannuksela M, 1989b (A6.12.6/10)
Reactions are stronger on the face than on other skins. The cheek is the most sensitive part of the face.

Lahti, 1980 A6.12.6/06)
The condition of the skin (neither scratching nor stripping the skin enhanced the contact urticarial reactions to benzoic acid.

**Studies on the general population**

**Young E, Patel S, Stoneham M, Rona R, Wilkinson JD, 1987 (A6.12.6/17)**
A large scale study in the area of Wycombe, Great Britain including questionnaires and interviewing was undertaken. Persons for whom at the end of the interviewing phase a real intolerance to certain substances could be assumed were included in an oral provocation test after remaining on an elimination diet. The trial regime involved taking five different low dose capsules during the first ten days, alternating with lactose placebo capsules and then taking five high dose capsules, alternating with placebo during the next ten days. The capsules contained combinations of chemicals, e.g. 50 mg aspirin and 10 mg sodium benzoate (low dose) and 300 mg aspirin and 100 mg sodium benzoate (high dose).

**Summarized data of the large scale study in the area of Wycombe, Great Britain**

| Stage of assessment | No. of individuals involved | Respondents [1] | Non-respondents [1] |
|---|---|---|---|
| Questioning by Questionnaire | 30000 [2] (in 11388 households) | 18582 (62%) | 11418 (38%) |
| Called for interview | 1223 | 649 (53%) | 574 (47%) |
| Entered into trial | 132 | 81 (61%) | 51 (39%) |
| Individuals who reacted to a combination of chemicals [3] | | 3 (4%) | |

1) Respondents / non-respondents are these individuals who answered / not answered the questionnaires, took part / took not part in the interview and participated / not participated in the trial until the end
2) Estimated
3) Two adults reacted to annatto, a natural colour. The reaction of one five-year-old child was doubtful because of reacting to two food additives but to the placebo capsules as well.

**Result:**
No individual included in the oral provocation test reacted to the combination of aspirin and sodium benzoate. Therefore, the planned testing for each of the two

substances alone could be omitted. This means that no person showed sensitivity to benzoates in this study.

**Basketter DA, Wilhelm K-P, 1996 (A6.12.6/02)**
Double blind skin tests with unselected volunteers (200) were run with three known urticants (benzoic acid among these).
The substances were applied in chambers onto the skin for 20 min and assessed 30 min following initiation of the application. Benzoic acid was used in concentrations of 125 mM (1.5%, 306 µg/20 µl) and 500 mM (6.0%, 1220 µg/20 µl).
The NIICRs (mostly erythema) assessed ten minutes after direct contact of benzoic acid with the skin are summarised in the table. No significant correlation between age or sex and the degree of NIICR was found.

**Skin reactions with benzoic acid in an unselected population**

| | Erythema / oedema score [1] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Urticant | 0[2] | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Benzoic acid (125 mM) | 53/175 | 44/19 | 41/5 | 25/1 | 26/0 | 10/0 | 1/0 | 0/0 | 0/0 |
| Benzoic acid (500 mM) | 43/164 | 35/30 | 41/5 | 31/1 | 39/0 | 9/0 | 2/0 | 0/0 | 0/0 |

1) Number of individuals with specified grade of erythema / oedema
2) Erythema / oedema grading:
0: nothing visible
1: a marginal reaction, not sufficient to be classified as "slight"
2: perceptible erythema / oedema (swelling)
3: higher grade than "perceptible", not sufficient to be classified as "distinct"
4: distinct erythema / oedema (swelling)
5: higher grade than "distinct", not sufficient to be classified as "well developed"
6: well developed, may extend beyond site
7: higher grade than "well developed", not sufficient to be classified as "strong"
8: strong, deep erythema / strong, "blisterlike" oedema (swelling), both may extend beyond site

**Result:**
In this study, 10 minutes after direct contact of benzoic acid with the skin a relatively large group of unselected volunteers reacted at the concentrations applieds. This again indicates the big influence of the study design on the result.

**Sensitisation**

In a maximization test - developed for humans and described in fullness by Kligman (1966) - benzoic acid and related compounds did not show any sensitization potential in volunteers (Leyden & Kligman, 1977, A6.12.6/11). Sensitivity to benzoates used in cosmetic products is sometimes observable (Broeckx et al., 1987, A6.12.6/04).

X

## 3 SUMMARY AND CONCLUSION

| | |
|---|---|
| | Benzoic acid and its salts are known to cause pseudoallergic reactions (e.g. urticaria and asthma) which mimic signs and symptoms of allergic disorders but without underlying immunologic mechanisms, therefore also termed as non-immune immediate contact reactions (NIICRs).<br>Intensive testing (skin tests and oral provocation tests) has been carried out with benzoates in patients with a known or suspected sensitivity to pseudoallergic (or allergic) substances. The number of patients reacting and the strength of reaction depend on a variety of factors as the kind of test, the study design, the number of patients and the criteria for judging the reactions. Therefore an overall frequency of positive reactions in these tests cannot be given.<br>In the general population, in one study no person reacts sensitively to sodium benzoate in food. In another study a relatively large group of unselected volunteers reacted at the used concentrations 10 minutes after direct contact of benzoic acid. This again indicates the big influence of the study design on the result.<br>Sensitivity (but not allergenicity) to benzoates in cosmetics or at occupational exposure was occasionally reported.<br>In a maximization test - developed for humans - benzoic acid and related compounds did not show a sensitisation reaction in volunteers. |
| | **Evaluation by Competent Authorities** |
| | *Use separate "evaluation boxes" to provide transparency as to the comments and views submitted* |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | 2008/04/17 |

| | |
|---|---|
| **Evaluation of applicant's justification** | 1, Reference: Most of the cited references are on non-immune immediate contact reactions provoked by benzoic acid, not on allergenicity observations .<br>2, Results; Table "Oral provocation tests", study by Genton et al. (1985): The applicant's citation is not correct, as the study does not mention 6 of 33 humans having reacted with urticaria but 5 of 33 reacted with urticaria and 1 with asthma.<br>2, Results; Table "Oral provocation tests", study by Schaubschläger et al. (1991): The applicant's citation is not correct. The whole study group consisted of 29 subjects of whom 21 had a "suspicious history of sensitivity to food additives" (group 1) and 8 suffered "from abdominal discomfort, with no history of food sensitivity" (group 2, serving as controls). A positive response in the oral provocation test was described for 4 subjects in group 1 (4 of 21) and for none in group 2 (0 of 8).<br>2, Results; "The used vehicle", study by Ylipieti et al. (1989): The applicant's citation is not correct. Ylipieti et al. did not describe the patients in the study group as suffering from urticaria but from psoriasis, eczema and rosacea, respectively. Furthermore, the doses were not 10 ml but 10 µl. Finally, one vehicle was not mentioned to be synthetic lanolin but a synthetic lanolin *substitute*.<br>2, Results; Basketter DA, Wilhelm K-P, 1996 (A6.12.6/02): The mean scores were 0.16 (oedema) and 1.81 (erythema) after application of 125 mM benzoic acid and 0.22 (oedema) and 2.12 (erythema) after application of 500 mM benzoic acid.<br><br>An additional study was identified as a key study:<br>Frosch PJ & Kligman AM (1976). The chamber-scarification test for irritancy. Contact Dermatitis 2:314-324.<br>Material and methods: Benzoic acid (7.5 % , 15 % and 30% (only unscarified skin) in ethanol) was applied once daily for 3 days in aluminium chambers on scarified and unscarified skin on the forearm of 5-10 healthy volunteers and rated 24 h after the last exposure.<br>Result: The irritant threshold concentration of benzoic acid on unscarified skin was 30 %, on scarified skin 7.5 %. Applying a five-point grading system of 0-4, a moderate response (score between 1.5-2.4) was observed at 7.5 % on scarified skin. A marked response, leading to erosions, was observed at 15 % (score between 2.5-4) on scarified skin.<br><br>Revised version is adopted. |
| **Conclusion** | (Occupational) exposure to benzoic acid can lead to e.g. urticaria or asthma if a person is sensitive. From the literature, this reaction does not seem to be mediated immunologically. Study results concerning the prevalence of sensitivity to benzoic acid are inconsistent.<br>Applicant's version is acceptable. |
| **Remarks** | One case report describes anaphylactic shock after oral exposure to sodium-benzoate in a chemical worker (Pevny I et al. (1981): Excessive allergy due to benzoic acid followed by anaphylactic shock; Dermatosen, 29 (5), 123 – 130, article in German). |
| | **COMMENTS FROM OTHER MEMBER STATE** *(specify)* |
| **Date** | *Give date of comments submitted* |
| **Evaluation of applicant's justification** | *Discuss if deviating from view of rapporteur member state* |
| **Conclusion** | *Discuss if deviating from view of rapporteur member state* |
| **Remarks** | |

| Section A7.1.1.1.1<br>Annex Point IIA7.6.1.1 | Hydrolysis as a function of pH and identification of breakdown products | Official use only |
|---|---|---|
| | **1 REFERENCE** | |
| | Kellner G, 2008, Statement concerning Hydrolysis as a function of pH and identification of breakdown products<br>ChemCon GmbH, Kirchlinteln, Germany | |
| | **2 Results** | |
| | According to EC method C.7 and OECD guideline 111 hydrolysis refers to a reaction of a chemical RX with water. This reaction may be represented by the net exchange of the group X with OH:<br>RX + HOH ∏ ROH + HX<br>The rate at which the concentration of RX decreases is given by:<br>rate = k $[H_2O]$ · [RX]<br>Organic chemicals are only prone to hydrolysis, if water can react at any point in the molecule RX forming a carbon–oxygen bond ROH and cleaving a carbon-X bond.<br>Benzoic acid constitutes only of a benzene ring and a carboxylic acid group. Both types are unable to react with water and are resistant to hydrolysis.<br>Considering the above mentioned general knowledge on the mechanism of hydrolysis reactions, an experimental study for the determination of the hydrolysis rate of benzoic acid was not prepared. | |
| | **3 Conclusion** | |
| | Benzoic acid does not hydrolyse. | |
| | **Evaluation by Competent Authorities** | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | 2009/01/26 | |
| **Evaluation of applicant's justification** | Applicant's justification is applicable. | |
| **Conclusion** | Applicant's justification is acceptable. | |
| **Remarks** | The justification is supported by the reference: Lyman, W.J., William, F.R. and Rosenblatt, D.H.: Handbook of chemical property estimation methods; Am. Chem. Soc., Washington D.C. (1990), p. 7-4, table 7.1 | |
| | **COMMENTS FROM OTHER MEMBER STATE** *(specify)* | |
| **Date** | *Give date of comments submitted* | |
| **Evaluation of applicant's justification** | *Discuss if deviating from view of rapporteur member state* | |
| **Conclusion** | *Discuss if deviating from view of rapporteur member state* | |
| **Remarks** | | |

## Section A7.1.1.1.2
**Annex Point IIA7.6.2.2**

# Phototransformation in water including identity of transformation products

| | | Official use only |
|---|---|---|
| | **1 REFERENCE** | |
| | Deng Y et al., 2003, Separation and identification of photodegradation products of benzoic acid by capillary zone electrophoresis, Elsevier Journal of Chromatography A, 1013, 191-201 (A7.1.1.1.2/01)<br><br>Ogata Y, Tomizawa K, Yamashita Y, 1979, Photoinduced Oxidation of Benzoic Acid with Aqueous Hydrogen Peroxide, J.C.S. Perkin II, 9/775, 616-619 (A7.1.1.1.2/02)<br><br>Oussi D et al., 1998, Photodegradation of benzoic acid in aqueous solutions, Environmental Technology, 19, 995-960 (A7.1.1.1.2/03)<br><br>Ware GW, Crosby DG, Giles JW, (1980), Photodecomposition of DDA, Arch. Environm. Contam. Toxicol. Vol. 9, pp 135 – 146 (A7.1.1.1.2/04) | |
| | **2 RESULTS** | |
| **Deng Y et al., 2003 (A7.1.1.1.2/01)** | A capillary zone electrophoresis (CZE) method catalysed by dissolved iron (III) species with monochromatic light at a wavelength of 300 nm was developed for separation and identification of photodegradation products benzoic acid under irradiation at a wavelength of 300 nm. Parameters such as run buffer, applied voltage and injection time were optimised for the separation of benzoic acid and its photodegradation products. Linearity, limit of detection, and repeatability of migration time as well as peak area of the method were examined. Four reaction products, including salicylic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid were separated and identified by spiking the known compounds into the irradiated samples using the CZE method developed. The confirmation of the reaction products represents one of the key steps for proposing the possible reaction mechanisms involved in the Photodegradation of benzoic acid.<br>The role that iron plays in the photodegradation of benzoic acid and the identification of the reaction products provide useful information for proposing reaction mechanisms. Benzoic acid has two absorption bands at wavelengths of 228 and 274 nm, respectively. Therefore, direct photodegradation of benzoic acid would be difficult to proceed at 300 nm because the absorption bands of benzoic acid do not significantly overlap with the wavelength (300 nm) of the lamps. Indirect photodegradation, however, may occur as ferric hydroxide complexes $Fe(OH)_2^+$ and $Fe(OH)_4^-$ have weak absorption bands that extend into the wavelength region of 290-400 nm. The iron hydroxide complexes are considered to be one of the major photochemical precursors to hydroxyl radical (OH·) formation. | |
| **Oussi D et al., 1998 (A7.1.1.1.2/03)** | The photodegradation of benzoic acid in aqueous solutions using a medium pressure UV source has been studied. Benzoic acid concentration was determined by Reverse HPLC.<br>The degradation rate follows first order kinetics with respect to the radiation rate. A simple mechanism with three steps can explain the photochemical degradation of benzoic acid in aqueous solutions.<br>By means of the evaluation of the amount of photons absorbed by benzoic acid, the quantum yield (average between 240-300 nm) was obtained ($5x10^{-2}$ mol Einstein at 25°C and pH = 3.8).<br>The quantum yield for the photodecomposition of benzoic acid depends on the temperature and pH of the medium. The temperature has a positive effect on the decomposition rate (increasing the temperature from 25°C to 50°C increases the conversion rate by 20%), while augmentation of pH or initial concentration of benzoic acid show an inverse effect. | |

## Section A7.1.1.1.2 Phototransformation in water including identity of transformation products

**Annex Point IIA7.6.2.2**

| | |
|---|---|
| **Ogata Y, Tomizawa K, Yamashita Y, 1979 (A7.1.1.1.2/02)** | In the absence of $H_2O_2$ decomposition of benzoic acid did not occur after irradiation for 20 hours. |
| **Ware G.W, Crosby DG, Giles JW, 1980 (A7.1.1.1.2/04)** | Benzoic was proved to be stable in a photodecomposition experiment with 2,2-bis-(p-chlorophenyl) acetic acid (DDA). Benzoic acid was an endproduct of DDAC photodecomposition. |
| **s** | **3 CONCLUSION**<br>A direct phototransformation does not occur because benzoic acid is a very stable compound and at >290 nm there is no absorption.<br>In experiments with high energy input and added catalyzers photolysis could be observed and the degradation products identified as salicylic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid. The quantum yield for the photodecomposition of benzoic acid depends on the temperature and pH of the medium. The temperature has a positive effect on the decomposition rate (increasing the temperature from 25°C to 50°C increases the conversion rate by 20%), while augmentation of pH or initial concentration of benzoic acid shows an inverse effect.<br>In the absence of $H_2O_2$ decomposition of benzoic acid did not occur after irradiation for 20 hours. |

### Evaluation by Competent Authorities

Use separate "evaluation boxes" to provide transparency as to the comments and views submitted

**EVALUATION BY RAPPORTEUR MEMBER STATE**

| | |
|---|---|
| **Date** | 2009/03/17 |
| **Evaluation of applicant's justification** | Applicant's justification is applicable. |
| **Conclusion** | Applicant's justification is acceptable. |
| **Remarks** | The correct unit for the quantum yield is mol x einstein$^{-1}$ (ref. to Oussi D. et al., 1998). |

**COMMENTS FROM OTHER MEMBER STATE** *(specify)*

| | |
|---|---|
| **Date** | *Give date of comments submitted* |
| **Evaluation of applicant's justification** | *Discuss if deviating from view of rapporteur member state* |
| **Conclusion** | *Discuss if deviating from view of rapporteur member state* |
| **Remarks** | |

| | | | |
|---|---|---|---|
| **Section A7.1.1.2.1**<br>**Annex Point IIA7.6.1.1** | | **Biodegradability (ready)** | Official use only |
| | | | |
| | | **1 SUMMARY OF TESTS ACCORDING TO OECD 301B, 301D AND 301E (EQUIVALENT TO EC METHODS C.4-C, C.4-E AND C.4-B)** | **Official use only** |
| **1.1** | **Reference** | Lebertz H, 2008, Gutachten zur biologischen Abbaubarkeit von Natriumbenzoat in Standard-Abbautestverfahren gemäß OECD Richtlinien, SGS Institut Fresenius, Taunusstein, Germany; unpublished report no. report number, September 14, 2008 | |
| **Data protection** | | Yes | |
| 1.1.1 | Data owner | MENNO CHEMIE-VERTRIEB G.M.B.H., Norderstedt, Germany | |
| 1.1.2 | Criteria for data protection | Data submitted to the MS after 13. May 2000 on existing a.s. for the purpose of its entry into Annex I | |
| **1.2** | **Result** | Summary of sodium benzoate degradation data from studies according to OECD Guideline 301. Includes all tests performed by SGS Fresenius GmbH in the years 2001 - 2008 under GLP conditions or according to DIN EN ISO 17025.<br>**Biodegradability according OECD guideline 301B**<br>74 tests were performed, the mean biodegradability was 90.3%.<br>The result is not suitable for a quantitative calculation because during incubation benzoate is partly transformed into the bio mass and therefore not detected as evolved $CO_2$<br>**Biodegradability according OECD guideline 301D**<br>33 tests were performed, the mean biodegradability was 77.7%.<br>The result is not suitable for a quantitative calculation because during incubation benzoate is partly transformed into the bio mass and therefore not detected as oxygen demand.<br>**Biodegradability according OECD guideline 301E**<br>21 tests were performed, the mean biodegradability was 98.7%.<br>Compared to the two above mentioned tests, the OECD 301 E (Modified OECD Screening -DOC Die-Away test) is more suitable for a quantitative assessment because benzoate incorporated in the bio mass is taken into account as well.<br>However, for a reliable quantitative assessment a substance specific analytic would be necessary, which was not performed in any of the above reported test results.<br>Under environmental conditions as well as under the conditions of the mentioned OECD tests (pH 7.4 ± 0.2), benzoic acid is not present as undissociated acid, but depending on pH as benzoate. Therefore, for predicting the biodegradability of benzoic acid, a benzoate may be used as well. By convention the sodium salt is used as reference compound with known easy biodegradability in the OECD guideline 301 to check the activity of the inoculum for the investigation of ready biodegradability of chemicals.<br>The metabolic pathway is well known:<br>- hydroxylation from benzoate to protocatechuate<br>- protocatechuate in a six step reaction to succinate<br>- succinate within the citrate cycle to $CO_2$ .<br>There are no stable intermediates or metabolites. Benzoic acid is ultimately degraded to $CO_2$. | |

| | | |
|---|---|---|
| | **2 TEST ACORDING OECD 301C (EQUIVALENT TO EC METHOD C.4-F)** | |
| **2.1 Reference** | National Institute of Technology and Evaluation (NITE)<br>Biodegradation and Bioconcentration of Existing Chemical substances under the Chemical Substances Control Law<br>Information on the chemical published in the Official Bulletin of Economy, Trade and Industry (Former Title: The Official Bulletin of the Ministry of International Trade and Industry) published 1979/12/20 | |
| **Data protection** | No | |
| **2.2 Result** | **Biodegradability according MITI-I (OECD TG 301C)**<br>Indirect Analysis: BOD 85%<br>Direct Analysis: TOC 98%, UV-VIS 100% | |
| | **3 CONCLUSION** | |
| | Benzoic acid proved to be readily biodegradable in all tests performed according to OECD 301 B, OECD 301 C, OECD 301 D and OECD 301 E (equivalent to EC methods C.4-C, C.4-F, C.4-E and C.4-B). According to the EC method description (chapter I 3 Reference substances) sodium benzoate degrades in these methods even when no inoculum is deliberately added.<br>Benzoic acid can be classified as ready and ultimate biodegradable in aerobic aquatic environments. | |
| | **Evaluation by Competent Authorities** | |
| | *Use separate "evaluation boxes" to provide transparency as to the comments and views submitted* | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | 2009/02/26 | |
| **Evaluation of applicant's justification** | Test performance is not reported in sufficient detail to evaluate the deviations from international standard methods. In particular, there is no information on test conditions and controls. No graphs for data on biodegradation were included. No data on the compliance with the 10-d or the 14-d- window criteria, respectively, were reported,<br>**Deviations** The tests are not reported according to the OECD requirements (OECD 301 guideline, heading test report) including validity criteria. | |
| **Conclusion** | Benzoic acid is classified as "readily biodegradable". | |
| **Reliability** | 2 | |
| **Acceptability** | acceptable | |
| **Remarks** | In spite of a lack of detailed documentation of test performance and results, the summary could be accepted, because the applicant provides a plausible reasoning for the classification of benzoic acid as "readily biodegradable" which allows an expert judgement in a weight of evidence approach. | |
| | **COMMENTS FROM OTHER MEMBER STATE** (specify) | |
| **Date** | Give date of comments submitted | |
| **Evaluation of applicant's justification** | Discuss if deviating from view of rapporteur member state | |

| | |
|---|---|
| **Conclusion** | Discuss if deviating from view of rapporteur member state |
| **Remarks** | |

## Section 7.1.2.1.2 Anaerobic biodegradation
**Annex Point IIIA XII 2.1**

Official use only

### 1 REFERENCE

| | | |
|---|---|---|
| **1.1** | **Reference** | Shelton DR, Tiedje JM, 1984, General Method for Determining Anaerobic Biodegradation Potential, Departments of Crop and Soil Sciences and Microbiology and Public Health, Michigan State University, East Lansing, Michigan, USA<br>Applied and Environmental Microbiology, Apr. 1984, 850-857 (published) |
| **1.2** | **Data protection** | No |
| 1.2.1 | Data owner | Not applicable |
| 1.2.2 | Companies with letter of access | - |
| 1.2.3 | Criteria for data protection | No data protection / published data |

### 2 GUIDELINES AND QUALITY ASSURANCE

| | | |
|---|---|---|
| **2.1** | **Guideline study** | No, a simple, generalized method was refined and validated to test whether an organic chemical was susceptible to anaerobic degradation to $CH_4 + CO_2$. |
| **2.2** | **GLP** | No |
| **2.3** | **Deviations** | Not applicable |

### 3 METHOD

| | | |
|---|---|---|
| **3.1** | **Test material** | Benzoic acid as given in section 2 |
| 3.1.1 | Lot/Batch number | No lot/batch number available |
| 3.1.2 | Specification | As given in section 2 |
| 3.1.3 | Purity | >99% |
| 3.1.4 | Further relevant properties | - |
| 3.1.5 | Composition of Product | - |
| 3.1.6 | TS inhibitory to microorganisms | No |
| 3.1.7 | Specific chemical analysis | No analytical confirmation of the test substance. |
| **3.2** | **Reference substance** | p-Cresol and phthalic acid |
| 3.2.1 | Initial concentration of reference substance | 50 µg of C |
| **3.3** | **Testing procedure** | *Non-entry field* |
| 3.3.1 | Inoculum / test species | See table A7_1_2_1_2-1 |

## Section 7.1.2.1.2 Anaerobic biodegradation
**Annex Point IIIA XII 2.1**

| | | |
|---|---|---|
| 3.3.2 | Test system | See table A7_1_2_1_2-2 |
| 3.3.3 | Test conditions | See table A7_1_2_1_2-3 |
| 3.3.4 | Method of preparation of test solution | |
| 3.3.5 | Initial TS concentration | 50 µg of C |
| 3.3.6 | Duration of test | 8 weeks at 35°C or until biodegradation is complete. |
| 3.3.7 | Analytical parameter | $CO_2$ and $NH_4$<br>Measurement of gas production by a pressure transducer.<br>Methane production was quantified by gas chromatography with flame ionizations detector. |
| 3.3.8 | Sampling | *Give details on sampling intervals* |
| 3.3.9 | Intermediates/ degradation products | Not identified |
| 3.3.10 | Controls | Ethanol (easily degraded) as positive control |
| 3.3.11 | Statistics | Since all substrates are provided at 50 µg of C per ml, the theoretical gas yield from 100 ml of medium for all substrates is 10.5 ml. This gas will be divided between $CO_2$ and $CH_4$ based on the stoichiometry of the reaction which can be calculated by the Buswell equation. |
| o | | **4 RESULTS** |
| **4.1** | **Degradation of test substance** | *Non-entry field* |
| 4.1.1 | Degradation of TS in abiotic control | Not applicable |
| 4.1.2 | Degradation | > 75% of theoretical methane production |
| 4.1.3 | Graph | No data given |
| 4.1.4 | Other observations | - |
| 4.1.5 | Degradation of reference substance | Pattern of gas production |

## Section 7.1.2.1.2 Anaerobic biodegradation
**Annex Point IIIA XII 2.1**

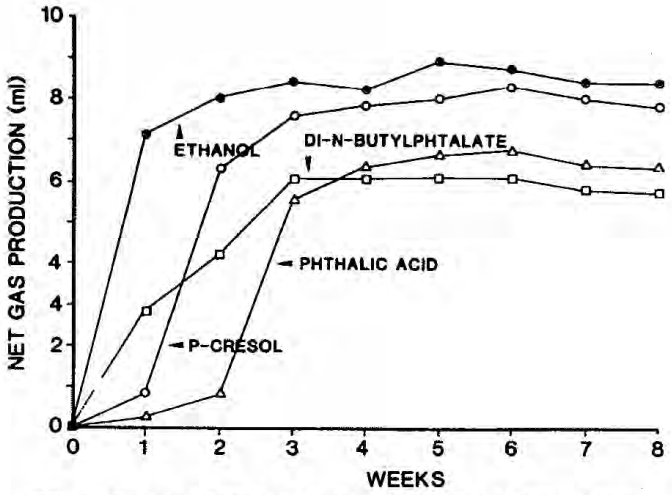



FIG. 2. Pattern of gas production ($CH_4$ + $CO_2$) from ethanol, *p*-cresol, phthalic acid, and di-*n*-butylphthalate incubated anaerobically with 10% digester sludge.

| | | |
|---|---|---|
| 4.1.6 | Intermediates/ degradation products | Not applicable |

**5 APPLICANT'S SUMMARY AND CONCLUSION**

| | | |
|---|---|---|
| **5.1** | **Materials and methods** | Sludge from primary or secondary anaerobic digesters with 15 – 30 day retentions time and total organic solids of 1 – 2% was diluted to 10% and incubated anaerobically wit 50 µl of C per ml of test chemical.<br>Gas production was measured by gas chromatography and by a pressure transducer. |
| **5.2** | **Results and discussion** | Benzoic acid was degradable. (>75% of the theoretical gas production was observed). |
| **5.3** | **Conclusion** | Benzoic acid is degradable under anaerobic conditions. Validity criteria can be considered as fulfilled.<br>The results from the anaerobic digesters can be extended to natural anaerobic environments. |
| 5.3.1 | Reliability | 2 |
| 5.3.2 | Deficiencies | Not applicable |

| **Evaluation by Competent Authorities** | |
|---|---|
| | Use separate "evaluation boxes" to provide transparency as to the comments and views submitted |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | 2008/05/18 |

## Section 7.1.2.1.2 Anaerobic biodegradation
**Annex Point IIIA XII 2.1**

| | |
|---|---|
| **Materials and Methods** | The applicant´s version is acceptable.<br>The study is comparable to OECD 311, "Anaerobic degradation in digested sludge" and is cited in this guideline. |
| **Results and discussion** | Applicant's version is adopted apart from the following comment:<br>Detailed data are missing. The included figure is wrong; it contains no data on benzoic acid. The following table should have been included instead: |

TABLE 2. Effect of substrate concentration on gas production in 10% sludge from the Jackson digester

| Substrate concn (µg of carbon per ml) | Mean % of theoretical degradation ± SD | | | |
|---|---|---|---|---|
| | Phenol | *p*-Cresol | Benzoic acid | Phthalic acid |
| 25 | 100 ± 9.9 | 98 | 97 ± 18.0 | 105 ± 4.0 |
| 50 | 104 ± 8.0 | 86 ± 11.3 | 92 ± 6.0 | 104 ± 18.7 |
| 100 | 106 ± 6.5 | 98 ± 5.5 | 96 ± 4.7 | 109 ± 3.7 |
| 200 | 113 ± 3.1 | 82 ± 4.5 | 98 ± 3.6 | 101 ± 1.5 |
| Background gas production (ml) | 17.4 | 31.4 | 24.5 | 28.4 |
| Theoretical gas production from 50 µg of carbon per ml (ml)[a] | 7.4 | 7.6 | 8.7 | 5.7 |

[a] Corrected for gas solubilities.

| | |
|---|---|
| **Conclusion** | Applicant's version is adopted with the following comment:<br>The biodegradation under anaerobic conditions in digested sludge of benzoic acid was above 75%, ranging from 92% ± 18% to 98% ±3.6%, of the theoretical yield within 8 weeks. |
| **Reliability** | 2 |
| **Acceptability** | acceptable<br>In spite of a lack in detail in documentation of the testing procedure. |
| **Remarks** | Additional information on the anaerobic biodegradability of benzoic acid and a specific metabolism pathway in *R. palustris* and *P. denitrificans* under anaerobic conditions has been provided by the applicant. The information is presented in Table A7_1_2_1_2-4, and filed in Doc III A7.1.2.1.2/02 and Doc III A7.1.2.1.2/03. |

| | |
|---|---|
| | **COMMENTS FROM** ... *(specify)* |
| **Date** | *Give date of comments submitted* |
| **Materials and Methods** | *Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.*<br>*Discuss if deviating from view of rapporteur member state* |
| **Results and discussion** | *Discuss if deviating from view of rapporteur member state* |
| **Conclusion** | *Discuss if deviating from view of rapporteur member state* |
| **Reliability** | *Discuss if deviating from view of rapporteur member state* |
| **Acceptability** | *Discuss if deviating from view of rapporteur member state* |
| **Remarks** | |

**Table A7_1_2_1_2-1: Inoculum / Test organism**

| Criteria | Details |
|---|---|
| Nature | Sludge from primary or secondary anaerobic digesters, 15 – 30 day retentions time |
| Species | - |
| Strain | - |
| Source | Waste treatment plant |
| Sampling site | Mid-Michigan communities |
| Laboratory culture | - |
| Method of cultivation | - |
| Preparation of inoculum for exposure | 10% homogeneous sludge solution with 50 µg of C per ml |
| Pretreatment | Stored at 4°C for 4 weeks |
| Initial cell concentration | Not applicable |

**Table A7_1_2_1_2-2: Test system**

| Criteria | Details |
|---|---|
| Culturing apparatus | Serum bottles of 160 ml capacity with new butyl rubber stoppers and aluminium crimp seals. |
| Number of replicates/concentration | 3 |
| Measuring equipment | Measurement of gas production by a pressure transducer.<br>Methane production was quantified by gas chromatography with flame ionizations detector |
| Oxidation reduction indicator | No |

**Table A7_1_2_1_2-3: Test conditions**

| Criteria | Details |
|---|---|
| Composition of medium | Per litre<br>Phosphate buffer:<br>0.27 g of KH2P04 and 0.35 g of K2HP04 (pH 7.0)<br>Mineral salts:<br>0.53 g of $NH_4CI$<br>75 mg of Ca $Cl_2$ x 2 $H_2O$<br>100 mg of $MgCl_2$ x 6 H2O<br>20 mg of $FeCI_2$ x 4 $H_2O$<br>Trace metals modified from Zehnder and Wuhrmann<br>0.5 mg of $MgCl_2$ x 4 $H_2O$<br>0.05 mg of $H_3BO_3$<br>0.05 mg of $ZnCl_2$<br>0.03 mg of 1 CuCl2<br>0.01 mg of $NaMo_4$ x 2 $H_2O$<br>0.5 mg of $CoCl_2$ x 6 $H_2O$<br>0.05 mg of $NiCl_2$ x 6 $H_2O$<br>0.05 mg of $Na_2SeO_3$ |
| Additional substrate | Yes<br>1.2 g NaCO3 and 0.5 g of Na2S x 9 H2O per litre |

| | |
|---|---|
| Solvent | - |
| Preparation of medium | Autoclaved for 5 – 10 minutes to drive off $O_2$ and then cooled to 35°C while sparging with a 10% $CO_2$-90% $N_2$ gas mixture passed through copper fillings at 300°C to remove traces of $O_2$. |
| Test temperature | 35°C |
| pH | No data given |
| Suspended solids concentration | Total organic solids of 1 – 2%. |
| Other relevant criteria | - |

**Table A7_1_2_1_2-4: Additional data anaerobic biodegradation**

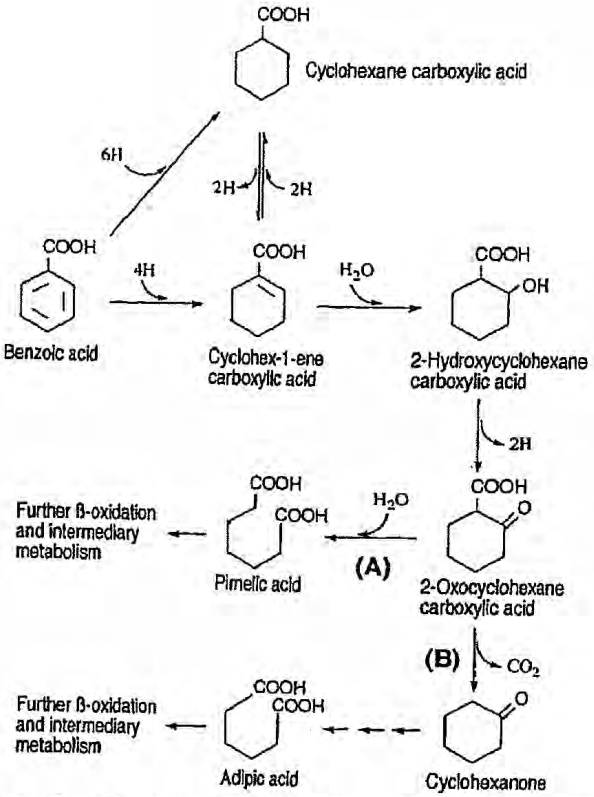
<table>
<tr><th>Method</th><th>Result</th><th>Reference</th></tr>
<tr><td>Serum bottle modification of the Hungate technique for growing anaerobes was used for methanogenic enrichments on benzoic acid. Mass balances on the conversion of organic carbon to $CO_2$ and $NH_4$ were determined.</td><td>Benzoic acid is degradable under anaerobic conditions
<table>
<tr><td>Acclimation lag (days)</td><td>Period of gas production</td><td>Conversion of substrate carbon to gas (%)</td></tr>
<tr><td>8 ± 0.5</td><td>18 ± 1.6</td><td>91 ± 7.8</td></tr>
</table>
n = 5</td><td>Healy JB, Young LY, 1979, (A7.1.2.1.2/02)</td></tr>
<tr><td>Pathway for anaerobic degradation of benzoic acid</td><td>Benzoic acid is degraded under anaerobic conditions by a mechanism quite distinct from the aerobic pathway.


Fig. 1. Proposed pathway for the anaerobic degradation of benzoic acid by *R. palustris* and *Paracoccus denitrificans*. The pathway is common to both microorganisms as far as 2-oxocyclohexane carboxylic acid. At this point in *R. palustris* (A), a hydrolytic cleavage occurs to yield pimelic acid, whereas in *P. denitrificans* (B), 2-oxocyclohexane carboxylic acid is decarboxylated, yielding cyclohexanone which is further metabolised to adipic acid. After Dutton and Evans [14] and Williams and Evans [16].</td><td>Elder JE, Kelly D, 1994 (A7.1.2.1.2/03)</td></tr>
</table>

## Section A7.1.3 Adsorption / Desorption screening test

**Annex Point IIA7.7**

| | | | Official use only |
|---|---|---|---|
| | | **1 REFERENCE** | |
| **1.1** | **Reference** | Kellner G, 2008, Statement concerning Adsorption/desorption Screening Test, ChemCon GmbH, Kirchlinteln, Germany, October 30, 2008 | |
| **Data protection** | | Yes | |
| 1.1.1 | Data owner | MENNO CHEMIE-VERTRIEB G.M.B.H., Norderstedt, Germany | |
| 1.1.2 | Companies with letter of access | - | |
| 1.1.3 | Criteria for data protection | Data submitted to the MS after 13. May 2000 on existing a.s. for the purpose of its entry into Annex I | |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** | |
| **2.1** | **Guideline study** | No | |
| **2.2** | **GLP** | No | |
| **2.3** | **Deviations** | Not applicable / QSAR model | |
| | | **3 MATERIALS AND METHODS** | |
| **3.1** | **Test material** | Benzoic acid (QSAR calculation performed with the molecule) | |
| 3.1.1 | Lot/Batch number | Not applicable / QSAR model | |
| 3.1.2 | Specification | Not applicable / QSAR model | |
| 3.1.3 | Purity | Not applicable / QSAR model | |
| 3.1.4 | Further relevant properties | Not applicable / QSAR model | |
| 3.1.5 | Method of analysis | Not applicable / QSAR model | |
| **3.2** | **Degradation products** | Not applicable / QSAR model | |
| 3.2.1 | Method of analysis for degradation products | Not applicable / QSAR model | |
| **3.3** | **Reference substance** | No | |
| 3.3.1 | Method of analysis for reference substance | Not applicable / QSAR model | |
| **3.4** | **Soil types** | Not applicable / QSAR model | |
| **3.5** | **Testing procedure** | Non-entry field | |
| 3.5.1 | OECD 121 | The experimental method described in OECD 121 uses HPLC for the estimation of the adsorption coefficient $K_{oc}$. The method is validated for several substances listed in the guideline and may be applied to other substances in the Log $K_{oc}$ range of 1.5 to 5.0. Benzoic acid is not listed. It is mentioned that the method may not work for moderate organic acids. Benzoic acid can be classified as moderate organic acid. Taking into account the known physico-chemical properties of benzoic acid (e.g. octanol-water partition coefficient, pKa, polarity) it can safely be deduced that in the HPLC system described in OECD 121 (moderately polar | |

## Section A7.1.3 Adsorption / Desorption screening test

**Annex Point IIA7.7**

| | | | |
|---|---|---|---|
| | | stationary phase, mobile phase methanol/water and methanol/citrate-buffer for the ionized and the non-ionized form respectively) the retention time of benzoic acid (as well as of other weak organic acids) will be much shorter then those of Atrazine (the most mobile validated substance) and in any case outside the validated range of Log $K_{oc}$ 1.5 to 5.0. Consequently, no study according to OECD 121 was performed with benzoic acid, as the failure to comply with the requirements for a valid study can be foreseen. However, a Log $K_{oc}$ < 1.5 can safely be predicted. | |
| 3.5.2 | OECD 106 | The experimental method described in OECD 106 uses different soil type samples which are mixed with the test article solution and agitated for some time. At steady state (maximum test article adsorption, up to 48 h) the mixture is separated and the test article concentration determined in the aqueous phase. For determining the desorption, the soil with the adsorbed test article is agitated with fresh aqueous $CaCl_2$ solution until a steady state is reached again. The aqueous phase is separated, analyzed for test article concentration, and the remaining soil treated in the same manner with fresh aqueous $CaCl_2$ solution. This procedure may be repeated several times, until all test article is desorbed or an irreversible adsorption is recognized. The whole test lasts for several days. If the test article is not stable during the test period, results become unreliable. During the adsorption part of the study it can not be differentiated between a test article decrease as a result of adsorption onto soil and a decrease as a result of degradation (abiotic or biotic). During the desorption step it can not be differentiated between test article not present in the aqueous solution because it is still adsorbed on the soil or degraded on the soil or degraded in the desorption solution. As in case of benzoic acid only weak adsorption is expected, the concentration changes in the aqueous solutions are expected to be quite small from step to step. In this case microbial degradation and not the adsorption/desorption will be the most influential parameter for the observed test article decrease. The use of radiolabelled benzoic acid does not overcome the problem because it can not be differentiated between the intact benzoic acid molecule and the various intermediate products of the microbial degradation. Heat sterilization of the soil in order to exclude microbial degradation will change the physico-chemical properties and texture of the soil and is therefore unsuitable. The guideline recommends temperatures of only 20 – 25°C (e.g. for drying soil samples). As conclusion, the determination of a valid $K_{oc}$ with benzoic acid, which is known to degrade very fast, is technically not feasible following the OECD 106 test guideline. Therefore, such a test was not performed with benzoic acid. | |
| 3.5.3 | QSAR models | Because the experimental determination of the $K_{oc}$ is not possible, for an estimation of the $K_{oc}$ a QSAR model has to be used. QSAR models take into account several known parameters of the individual test substance and by comparison with $K_{oc}$ values of similar chemical structures try to calculate a $K_{oc}$. Experience with several QSAR models during the last decades revealed, that there is no reliable model available for all chemical classes. Instead, for benzoic acid a model developed for similar chemical classes has to used.<br>A comparison of several models by Sabljic and Güsten, cited in the EU TGD on Risk Assessment, allows a predicting of the expected log $K_{oc}$ range depending on chemical classes. For organic acids a range between -1.0 and +5.0 resp. -0.5 to 4.0 with standard error range from 0.35 to 1.0 log units has been derived from a large number of experimental and QSAR determinations. Models use known parameters (e.g. log $P_{ow}$ 1.87 for benzoic acid) as base for their calculations. However, as the | X |

| | | |
|---|---|---|
| | | solubility of benzoic acid changes with pH, the QSAR models should predict results as a range of $K_{oc}$ values depending on pH. Two models were used and compared, as given in 3.6.5 and 3.6.6. |
| **3.6** | **Test performance** | Non-entry field |
| 3.6.1 | Preliminary test | No (technically not feasible) |
| 3.6.2 | Screening test: Adsorption | No (technically not feasible) |
| 3.6.3 | Screening test: Desorption | No (technically not feasible) |
| 3.6.4 | HPLC-method | No (expected value outside validated range) |
| 3.6.5 | EPI Suite vers. 3.20 (February 2007) by US EPA uses the PCKOCWIN v1.66 module | "The Soil Adsorption Coefficient Program (PCKOCWIN) estimates the soil adsorption coefficient (Koc) of organic compounds. Koc can be defined as "the ratio of the amount of chemical adsorbed per unit weight of organic carbon (oc) in the soil or sediment to the concentration of the chemical in solution at equilibrium" (Lyman, 1990); it is represented by the following equation (Lyman, 1990):<br>Koc = (µg adsorbed/g organic carbon) / (µg/mL solution)<br>Koc provides an indication of the extent to which a chemical partitions between solid and solution phases in soil, or between water and sediment in aquatic ecosystems. Estimated values of Koc are often used in environmental fate assessment because measurement of Koc is expensive. Traditional estimation methods rely upon the octanol/water partition coefficient or related parameters, but recently the first-order molecular connectivity index (1-MCI) has been used successfully to predict Koc values for hydrophobic organic compounds (Sabljic, 1984, 1987; Bahnick and Doucette, 1988). PCKOCWIN uses 1-MCI and a series of group contribution factors to predict Koc. The group contribution method outperforms traditional estimation methods based on octanol/water partition coefficients and water solubility." (quote from program description) |
| 3.6.6 | Advanced Chemistry Development, Inc., Toronto ON, Canada, module ACD/Adsorption Coeff. 8.02 | ACD/Labs (Advanced Chemistry Development, Inc., Toronto ON, Canada) uses the ACD/Adsorption Coeff. 8.02 module. The full printout of the calculation for benzoic acid is cited:<br>"The organic carbon adsorption coefficient, Koc, is the extent to which an organic chemical partitions itself between the solid and solution phases of water-saturated or unsaturated soil, runoff water or sediment. It is determined by several physical and chemical properties of both the chemical and the soil (or sediment). In most cases, it is possible to express the tendency of a chemical to be adsorbed in terms of the parameter Koc, which is largely independent of the properties of the soil or sediment. The Koc value may be thought of as the ratio of the amount of chemical adsorbed per unit weight of organic carbon (oc) in the soil or sediment to the concentration of the chemical in solution at equilibrium:<br>Koc = µg adsorbed / g organic carbon / µg / mL solution<br>**4 RESULTS** |
| 4.1 | EPI Suite vers. 3.20 (February 2007) by US EPA uses the | "----------PCKOCWIN v1.66 Results----------<br>First Order Molecular Connectivity: 4.305 |

## Section A7.1.3 Adsorption / Desorption screening test

**Annex Point IIA7.7**

| | | |
|---|---|---|
| | PCKOCWIN v1.66 module | Non-Corrected Log Koc: 2.9123<br>Fragment Correction(s):<br>* Organic Acid (-CO-OH) -1.7512<br>Corrected Log Koc: 1.1611<br>Estimated Koc: 14.49<br>NOTE:<br>The Koc of this structure may be sensitive to pH! The estimated Koc represents a best-fit to the majority of experimental values; however, the Koc may vary significantly with pH."(end of quote)<br>The predicted Koc is valid only for one (however not given) pH value. Nevertheless, the value Koc 14.5 allows a gross estimate of the expected range. |
| 4.1.1 | Advanced Chemistry Development, Inc., Toronto ON, Canada, module ACD/Adsorption Coeff. 8.02 | Adsorption coefficient (Koc) for benzoic acid / benzoate<br>pH = 0.0 ; Koc = 255 ; log(Koc) = 2.4 ± 1.0<br>pH = 1.0 ; Koc = 255 ; log(Koc) = 2.4 ± 1.0<br>pH = 2.0 ; Koc = 254 ; log(Koc) = 2.4 ± 1.0<br>pH = 3.0 ; Koc = 240 ; log(Koc) = 2.4 ± 1.0<br>pH = 4.0 ; Koc = 157 ; log(Koc) = 2.2 ± 1.0<br>pH = 5.0 ; Koc = 35.2 ; log(Koc) = 1.5 ± 1.0<br>pH = 6.0 ; Koc = 4.18 ; log(Koc) = 0.6 ± 1.0<br>pH = 7.0 ; Koc ~ 1<br>pH = 8.0 ; Koc ~ 1<br>pH = 9.0 ; Koc ~ 1<br>pH = 10.0 ; Koc ~ 1<br>pH = 11.0 ; Koc ~ 1<br>pH = 12.0 ; Koc ~ 1<br>pH = 13.0 ; Koc ~ 1<br>pH = 14.0 ; Koc ~ 1<br>The predicted Koc for the benzoic acid changes from the non-ionised molecule (pH 0 – 2, low water solubility) over the gradually ionised molecule (pH 3 – 7, water solubility increases) to the completely ionised benzoate (pH > 7, high water solubility).<br>To check the reliability of ACD/Adsorption Coeff. 8.02 module, Koc values for the chemical substances benzene, toluene, benzyl alcohol, formic acid, acetic acid and phenylacetic acid have been calculated, too. All these molecules from a formal point of view can be regarded as similar to benzoic acid or form a part of the benzoic acid molecule. Summarizing the obtained results from the reliability check, the outcome and changes in all predicted values can be easily explained, are plausible and are in accordance with the professional knowledge of a trained chemist. |
| **4.2** | **Calculations** | Non-entry field |
| 4.2.1 | Ka , Kd | Not applicable / QSAR model |
| 4.2.2 | $Ka_{oc}$ , $Kd_{oc}$ | Not applicable / QSAR model |
| **4.3** | **Degradation product(s)** | Not applicable / QSAR model |

## 5 APPLICANT'S SUMMARY AND CONCLUSION

| | | |
|---|---|---|
| **5.1** | **Materials and** | The experimental determination of the Koc utilising the OECD |

## Section A7.1.3 Adsorption / Desorption screening test

**Annex Point IIA7.7**

**methods**

guidelines 106 or 121 is not feasible.
For an estimation of the $K_{oc}$ of benzoic acid two QSAR models have been be used.

**5.2 Results and discussion**

The QSAR model PCKOCWIN v1.66 estimates a Koc of 14.5 (log Koc 1.16), but does not take into account the changes caused by pH variation. The value, therefore, may be only used as an estimate of the range of the Koc, but is sufficient to classify benzoic acid as substance with high mobility in soil.

The QSAR model ACD/Adsorption Coeff. 8.02 module calculates the Koc with the corresponding pH:

| | | |
|---|---|---|
| pH = 0.0 | Koc = 255 | log Koc = 2.4 ± 1.0 |
| pH = 1.0 | Koc = 255 | log Koc = 2.4 ± 1.0 |
| pH = 2.0 | Koc = 254 | log Koc = 2.4 ± 1.0 |
| pH = 3.0 | Koc = 240 | log Koc = 2.4 ± 1.0 |
| pH = 4.0 | Koc = 157 | log Koc = 2.2 ± 1.0 |
| pH = 5.0 | Koc = 35.2 | log Koc = 1.5 ± 1.0 |
| pH = 6.0 | Koc = 4.18 | log Koc = 0.6 ± 1.0 |
| pH = 7.0 and above | Koc ~ 1 | calculation not possible |

A check with similar compounds revealed no inconsistencies within the calculation method. And there are no inconsistencies to the PCKOCWIN value of log Koc 1.16, the estimate from the OECD 121 (log Koc < 1.5) and the range for organic acids between -1.0 and +5.0 respectively -0.5 to 4.0, mentioned in the EU TGD on Risk Assessment. Therefore, we recommend to use the values predicted by the ACD/Adsorption Coeff. 8.02 module for subsequent calculations of the environmental behaviour.

Nevertheless, as indicated by the error range ± 1.0 given for all values, care must be taken in interpreting quantitative results based an these estimated values. However, this is a general draw back of all QSAR models. As a qualitative statement, benzoic acid is regarded to be a highly mobile substance in soil.

5.2.1 Adsorbed a.s. [%] Not applicable / QSAR model

5.2.2 $K_a$ Not applicable / QSAR model

5.2.3 $K_d$ Not applicable / QSAR model

5.2.4 $Ka_{oc}$ Not applicable / QSAR model

5.2.5 Ka/Kd Not applicable / QSAR model

5.2.6 Degradation products (% of a.s.) Not applicable / QSAR model

**5.3 Conclusion**

The log Koc of benzoic acid changes with pH.

| | | |
|---|---|---|
| pH = 0.0 | Koc = 255 | log Koc = 2.4 ± 1.0 |
| pH = 1.0 | Koc = 255 | log Koc = 2.4 ± 1.0 |
| pH = 2.0 | Koc = 254 | log Koc = 2.4 ± 1.0 |

## Section A7.1.3 Adsorption / Desorption screening test

**Annex Point IIA7.7**

| | | |
|---|---|---|
| pH = 3.0 | Koc = 240 | log Koc = 2.4 ± 1.0 |
| pH = 4.0 | Koc = 157 | log Koc = 2.2 ± 1.0 |
| pH = 5.0 | Koc = 35.2 | log Koc = 1.5 ± 1.0 |
| pH = 6.0 | Koc = 4.18 | log Koc = 0.6 ± 1.0 |
| pH = 7.0 and above | Koc ~ 1 | calculation not possible |

Benzoic acid is regarded to be a highly mobile substance in soil at environmentally relevant pH values.

5.3.1 Reliability 2

5.3.2 Deficiencies QSAR model formally not validated

## Evaluation by Competent Authorities

**EVALUATION BY RAPPORTEUR MEMBER STATE**

**Date** 2009/03/17

**Materials and Methods** Applicant's version is accepted.

**Results and discussion** Applicant's version is adopted and is supported by the following information:
The comparison of several models (3.5.3) by Sabljic and Güsten, cited in the EU TGD on Risk Assessment (2003), Part III, Chapter 4.3, Table 4, allows a predicting of the expected log $K_{oc}$ range depending on chemical classes. RMS used the software PropertEst (Version 3.2.3) that refers to the above mentioned models (Sabljic, A. et al; Chemosphere 31(1995) 4489-4514) for estimation of $K_{oc}$. The model No. 6 (checked on 36 alcohols and organic acids) calculates in dependence of $logP_{ow}$ equal to 1.87 a $logK_{oc}$ of 1.38. For organic acids with $logP_{ow}$ range between -0.5 to 4.0 model No. 15 estimates a comparable $logK_{oc}$ of 1.44.

**Conclusion** Applicant's version is adopted.

**Reliability** 2

**Acceptability** acceptable

**Remarks**

**COMMENTS FROM ...**

**Date** Give date of comments submitted

**Materials and Methods** Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.
Discuss if deviating from view of rapporteur member state

**Results and discussion** Discuss if deviating from view of rapporteur member state

**Conclusion** Discuss if deviating from view of rapporteur member state

**Reliability** Discuss if deviating from view of rapporteur member state

**Acceptability** Discuss if deviating from view of rapporteur member state

**Remarks**

## Section A7.2.1 Aerobic degradation in soil, initial study
**Annex Point IIIA 12.1.1**

| | | Official use only |
|---|---|---|
| | **1 REFERENCE** | |
| | Verschueren, K, Handbook of Environmental Data on Organic Chemicals, 4th ed., Vol 1, 2001, Wiley & Sons, page286-287 (A7.2.1) | |
| | **2 RESULTS** | |
| | Verschueren K, 2001 (A7.2.1)<br>Biodegradation half lives in nonadapted aerobic subsoil (loam and sand): 0.30 days.<br>Decomposition period by a soil microflora: 1 day<br>99% COD removal at 88.5 mg COD/g dry inoculum/h (conditions 20°C, benzoic acid is sole carbon source, adapted microflora)<br>**3 CONCLUSION**<br>Benzoic acid can be classified as readily biodegradable in soil.<br>Benzoic acid is a compound naturally occurring in soil and plants. As a consequence, a benzoic acid degrading microbial population is widely present. | |
| | **Evaluation by Competent Authorities** | |
| | *Use separate "evaluation boxes" to provide transparency as to the comments and views submitted* | |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** | |
| **Date** | 2009/11/16 | |
| **Evaluation of applicant's justification** | The biodegradation half lives of benzoic acid in non-adapted aerobic subsoil (loam and sand) were reported to be 0.30 days under test conditions (20°C).<br>The reference cited in "Verschueren, K, Handbook of Environmental Data on Organic Chemicals" is "Van Beelen,P., and Peijnenburg, W. J. G. M., „De Afbraak van Organische Stoffen in het Grondwater" RIVM, rep. No. 718604002, The Netherlands, May 1989; „Characterizing the aerobic and anaeobic microbial activities in surface and subsurface Soils" – Thomas E. Ward, 1984"<br>Applicant's version is applicable. | |
| **Conclusion** | The applicant's version is adopted. | |
| **Remarks** | | |
| | **COMMENTS FROM OTHER MEMBER STATE** *(specify)* | |
| **Date** | *Give date of comments submitted* | |
| **Evaluation of applicant's justification** | *Discuss if deviating from view of rapporteur member state* | |
| **Conclusion** | *Discuss if deviating from view of rapporteur member state* | |

## Section A7.2.1 Aerobic degradation in soil, initial study

**Annex Point IIIA 12.1.1**

| Remarks |
| --- |

## Section A7.2.3 Adsorption and desorption in three soil types
**Annex Point IIIA XII 1.2**

Official use only

### 1 REFERENCE

| | | |
|---|---|---|
| **1.1** | **Reference** | Stolpe NB et al., 1993, Mobility of Aniline, Benzoic Acid and Toluene in Four Soils and Correlation with Soil Properties, Department of Agronomy, Department of Biochemistry, University of Nebraska-Lincoln, Lincoln, Nebraska, USA<br>Environmental Pollution 81, 287-295 (published) |
| **1.2** | **Data protection** | No |
| 1.2.1 | Data owner | Not applicable |
| 1.2.2 | Companies with letter of access | - |
| 1.2.3 | Criteria for data protection | No data protection / published data |

### 2 GUIDELINES AND QUALITY ASSURANCE

| | | |
|---|---|---|
| **2.1** | **Guideline study** | No. Soil properties and retention mechanisms affecting the relative mobility of benzoic acid in four soils were delineated in laboratory studies. The effect of benzoic acid was also determined on effective cation exchange capacity (ECEC) of the soils. |
| **2.2** | **GLP** | No |
| **2.3** | **Deviations** | Not applicable |

### 3 METHOD

| | | |
|---|---|---|
| **3.1** | **Test material** | Benzoic acid and [$^{14}$C]-benzoic acid<br>Aniline and [$^{14}$C]-aniline<br>Toluene and [$^{14}$C]-toluene |
| 3.1.1 | Lot/Batch number | Benzoic acid (Sigma. no. B-32.S0), [$^{14}$C ]-benzoic acid radiolabelled (Sigma no. 29, 717-8; 4 81 x $10^{11}$ Bq mol$^{-1}$) in reagent grade methanol (Fisher no. A412-4)<br>Aniline (Fisher no. A 740-500), [$^{14}$C]-aniline (Sigma no. 31, 178-2; 5 37 x $10^{11}$ Bq mol$^{-1}$) in deionized water<br>Toluene (Fisher no. T324-S00), [$^{14}$C]-toluene (Sigma no. 31, 435-8; 1 81 x $10^{11}$ Bq mol$^{-1}$) in reagent grade toluene |
| 3.1.2 | Specification | As given in section 2 |
| 3.1.3 | Purity | >99% |
| 3.1.4 | Further relevant properties | - |
| 3.1.5 | Composition of Product | - |
| 3.1.6 | TS inhibitory to microorganisms | No |
| 3.1.7 | Specific chemical analysis | $^{14}$C activity was determined with liquid scintillation |

## Section A7.2.3 Adsorption and desorption in three soil types
**Annex Point IIIA XII 1.2**

| | | |
|---|---|---|
| **3.2** | **Reference substance** | No reference substance |
| 3.2.1 | Initial concentration of reference substance | - |
| **3.3** | **Testing procedure** | *Non-entry field* |
| 3.3.1 | Soil types | See table A7_2_3-1<br>Cecil sandy loam (clayey, kaolinic, thermic Typic Hapludults)<br>Holdrege silt loam (fine-silty, mixed mesic Typic Argiustolls)<br>Sharpsburg silty clay (fine, montmorillonitic, mesic Typic Argiudolls)<br>Valentine fine sand( mixed mesic Typic Upstipsaments)<br>Samples were collected from summit, backslope and footslope positions of mapping units for each soil type. |
| 3.3.2 | Mobility determination | Soils were dry sieved to <500 µm for thin-layer chromatography (TLC) preparation. Soil slurries were spread over 5 X 20 cm glass slides using the 1 mm thickness of the TLC spreader and air-dried. A razor blade was used to trim the edges and horizontally score the plates 17 cm from the bottom so the capillary front would stop at that position. Five microliters of [$^{14}$C]-aniline or [$^{14}$C ]-benzoic acid solution (1346 and 377 Bq, respectively) were spotted 2 cm from the lower edge of the TLC plates to provide a 15-cm development zone. The plates were developed with deionized water in a TLC tank and air-dried. The location of $^{14}$C-activity on the plates was determined with a Bioscan System 300 Imaging Scanner. Relative mobility ($R_F$) values were calculated from the mean position of $^{14}$C-activity as determined b the first ordinary statistical moment, divided by the distance of plate development 15 cm. Triplicate plates were used for each treatment.<br>[$^{14}$C]-toluene mobility was measured using soil chromatographic columns. |
| 3.3.3 | Mineralogy and organic matter determinations | X-ray diffraction of powder-mounted whole soil and oriented clay was used to determine mineralogy. Oriented clay samples were treated with aniline (pure liquid), benzoic acid solution (0.1 M benzoic acid -0.1 M NaOH), or toluene (pure liquid) to determine whether the chemicals were sorbed into interlayers of the clays. Mg-saturated clays were air-dried on glass slides, and two or three drops of synthetic organic liquid or solution were placed on the edge of the clay film and allowed to diffuse until the entire clay film was moist. The slides were stored in desiccators containing the corresponding chemical. The samples were analyzed with a Rigaku Geigerflex X-ray diffractometer, with a strip chart recorder. the unit was set at 40 kV and 30 mA across he X-ray tube which emitted Cu-K α radiation. Scan speed was 8° 20 min$^{-1}$. The instrument chamber that held the samples during scanning contained 2 ml excess synthetic organic liquid or solution in a small glass vial to minimize evaporation of the sacs from the clay samples.<br>A fractionation procedure was used to determine the sorption sites of [$^{14}$C]-SOCs in organic matter of the soils. The fulvic acid (FA) fraction is soluble in both alkaline and acidic solutions, and has phenolic hydroxyl and carboxyl functional groups. The humic acid (HA) fraction is alkaline soluble and insoluble in acidic solutions, and is less oxidized than the FA fraction with larger aliphatic molecular structures. The humin fraction is insoluble in both alkaline and acidic solutions.<br>Soil samples of 1 g (<500 µm) were weighed into Teflon centrifuge tubes (50 ml volume) and treated with 5 mL [$^{14}$C]-aniline, [$^{14}$C]-benzoic acid, or [$^{14}$C]-toluene. The tubes were capped for 1 h and shaken |

| | | |
|---|---|---|
| | | periodically. Then 5 mL of deionized water was added to the tubes. The tubes were capped, shaken for 5 min, and centrifuged 5 min at 1500 rpm. $^{14}$C-activity of unbound SOC in the supernatant was measured by combining 1 ml of the supernatant with 10 mL ACS (aqueous counting scintillant) in a scintillation vial, with mixing and settling as previously described, and counting each vial for 2 min in the scintillation counter. Then 1 mL 2.5M NaOH was added to the same tubes to make the solutions 0.5M NaOH and the tubes were shaken overnight. The tubes were centrifuged and $^{14}$C-activily of SOC bound in The HA and FA fractions (plus unbound SOC) was measured in a 1-mL aliquot of the supernatant in 20 mL ACS. To acidify the remaining supernatant, 1 mL 2.5M HCl was added to the tubes. The tubes were shaken for 5 min, and centrifuged for 5 min at 1500 rpm. $^{14}$C-activity of SOC bound in the FA fraction (plus unbound SOC) was measured by combining a 1-mL aliquot of the supernatant with 10 mL ACS. The $^{14}$C-activity in the first water extract (unbound SOC) was subtracted from $^{14}$C-activity of subsequent extracts. Total $^{14}$C-activity of the FA fraction extract was calculated to include $^{14}$C-activity of the FA fraction that was removed with the previous 1 mL HA fraction + FA fraction aliquot. |
| 3.3.4 | Effect of SOCs (synthetic organic compounds) on ECEC (effective cation exchange capacity) | Soils were treated with reagent grade aniline, benzoic acid, or toluene to determine their effect on ECEC and total extractable bases. Soil (10 g) was placed into 125-ml Erlenmeyer flasks and treated with 10 ml of aniline (pure liquid), benzoic acid solution at pH 7 (0.8M benzoic acid-0.8M NaOH for whole soil ECEC, or 0.8M benzoic acid-0.4M $Ba(OH)_2$ for summation of bases), or toluene (pure liquid). The flasks were sealed with rubber stoppers and shaken for30 min on a wrist action shaker, opened and placed on a steam bath to evaporate the samples to near dryness. The samples were analyzed for ECEC and summation bases as previously described. |
| 3.3.5 | Infrared analysis | Diffuse reflectance IR spectroscopy was used to elucidate bonding mechanisms between soils and SOCs. Summit soil samples (10 g) were treated with 10 mL aniline (pure liquid), benzoic acid solution (0.8M benzoic acid-0.8M NaOH), or toluene (pure liquid) as previously described, and evaporated to dryness on a steam bath. The samples were lightly ground with an agate mortar and pestle and analyzed in a Mattson 4020 series FT-IR with a Hg-Cd Telluride (MCT) detector. The instrument was equipped with a Barnes Analytical/Spectra-Tech Diffuse Reflectance Accessory. |
| 3.3.6 | Initial TS concentration | 5 µL [$^{14}$C]-benzoic acid = 377 Bq<br>5 µL [$^{14}$C]-aniline = 1346 Bq<br>5 µL [$^{14}$C]-toluene = 2086 Bq |
| 3.3.7 | Intermediates/ degradation products | Not identified |
| 3.3.8 | Statistics | Data were interpreted using analysis of variance (ANOVA), least significant difference (LSD) of means, and correlation analysis (Steel & Torrie, 1980), |
| | | **4 RESULTS** |
| **4.1** | **Mobility, mechanism and Effective cation exchange capacity (ECEC)** | |

## Section A7.2.3 Adsorption and desorption in three soil types
**Annex Point IIIA XII 1.2**

| | | |
|---|---|---|
| 4.1.1 | Relative mobility ($R_F$) as affect by landscape position and soil type | See table A7_2_3-2<br>Benzoic acid was more mobile than aniline and toluene in Holdrege, Sharpsburg and Valentine soils (averaged over all landscape positions). Benzoic acid was most mobile in the footslope sample of the Cecil soil, and in the summit samples of the Sharpsburg and Valentine soils. |
| 4.1.2 | Correlation with soil properties | Benzoic acid mobility was positively correlated with soil pH, and negatively correlated with Al and Fe contents, and EAEC. The pH-dependent mobility of benzoic acid may be due to the effect of pH on speciation (dissociated or undissociated) of the benzoic acid and pH-dependent charge of the soils. Lower mobility in the Cecil soil may be partially attributed to retention of benzoate anions onto positively charged surfaces of Fe and Al oxides, but other retention mechanisms are possible.<br>Removal of organic matter increased mobility of benzoic acid in all soils. |
| 4.1.3 | Recovery of $^{14}$C chemicals added to whole and organic free soils | See table A7_2_3-3<br>Aqueous extraction of soils treated with the $^{14}$C-SOCs indicated benzoic acid was least strongly sorbed. |
| 4.1.4 | Graph | Retention mechanism<br>The absence of a carbonyl absorption band at 1700 $cm^{-1}$ in the diffuse reflectance IR of benzoic-acid treated Cecil soil indicated dissociation of the acid (see graph below). Bands at 3025 and 1598 $cm^{-1}$ were respectively (Fig. 2), Bands at 3025 and 1598 $cm^{-1}$were respectively assigned to =C-H and C=C stretching vibrations within the aromatic ring. Bands at 1556 and 1408 $cm^{-1}$ were respectively assigned to asymmetrical and symmetrical stretching vibrations of the carboxylate group, and were indicative of weak physical sorption through water bridging (hydrogen bonding) to Fe oxides. Lower Fe content or the Holdrege, Sharpsburg and Valentine soils may have contributed to weaker bonding and greater mobility of benzoic acid in those soils. Lower Al and Fe contents also would decrease the retention of benzoate anions to positively charged surfaces of Fe and Al oxides. |

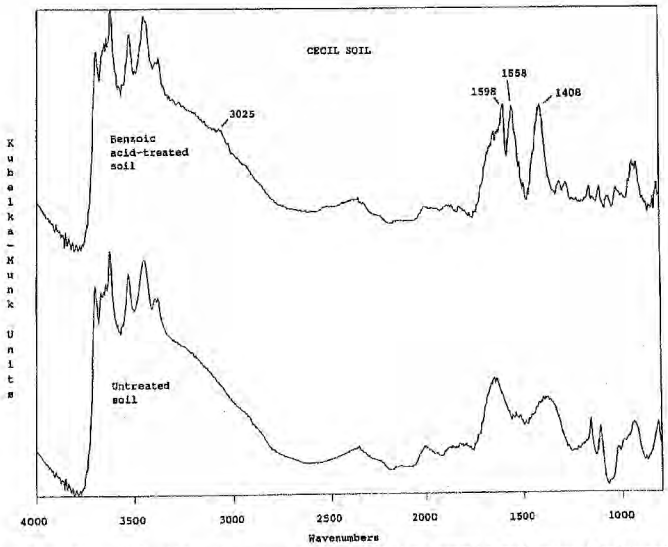



Fig. 2. Diffuse reflectance infrared absorption spectra of Cecil soil (whole soil, summit sample), untreated and treated with benzoic acid.

## Section A7.2.3 Adsorption and desorption in three soil types
**Annex Point IIIA XII 1.2**

| | | |
|---|---|---|
| 4.1.5 | Effective cation exchange capacity (ECEC) | See table A7_2_3-4<br>Benzoic acid and toluene treatment did not affect ECEC of the soils. Soils treated with aniline or benzoic acid had slightly lower total exchangeable bases than untreated soils, but the differences were significant only for the Cecil soils. |
| 4.1.6 | Intermediates/ degradation products | Not applicable |

**5 APPLICANT'S SUMMARY AND CONCLUSION**

| | | |
|---|---|---|
| **5.1** | **Materials and methods** | Soil properties and retention mechanisms affecting the relative mobility of benzoic aid were delineated in laboratory studies in four soils:<br>Cecil sandy loam (clayey, kaolinic, thermic Typic Hapludults)<br>Holdrege silt loam (fine-silty, mixed mesic Typic Argiustolls)<br>Sharpsburg silty clay (fine, montmorillonitic, mesic Typic Argiudolls)<br>Valentine fine sand( mixed mesic Typic Upstipsaments).<br>[$^{14}$C]-benzoic acid was developed on TLC plates covered with the respective soils. Relative mobility ($R_F$) values were calculated from the mean position of $^{14}$C-activity. |
| **5.2** | **Results and discussion** | Benzoic acid was most mobile compared to aniline and toluene, but was retained in the Cecil soil by hydrogen bonds to Fe oxides.<br>Changes in soil properties among soil series and topographic positions affected the mobility of benzoic acid by affecting the magnitude of potential bonding mechanisms between soils and SOCs.<br>Benzoic acid was retained through hydrogen bonds to Fe oxides in the Cecil soil, but retention through anion exchange was possible. Benzoic acid also may have been bound to HA fractions of organic matter through van der Waals forces and hydrogen bonds. |
| **5.3** | **Conclusion** | Mobility of benzoic acid depends on soil type and properties (organic carbon, clay content, pH). Benzoic acid is moderately mobile in soil. Validity criteria can be considered as fulfilled. |
| 5.3.1 | Reliability | 2 |
| 5.3.2 | Deficiencies | Not applicable |

| **Evaluation by Competent Authorities** | |
|---|---|
| | Use separate "evaluation boxes" to provide transparency as to the comments and views submitted |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | 2009/03/18 |
| **Materials and Methods** | Applicant's version is accepted. |
| **Results and discussion** | Applicant's version is accepted. |
| **Conclusion** | Applicant's version is adopted. The conclusion about mobility in soil supports the statement of study mentioned in III-A 7.1.3 which forecasts the pH-dependence of mobility in soil on the basis of QSAR calculation. |
| **Reliability** | Arising from the study object, only a statement can be met to the soil mobility of Benzoic acid in comparison to the soil mobility of Aniline and Toluene. |

## Section A7.2.3 Adsorption and desorption in three soil types
**Annex Point IIIA XII 1.2**

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

| | |
|---|---|
| | **COMMENTS FROM ...** *(specify)* |
| **Date** | *Give date of comments submitted* |
| **Materials and Methods** | *Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.*<br>*Discuss if deviating from view of rapporteur member state* |
| **Results and discussion** | *Discuss if deviating from view of rapporteur member state* |
| **Conclusion** | *Discuss if deviating from view of rapporteur member state* |
| **Reliability** | *Discuss if deviating from view of rapporteur member state* |
| **Acceptability** | *Discuss if deviating from view of rapporteur member state* |
| **Remarks** | |

## Table A7_2_3-1 Soil properties

**Table 1. Selected physical and chemical properties for Cecil, Holdrege, Sharpsburg, and Valentine soils. Samples were taken from summit, backslope, and footslope positions of the landscape**

| Soil | Sand (%) | Silt (%) | Clay (%) | g kg$^{-1}$ | | | $10^3$ m$^2$ kg$^{-1}$ | | cmol (+) kg$^{-1}$ | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Organic carbon | Al | Fe | Surface area | Surface area (−OM)[c] | ECEC[a] | ECEC (−OM) | EAEC[b] | Sum bases | Total acidity | Carboxyl acidity | pH |
| Cecil | | | | | | | | | | | | | | | |
| Summit | 56·5 | 12·8 | 30·7 | 8·4 | 3·7 | 42·1 | 55·3 | 43·5 | 10·4 | 8·3 | 0·3 | 2·9 | 4·5 | 1·4 | 5·7 |
| Backslope | 64·7 | 15·4 | 19·9 | 10·4 | 2·4 | 27·1 | 33·2 | 24·4 | 7·5 | 5·5 | 0·1 | 2·6 | 3·7 | 1·4 | 5·6 |
| Footslope | 73·3 | 16·0 | 10·7 | 9·7 | 1·4 | 13·5 | 20·7 | 11·8 | 5·4 | 3·5 | nd[d] | 2·0 | 2·5 | 1·2 | 5·7 |
| Holdrege | | | | | | | | | | | | | | | |
| Summit | 17·7 | 62·9 | 19·4 | 17·7 | 0·8 | 3·3 | 96·8 | 88·8 | 22·6 | 16·4 | nd | 16·2 | 4·9 | 1·8 | 6·5 |
| Backslope | 13·1 | 59·9 | 27·0 | 13·1 | 0·9 | 2·9 | 153·9 | 138·9 | 27·8 | 23·9 | 0·1 | 21·9 | 3·2 | 1·1 | 6·6 |
| Footslope | 14·7 | 59·6 | 25·7 | 15·8 | 0·9 | 3·4 | 124·2 | 121·0 | 24·6 | 21·5 | nd | 18·4 | 5·2 | 1·8 | 6·1 |
| Sharpsburg | | | | | | | | | | | | | | | |
| Summit | 5·7 | 61·9 | 32·4 | 15·7 | 1·5 | 8·7 | 146·6 | 127·7 | 25·6 | 25·3 | 0·1 | 17·0 | 5·6 | 2·4 | 5·9 |
| Backslope | 4·2 | 59·2 | 36·6 | 13·1 | 1·5 | 10·2 | 129·9 | 128·1 | 31·8 | 29·4 | 0·1 | 22·1 | 6·5 | 2·4 | 5·6 |
| Footslope | 6·7 | 64·8 | 28·5 | 18·2 | 1·3 | 6·9 | 193·4 | 153·5 | 26·6 | 24·1 | 0·1 | 17·6 | 6·9 | 3·2 | 5·7 |
| Valentine | | | | | | | | | | | | | | | |
| Summit | 97·1 | 1·3 | 1·6 | 0·7 | 0·2 | 0·5 | 14·5 | 10·4 | 2·5 | 2·1 | nd | 1·6 | 0·6 | 0·2 | 6·6 |
| Backslope | 94·9 | 2·9 | 2·2 | 4·0 | 0·2 | 0·7 | 16·8 | 10·4 | 3·8 | 2·7 | nd | 2·2 | 1·0 | 0·7 | 6·4 |
| Footslope | 92·1 | 4·4 | 3·5 | 4·8 | 0·3 | 0·8 | 22·9 | 22·9 | 5·7 | 4·0 | nd | 3·1 | 2·0 | 0·9 | 6·0 |

[a] ECEC = Effective cation exchange capacity.
[b] EAEC = Effective anion exchange capacity.
[c] −OM = Organic-free soils.
[d] nd = None detected.

## Table A7_2_3-2 Relative mobility ($R_F$) as affect by landscape position and soil type

| Soil | $R_F$ (Whole soil) | $R_F$ (Organic-free soil) |
|---|---|---|
| All landscape positions | | |
| Cecil | 0.12 | 0.77 |
| Holdrege | 0.60 | 0.89 |
| Sharpsburg | 0.46 | 0.82 |
| Valentine | 0.73 | 0.86 |
| LSD | 0.07 | 0.07 |
| Cecil | | |
| Summit | 0.10 | 0.60 |
| Backslope | 0.12 | 0.86 |
| Footslope | 0.15 | 0.84 |
| LSD | 0.03 | 0.06 |
| Holdrege | | |
| Summit | 0.55 | 0.90 |
| Backslope | 0.58 | 0.92 |
| Footslope | 0.66 | 0.84 |
| LSD | NSD | NSD |
| Sharpsburg | | |
| Summit | 0.52 | 0.86 |
| Backslope | 0.44 | 0.78 |
| Footslope | 0.40 | 0.82 |
| LSD | 0.07 | 0.05 |
| Valentine | | |
| Summit | 0.86 | 0.87 |
| Backslope | 0.70 | 0.88 |
| Footslope | 0.64 | 0.82 |
| LSD | 0.05 | NSD |
| | | |

LSD = Least significant difference

NSD = No significant difference

**Table A7_2_3-3 Recovery of $^{14}C$ chemicals added to whole and organic free soils**

| Soil | Whole soil | | | | Organic-free soil | |
|---|---|---|---|---|---|---|
| | Aqueous extract | Humic acid fraction | Fulvic acid | Not recovered | Aqueous extract | Not recovered |
| Cecil | 84.6 | 6.6 | 2.7 | 6.1 | 93.5 | 6.5 |
| Holdrege | 90.9 | 0.9 | nd | 8.2 | 94.4 | 5.6 |
| Sharpsburg | 91.8 | 1.7 | nd | 6.5 | 95.5 | 4.5 |
| Valentine | 94.0 | 2.7 | 0.9 | 2.4 | 98.9 | 1.1 |

nd = none detected

**Table A7_2_3-4 Effective cation exchange capacities (ECEC) and summation of extractable bases**

| Soil | Whole soil (cmol (+) $kg^{-1}$ soil) | | Organic free soil (cmol (+) $kg^{-1}$ soil) | |
|---|---|---|---|---|
| | Untreated | Benzoic acid | Untreated | Benzoic acid |
| Cecil | 7.8 | 6.8 | 5.8 | 5.1 |
| Holdrege | 25.0 | 21.8 | 20.6 | 19.5 |
| Sharpsburg | 28.0 | 23.4 | 26.3 | 24.7 |
| Valentine | 4.0 | 3.6 | 2.9 | 2.5 |
| LSD | 2.2 | 2.2 | 2.4 | 2.6 |

LSD = Least significant difference

NSD = No significant difference

**Section A7.3.1**
**Annex Point IIIA 7.5**

# Phototransformation in air

Official use only

## 1 REFERENCE

Atmospheric Oxidation Program for Microsoft Windows (AOPWIN) version 1.92, U.S. Environmental Protection Agency

The Atmospheric Oxidation Program for Microsoft Windows (AOPWIN) estimates the rate constant for the atmospheric, gas-phase reaction between photochemically produced hydroxyl radicals and organic chemicals. The rate constants estimated by the program are then used to calculate atmospheric half-lives for organic compounds based upon average atmospheric concentrations of hydroxyl radicals.

The estimation methods used by the Atmospheric Oxidation Program are based upon the structure-activity relationship (SAR) methods developed by Dr. Roger Atkinson and co-workers.

Preferences: 24 hr day, 5 E+5 OH radicals/$cm^3$

## 2 RESULTS

Printout of the AOPWIN program:

| | |
|---|---|
| SMILES: | O=C(O)c(cccc1)c1 |
| CHEM : | Benzoic acid |
| MOL FOR: | C7 H6 O2 |
| MOL WT : | 122.12 |

SUMMARY (AOP v1.92): HYDROXYL RADICALS

| | |
|---|---|
| Hydrogen Abstraction = | 0.0000 E-12 cm3/molecule-sec |
| Reaction with N, S and –OH = | 0.5200 E-12 cm3/molecule-sec |
| Addition to Triple Bonds = | 0.0000 E-12 cm3/molecule-sec |
| Addition to Olefinic Bonds = | 0.0000 E-12 cm3/molecule-sec |
| Addition to Aromatic Rings = | 0.7220 E-12 cm3/molecule-sec |
| Addition to Fused Rings = | 0.0000 E-12 cm3/molecule-sec |
| OVERALL OH Rate Constant = | 1.2420 E-12 cm3/molecule-sec |
| HALF-LIFE = | 12.918 Days (24-hr day; 0.5E6 OH/cm3) |

Experimental Database: NO Structure Matches

## 3 CONCLUSION

Benzoic acid has an estimated half life of 12.9 days in sunlight when considering OH radicals.

Direct photolysis is considered not to be a relevant breakdown process as can be deducted from the photolysis experiments in aqueous solutions.

**Section A7.3.1**
**Annex Point IIIA 7.5**

## Phototransformation in air

| | |
|---|---|
| | |
| **Evaluation by Competent Authorities** | |
| | *Use separate "evaluation boxes" to provide transparency as to the comments and views submitted* |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | 2009/01/20 |
| **Evaluation of applicant's justification** | Applicant's version is accepted. |
| **Conclusion** | Applicant's version is adopted. |
| **Reliability** | 1 |
| **Acceptability** | acceptable |
| **Remarks** | Applicant's version is adopted. The rate constant for degradation in air evaluates to 1.28 $h^{-1}$ leading to a half-life of 12.9 d and a value of 18.6 d for the chemical lifetime in the troposphere. |
| | |
| | **COMMENTS FROM OTHER MEMBER STATE** *(specify)* |
| **Date** | *Give date of comments submitted* |
| **Evaluation of applicant's justification** | *Discuss if deviating from view of rapporteur member state* |
| **Conclusion** | *Discuss if deviating from view of rapporteur member state* |
| **Remarks** | |

## Section A7.4.1.2 Acute toxicity to invertebrates

**Annex Point IIA7.1**

Official use only

| | | |
|---|---|---|
| | | **1 REFERENCE** |
| **1.1** | **Reference** | Jonas W, 1998b, Acute Immobilisation Test on *Daphnia magna* (Semi static Test Procedure). Test Substance: Benzoic Acid, SGS Natec Institut für naturwissenschaftlich technische Dienste GmbH, Hamburg, Germany; unpublished report no. NA 98 9408/2, July 10, 1998 |
| **1.2** | **Data protection** | Yes |
| 1.2.1 | Data owner | MENNO CHEMIE-VERTRIEB G.M.B.H., Norderstedt, Germany |
| 1.2.2 | Companies with letter of access | - |
| 1.2.3 | Criteria for data protection | Data submitted to the MS after 13. May 2000 on existing a.s. for the purpose of its entry into Annex I |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** |
| **2.1** | **Guideline study** | Yes, OECD 202 (1984) |
| **2.2** | **GLP** | Yes |
| **2.3** | **Deviations** | No |
| | | **3 MATERIALS AND METHODS** |
| **3.1** | **Test material** | Benzoic acid (As given in section 2) |
| 3.1.1 | Lot/Batch number | 3/48 |
| 3.1.2 | Specification | As given in section 2 |
| 3.1.3 | Purity | 99.0 – 100.5% |
| 3.1.4 | Composition of Product | - |
| 3.1.5 | Further relevant properties | - |
| 3.1.6 | Method of analysis | Determination of benzoic acid in MENNO Florades by HPLC, Analytical method C 17.2 (see 4.1 Analytical methods) |
| **3.2** | **Preparation of TS solution for poorly soluble or volatile test substances** | Not applicable |
| **3.3** | **Reference substance** | No |
| 3.3.1 | Method of analysis for reference substance | Not applicable |
| **3.4** | **Testing procedure** | *Non-entry field* |
| 3.4.1 | Dilution water | Elendt M4 Medium, see table A7_4_1_2-2 |
| 3.4.2 | Test organisms | *Daphnia magna*, see table A7_4_1_2-3 |
| 3.4.3 | Test system | Semi static, see table A7_4_1_2-4 |
| 3.4.4 | Test conditions | Test conditions in tabular form see table A7_4_1_2-5 |

## Section A7.4.1.2 Acute toxicity to invertebrates

**Annex Point IIA7.1**

| | | |
|---|---|---|
| 3.4.5 | Duration of the test | 48 hours |
| 3.4.6 | Test parameter | Immobilisation, clinical signs |
| 3.4.7 | Sampling | 24 and 48 hours |
| 3.4.8 | Monitoring of TS concentration | Yes, at 0, 24 and 48 hours |
| 3.4.9 | Statistics | Probit method |

**4 RESULTS**

| | | |
|---|---|---|
| **4.1** | **Limit Test** | Not performed |
| 4.1.1 | Concentration | - |
| 4.1.2 | Number/ percentage of animals showing adverse effects | - |
| 4.1.3 | Nature of adverse effects | - |
| **4.2** | **Results test substance** | *Non-entry field* |
| 4.2.1 | Initial concentrations of test substance | 12, 26, 55 and 120 mg Benzoic acid/L. |
| 4.2.2 | Actual concentrations of test substance | **Summary of analytical results** |

| Nominal concentration Benzoic acid/L | % of nominal after 0 h | % of nominal after 24 h | % of nominal after 48 h |
|---|---|---|---|
| control | n.a. | n.a. | n.a. |
| 12 | 101 | 97 | 100 |
| 26 | 101 | 106 | 102 |
| 55 | 99 | 102 | 98 |
| 120 | 99 | 102 | 98 |

The concentrations in the test container corresponded well with the nominal values. The overall average of measured concentrations was 101% of the nominal concentrations (variation coefficient = ±2%).

| | | |
|---|---|---|
| 4.2.3 | Effect data (Mortality) | Immobilisation was observed at the highest concentration after 48 hours, see table A7_4_1_2-6<br>$LC_0$, $LC_{50}$ and $LC_{100}$ values for at least 24 and 48 h (including 95 % c.l.; see table A7_4_1_2-7 |
| 4.2.4 | Concentration / response curve | Not applicable |
| 4.2.5 | Other effects | No other effects |
| **4.3** | **Results of controls** | Immobilised animals / controls |
| 4.3.1 | Number/ | |

| **hours** | 24 | 48 |
|---|---|---|

## Section A7.4.1.2 Acute toxicity to invertebrates

**Annex Point IIA7.1**

| | | | | |
|---|---|---|---|---|
| | percentage of animals showing adverse effects | **Animals** | 0 | 0 |
| 4.3.2 | Nature of adverse effects | Not applicable | | |
| **4.4** | **Test with reference substance** | Not performed | | |
| 4.4.1 | Concentrations | - | | |
| 4.4.2 | Results | - | | |

**5 APPLICANT'S SUMMARY AND CONCLUSION**

**5.1 Materials and methods**

Benzoic acid was assessed for its toxicity effects in a semi static acute toxicity test on *daphnia magna*. In each test solution twenty Daphnia magna were exposed under semi static conditions to nominal concentrations ranging from 12 to 120 mg Benzoic acid/L. The test was carried out following OECD guideline 202.

**5.2 Results and discussion**

Highest tested conc. without toxic effect (NOEC)

| | |
|---|---|
| 24 h: | >120 mg |
| 48 h: | 55 mg |

The $EC_{50}$ is the concentration at which 50% of the animals were dead. This value was not reached in this study. Therefore the $EC_{50}$ could not be determined.

5.2.1 $LC_0$

| | |
|---|---|
| 24 h: | 120 mg/L |
| 48 h: | 55 mg/L |

5.2.2 $LC_{50}$ >120 mg/L

5.2.3 $LC_{100}$ >120 mg/L

**5.3 Conclusion**

Benzoic acid is not acute toxic to *Daphnia magna.*

Validity criteria can be considered as fulfilled, see table A7_4_1_2-8

5.3.1 Other Conclusions -

5.3.2 Reliability 1

5.3.3 Deficiencies No

| **Evaluation by Competent Authorities** | |
|---|---|
| | Use separate "evaluation boxes" to provide transparency as to the comments and views submitted |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | 2008/02/12 |
| **Materials and Methods** | Applicant's version is acceptable. |
| **Results and discussion** | Applicant's version can be adopted. |
| **Conclusion** | Applicant's version can be adopted. |

## Section A7.4.1.2 Acute toxicity to invertebrates

**Annex Point IIA7.1**

| | |
|---|---|
| **Reliability** | 1 |
| **Acceptability** | acceptable |
| **Remarks** | - |
| | **COMMENTS FROM ...** |
| **Date** | *Give date of comments submitted* |
| **Materials and Methods** | *Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.*<br>*Discuss if deviating from view of rapporteur member state* |
| **Results and discussion** | *Discuss if deviating from view of rapporteur member state* |
| **Conclusion** | *Discuss if deviating from view of rapporteur member state* |
| **Reliability** | *Discuss if deviating from view of rapporteur member state* |
| **Acceptability** | *Discuss if deviating from view of rapporteur member state* |
| **Remarks** | |

**Table A7_4_1_2-1: Preparation of TS solution for poorly soluble or volatile test substances**

| Criteria | Details |
|---|---|
| Dispersion | Not applicable |
| Vehicle | Not applicable |
| Concentration of vehicle | Not applicable |
| Vehicle control performed | Not applicable |
| Other procedures | Not applicable |

**Table A7_4_1_2-2: Dilution water at the beginning of the study**

| Criteria | Details |
|---|---|
| Source | Elendt M4 Medium as given in Draft OECD-guideline 202 Part II |
| Hardness | 185 mg $CaCO_3$/L |
| pH | 8.2 |
| Oxygen content | 92 - 96 (%Saturation) |
| Conductivity adjusted to 20°C | No data given |
| Holding water different from dilution water | No |

**Table A7_4_1_2-3: Test organisms**

| Criteria | Details |
|---|---|
| Species/strain | *Daphnia magna* / clone 5 (=cloneA) |
| Source | Institut für Wasser-, Boden- und Lufthygiene, Berlin, Germany |
| Age | Less than 24 hours at test start |
| Breeding method | Laboratory culture of the green algae (Scenedesmus subspicatus), used after two days growth |
| Amount of food | Not applicable |
| Feeding frequency | No feeding during test |
| Pretreatment | Data not given |
| Feeding of animals during test | No feeding during test |

**Table A7_4_1_2-4: Test system**

| Criteria | Details |
|---|---|
| Test type | Semi static |
| Renewal of test solution | Daily |
| Volume of test vessels | 1 L glass beakers |
| Volume/animal | 200 mL / animal |
| Number of animals/vessel | 5 |
| Number of vessels/ concentration | 4 |

| | |
|---|---|
| Test performed in closed vessels due to significant volatility of TS | Not applicable |

**Table A7_4_1_2-5: Test conditions**

| Criteria | Details |
|---|---|
| Test temperature | Test temperature: 20.5°C |
| Dissolved oxygen | Start of test: 96 End of test: 92 (% Saturation) |
| pH | Start of test: 8.02 End of test: 8.02 |
| Adjustment of pH | No |
| Aeration of dilution water | No |
| Intensity of irradiation | 1000 – 4000 lux |
| Photoperiod | 16 hours light (1000 – 4000 lux), 8 hours darkness |

**Table A7_4_1_2-6: Immobilisation data**

| Test-Substance Concentration (nominal) [mg/l] | Immobilisation | | | |
|---|---|---|---|---|
| | Number | | Percentage | |
| | 24 h | 48 h | 24 h | 48 h |
| 0 (Control) | 0 | 0 | 0 | 0 |
| 12 | 0 | 0 | 0 | 0 |
| 26 | 0 | 0 | 0 | 0 |
| 55 | 0 | 0 | 0 | 0 |
| 120 | 0 | 4 | 0 | 25 |
| **Temperature [°C]** | 20.5°C | 20.5°C | | |
| **pH** | 8.02 | 8.02 | | |
| **Oxygen [%Saturation]** | 96 | 92 | | |

**Table A7_4_1_2-7: Effect data (nominal concentrations)**

The $EC_{50}$ is the concentration at which 50% of the daphnia were immobilised. This value was not reached in this study. Therefore the $EC_{50}$ could not be determined. Following 48 hours of exposure, the no observed effect concentration for this study is 55 mg

| | 24 h [mg/l][1] | 95 % c.l. | 48 h [mg/l][1] | 95 % c.l. |
|---|---|---|---|---|
| **$LC_0$** | 120 | - | 55 | - |
| **$LC_{50}$** | >120 | - | >120 | - |
| **$LC_{100}$** | >120 | - | >120 | - |

**Table A7_4_1_1-8: Validity criteria for acute fish test according to OECD Guideline 203**

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

| | | |
|---|---|---|
| Criteria for poorly soluble test substances | | |
| | | |
| | | |

**Table A7_4_1_2-8: Additional data / acute toxicity to daphnia magna**

| Result | Reference |
|---|---|
| Benzoic acid<br>48h $EC_{50}$ (95%C.I.):<br>7.04 mmol/L (=860 mg/L) | Kamaya Y, Fukaya Y, Suzuki K, 2005, (A7.4.1.2/02) |
| Benzoic acid<br>$LC_0$ (24 h) 540 mg/L, neutralized solution<br>$LC_{50}$ (24 h) 1540 mg/L, calculated, neutralized solution | Bringmann G Kühn R, 1977 (A7.4.1.2/03) |
| Sodium salt: $LC_{50}$ (96 h) > 100 mg/L, | Ewell WS, Gorsuch JW, Kringle RO, Robillard KA, Spiegel RC, 1986 (A7.4.1.2/03) |
| Benzoic acid<br>"Immobilization threshold": 146 mg/L (prolonged (none defined) exposure in lake Erie water at 25 °C) | McKee JE, Wolf HW, 1963 (A7.4.1.1/03) |

# Section A7.4.1.3 Growth inhibition test on algae

**Annex Point IIA7.1**

Official use only

| | | |
|---|---|---|
| | | **1 REFERENCE** |
| **1.1** | **Reference** | Lebertz H, 2008, Study on the 'toxicity towards algae' of Benzoic Acid according to OECD-Test Guideline 201 (Algae, Growth Inhibition Test) Version dated 23-Mar-2006, SGS Institut Fresenius, Taunusstein, Germany; unpublished report no. IF-08/01196420, October 17, 2008 |
| **1.2** | **Data protection** | Yes |
| 1.2.1 | Data owner | MENNO CHEMIE-VERTRIEB G.M.B.H., Norderstedt, Germany |
| 1.2.2 | Companies with letter of access | - |
| 1.2.3 | Criteria for data protection | Data submitted to the MS after 13. May 2000 on existing a.s. for the purpose of its entry into Annex I |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** |
| **2.1** | **Guideline study** | Yes, OECD 201 (Version dated 23-Mar-2006) |
| **2.2** | **GLP** | Yes |
| **2.3** | **Deviations** | No |
| | | **3 MATERIALS AND METHODS** |
| **3.1** | **Test material** | Benzoic acid (As given in section 2) |
| 3.1.1 | Lot/Batch number | 35361068 |
| 3.1.2 | Specification | As given in section 2 |
| 3.1.3 | Purity | 99.99%, Certificate of analysis dated 25-October-2004 |
| 3.1.4 | Composition of Product | - |
| 3.1.5 | Further relevant properties | - |
| 3.1.6 | Method of analysis | For the Certificate of Analysis: Acidimetric Titration of dried substance<br>For the determination of Benzoic acid in the aqueous test solutions of the algae toxicity test: HPLC with UV/Vis detection |
| **3.2** | **Preparation of TS solution for poorly soluble or volatile test substances** | Not applicable, because test article is neither poorly soluble nor volatile. . However, the pH of the test item solution was after dissolution adjusted to 7.8 to avoid effects caused by the acidity of the test article.<br>Remark: Increasing the buffer concentration in the test media (instead of adjusting the test article solution) resulted in inhibitory effects to the algae and is therefore not suitable to mask the effects of the acidity. |
| **3.3** | **Reference substance** | No |
| 3.3.1 | Method of analysis for reference substance | Not applicable |
| **3.4** | **Testing procedure** | *Non-entry field* |

## Section A7.4.1.3 Growth inhibition test on algae

**Annex Point IIA7.1**

3.4.1 Culture medium: As required in the OECD Guideline 201 (2006)

**Stock solutions**

3.4.1.1 Mineral Nutrient Salts

| g/L deionised water | Component |
|---|---|
| 1.50 | $NH_4Cl$ |
| 1.20 | $MgCl_2$ x 6 $H_2O$ |
| 1.80 | $CaCl_2$ x 2 $H_2O$ |
| 1.50 | $MgSO_4$ x 7 $H_2O$ |
| 0.16 | $KH_2PO_4$ |

3.4.1.2 Mineral Nutrient Salts

| g/L deionised water | Component |
|---|---|
| 0.08 | $FeCl_3$ x 6 $H_2O$ |
| 0.10 | $Na_2EDTA$ x 2 $H_2O$ |

3.4.1.3 Trace Elements

| mg/L deionised water | Component |
|---|---|
| 185 | $H_3BO_3$ |
| 415 | $MnCl_2$ x 4 $H_2O$ |
| 3 | $ZnCl_2$ |
| 1.5 | $CoCl_2$ x 6 $H_2O$ |
| 0.01 | $CuCl_2$ x 2 $H_2O$ |
| 7 | $Na_2MoO_4$ x 2 $H_2O$ |

3.4.1.4 Sodium Hydrogen carbonate

| g/L deionised water | Component |
|---|---|
| 1.00 | $NaHCO_3$ |

To avoid microbial degradation, all stock solutions used in the study were heat sterilised by autoclaving. Solutions with the test item were sterilized by membrane filtrating and subsequently handled under aseptic conditions in a laminar flow clean bench with HEPA filter. All equipment in contact with the solutions (e.g. beaker, spatula, pipette) was heat sterilized prior to use (if appropriate) and kept under the clean bench to avoid secondary infection.

3.4.2 Test organisms: *Desmodesmus subspicatus CHODAT*, see table A7_4_1_3-2, cultivated under aseptic conditions and checked for fungal and bacterial contamination

3.4.3 Test system: See table A7_4_1_3-3

3.4.4 Test conditions: See table A7_4_1_3-4 under aseptic conditions in a laminar flow clean bench with HEPA filter

3.4.5 Duration of the: 72 hours

## Section A7.4.1.3 Growth inhibition test on algae

**Annex Point IIA7.1**

| | | |
|---|---|---|
| | test | |
| 3.4.6 | Test parameter | Inhibition of the yield and growth rate<br>(Yield = Cell number at time $T_{72h}$ - cell number at time $T_0$)<br>Measure for biomass of algae: absorbance of the algae culture, measured at 578 nm. |
| 3.4.7 | Sampling | 0, 25, 48 and 72 hours |
| 3.4.8 | Monitoring of TS concentration | Yes, at 0 and 72 hours |
| 3.4.9 | Statistics | Probit method |

**4 RESULTS**

| | | |
|---|---|---|
| **4.1** | **Limit Test** | Not performed |
| 4.1.1 | Concentration | - |
| 4.1.2 | Number/ percentage of animals showing adverse effects | - |
| 4.1.3 | Nature of adverse effects | - |
| **4.2** | **Results test substance** | *Non-entry field* |
| 4.2.1 | Initial concentrations of test substance | Screening test: 1, 10, 100 and 1000 mg Benzoic acid/L<br>Main Test: 100, 200, 400, 800 and 1000 mg Benzoic acid/L |
| 4.2.2 | Actual concentrations of test substance | **Summary of analytical results** |

| Nominal concentration mg/L | Measured concentration mg/L | Net values (net-control) | % value of $t_0$ | % value of nominal concentration |
|---|---|---|---|---|
| **$t_0$** | | | | |
| Control | n.a. | n.a. | n.a. | n.a. |
| 100 | 98 | 98 | - | 98 |
| 200 | 211 | 211 | - | 106 |
| 400 | 429 | 429 | - | 107 |
| 800 | 808 | 808 | - | 101 |
| 1000 | 1061 | 1061 | - | 106 |
| **$t_{72\,h}$** | | | | |
| Control | n.a. | n.a. | n.a. | n.a. |
| 100 | 110 | 110 | 112 | 110 |
| 200 | 216 | 216 | 102 | 108 |
| 400 | 399 | 399 | 93 | 100 |

## Section A7.4.1.3 Growth inhibition test on algae

**Annex Point IIA7.1**

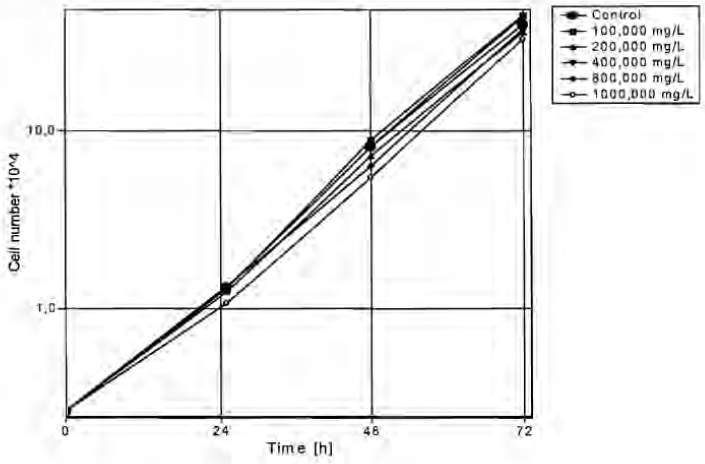
| | | | | |
|---|---|---|---|---|
| 800 | 818 | 818 | 101 | 102 |
| 1000 | 1037 | 1037 | 98 | 104 |

Recoveries of the theoretical concentrations were higher than 80%, and therefore the effective concentrations are based on the nominal concentrations.

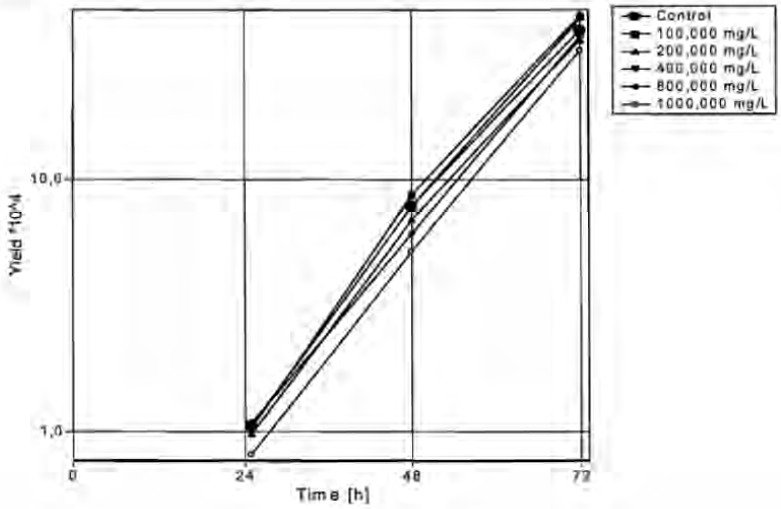
Benzoic acid was stable during the incubation phase of 72 hours as a result of the strictly observed aseptic conditions (described under point 3.4.1, 3.4.2 and 3.4.3 above).

4.2.3 Curves

Cell number in *Desmodesmus subspicatus* as dependent on test item concentration and time.




Yield in *Desmodesmus subspicatus* as dependent on test item concentration and time.




4.2.4 Cell concentration data

Cell concentration data see table A7_4_1_3-5

**Inhibition Values (Main Test)**

| Nominal Concentration | Growth rate | Inhibition of the Growth rate after | Yield after 72 h | Inhibition of the Yield after |
|---|---|---|---|---|

## Section A7.4.1.3 Growth inhibition test on algae

**Annex Point IIA7.1**

| (mg A.I./L) | ($day^{-1}$) | 72 h (%) | | 72 h (%) |
|---|---|---|---|---|
| 0 | 1.662 | - | $3.92*10^5$ | - |
| 100 | 1.703 | -2.5 | $4.43*10^5$ | -13.3 |
| 200 | 1.630 | 2.0 | $3.55*10^5$ | 9.4 |
| 400 | 1.696 | -2.1 | $4.34*10^5$ | -10.8 |
| 800 | 1.644 | 1.1 | $3.71*10^5$ | 5.3 |
| 1000 | 1.601 | 3.6 | $3.26*10^5$ | 16.7 |

4.2.5 Effect data (cell multiplication inhibition)

**Toxic effects**

| On the Basis of the Nominal Concentrations tested (mg/L) | | | |
|---|---|---|---|
| | **Yield (0-72h)** | **Growth rate (0-72h)** | **Section-by-section Growth rate (48-72h)** |
| EC10<br>95%-CL lower<br>upper | 829<br>n.d.<br>n.d. | n.d.<br>n.d.<br>n.d. | n.d.<br>n.d.<br>n.d. |
| EC20<br>95%-CL lower<br>upper | n.d.<br>n.d.<br>n.d. | n.d.<br>n.d.<br>n.d. | n.d.<br>n.d.<br>n.d. |
| EC50<br>95%-CL lower<br>upper | n.d. (>1000)<br>n.d.<br>n.d. | n.d. (>1000)<br>n.d.<br>n.d. | n.d. (>1000)<br>n.d.<br>n.d. |
| LOEC<br>NOEC | 800<br>400 | 800<br>400 | >1000<br>>1000 |

n.d. = not determined due to mathematical reasons

4.2.6 Other effects: No other effects

**4.3 Results of controls**: See table A7_4_1_3-5

**4.4 Test with reference substance**: Not performed

4.4.1 Concentrations: -

4.4.2 Results: -

## 5 APPLICANT'S SUMMARY AND CONCLUSION

**5.1 Materials and methods**

The chronic toxicity of the test substance Benzoic acid towards algae was tested according to OECD-Test Guideline 201, performed with the pH-adjusted aqueous solution of the acidic test item under aseptic conditions. The toxic effects were investigated by determination of the inhibition of the growth rate of the algae, the yield during the exposure period of 72 hours.

The algae were exposed to nominal concentrations ranging from 100 to 1000 mg Benzoic acid/L.

**5.2 Results and**

## Section A7.4.1.3 Growth inhibition test on algae

**Annex Point IIA7.1**

| | | | |
|---|---|---|---|
| | **discussion** | | |
| 5.2.1 | NOEC | 400 mg Benzoic acid/L | |
| 5.2.2 | LOEC | 800 mg Benzoic acid/L | |
| 5.2.3 | $LC_{50}$ | > 800 mg Benzoic acid/L | X |
| 5.2.4 | $LC_{10}$ | 829 mg Benzoic acid/L | |
| **5.3** | **Conclusion** | Benzoic acid is not toxic to algae<br>Validity criteria can be considered as fulfilled, see table A7_4_1_3-6.<br>In contrast to published toxicity data and another own test supplied in this dossier, in this study the pH was adjusted at study start to physiological conditions for the test organisms (approximately pH 8). This allows differentiation between the inherent toxicity of the benzoic acid molecule and the effects caused by lowering the pH by acidic test articles like benzoic acid. | |
| 5.3.1 | Other Conclusions | - | |
| 5.3.2 | Reliability | 1 | |
| 5.3.3 | Deficiencies | No | |

| **Evaluation by Competent Authorities** | |
|---|---|
| | Use separate "evaluation boxes" to provide transparency as to the comments and views submitted |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | 2009/05/13 |
| **Materials and Methods** | Applicant's version is acceptable. |
| **Results and discussion** | Applicant's version can be adopted with the following comment:<br>5.2.3: EC50 > 1000 mg/L. |
| **Conclusion** | Applicant's version can be adopted. |
| **Reliability** | 1 |
| **Acceptability** | acceptable |
| **Remarks** | |
| | **COMMENTS FROM ...** |
| **Date** | *Give date of comments submitted* |
| **Materials and Methods** | *Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.*<br>*Discuss if deviating from view of rapporteur member state* |
| **Results and discussion** | *Discuss if deviating from view of rapporteur member state* |
| **Conclusion** | *Discuss if deviating from view of rapporteur member state* |
| **Reliability** | *Discuss if deviating from view of rapporteur member state* |
| **Acceptability** | *Discuss if deviating from view of rapporteur member state* |

## Section A7.4.1.3 Growth inhibition test on algae

**Annex Point IIA7.1**

| Remarks |
| --- |

**Table A7_4_1_3-1: Preparation of TS solution for poorly soluble or volatile test substances**

| Criteria | Details |
|---|---|
| Dispersion | Not applicable |
| Vehicle | Not applicable |
| Concentration of vehicle | Not applicable |
| Vehicle control performed | Not applicable |
| Other procedures | Not applicable |

**Table A7_4_1_3-2: Test organisms**

| Criteria | Details |
|---|---|
| Species/strain | *Desmodesmus subspicatus CHODAT,* |
| Strain | 86.81 |
| Source | Sammlung von Algenkulturen, Pflanzenphysiologisches Institut der Universität Göttingen (SAG) |
| Laboratory culture | Yes |
| Method of cultivation | according OECD Guideline 201 |
| Pre treatment | 2 days prior to starting the test a pre-culture was inoculated using the algal stock culture. This culture was prepared in "pre-culture medium" using 100 ml of the stock solution 3.4.1.1 and 10 ml of each stock solution 3.4.1.2 and 3.4.1.3 and 1 g $NaHCO_3$ in 880 ml ultrapure water. This "intermediate dilution" was used for the pre-culture at a final concentration of 50 mL/500 mL prepared in ultrapure water. |
| Initial cell concentration | 2.7 x $10^3$ cells/ml |

**Table A7_4_1_3-3: Test system**

| Criteria | Details |
|---|---|
| Volume of culture flasks | 50 ml |
| Culturing apparatus | Constant conditions of 23.3 to 23.8 °C<br>To assure the input of $CO_2$, the test solutions were stirred for the duration of 15 minutes per hour. |
| Light quality | ≥120 µE/$m^2$s (≈8000 Lux) |
| Number of vessels / concentration | Screening test: 5 replicates<br>Main test: 7 replicates |
| Test performed in closed vessels due to significant volatility of TS | Not applicable |

**Table A7_4_1_3-4: Test conditions**

<table>
<tr><th>Criteria</th><th>Details</th></tr>
<tr><td>Test temperature</td><td>23.3 to 23.8 °C, main 23.5 °C</td></tr>
<tr><td>pH</td><td>Screening test
<table>
<tr><td>Test solution/ Concentration Test item</td><td colspan="2">pH at</td></tr>
<tr><td></td><td>$T_0$</td><td>$T_{72h}$</td></tr>
<tr><td>Control with algal inoculation</td><td>8.21</td><td>8.96</td></tr>
<tr><td>Control without algal inoculation</td><td>8.19</td><td>8.22</td></tr>
<tr><td>1.0 mg/L with algal inoculation</td><td>8.20</td><td>8.97</td></tr>
<tr><td>1.0 mg/L without algal inoculation</td><td>8.20</td><td>8.20</td></tr>
<tr><td>10.0 mg/L with algal inoculation</td><td>8.17</td><td>8.80</td></tr>
<tr><td>10.0 mg/L without algal inoculation</td><td>8.16</td><td>8.19</td></tr>
<tr><td>100.0 mg/L with algal inoculation</td><td>8.18</td><td>8.95</td></tr>
<tr><td>100.0 mg/L without algal inoculation</td><td>8.16</td><td>8.20</td></tr>
<tr><td>1000.0 mg/L with algal inoculation</td><td>8.17</td><td>8.81</td></tr>
<tr><td>1000.0 mg/L without algal inoculation</td><td>8.16</td><td>8.27</td></tr>
</table>
Main test
<table>
<tr><td>Test solution/ Concentration Test item</td><td colspan="4">pH at</td></tr>
<tr><td></td><td>$T_0$</td><td>$T_{25h}$</td><td>$T_{48h}$</td><td>$T_{72h}$</td></tr>
<tr><td>Control with algal inoculation</td><td>8.04</td><td>8.11</td><td>8.38</td><td>9.16</td></tr>
<tr><td>Control without algal inoculation</td><td>8.03</td><td>8.00</td><td>8.07</td><td>8.16</td></tr>
<tr><td>100.0 mg/L with algal inoculation</td><td>8.06</td><td>8.12</td><td>8.39</td><td>9.37</td></tr>
<tr><td>100.0 mg/L without algal inoculation</td><td>8.04</td><td>8.09</td><td>8.13</td><td>8.18</td></tr>
<tr><td>200.0 mg/L with algal inoculation</td><td>8.02</td><td>8.17</td><td>8.39</td><td>9.18</td></tr>
<tr><td>200.0 mg/L without algal inoculation</td><td>8.02</td><td>8.16</td><td>8.19</td><td>8.18</td></tr>
<tr><td>400.0 mg/L with algal inoculation</td><td>8.01</td><td>8.17</td><td>8.40</td><td>9.27</td></tr>
<tr><td>400.0 mg/L without algal inoculation</td><td>8.00</td><td>8.16</td><td>8.19</td><td>8.18</td></tr>
<tr><td>800.0 mg/L with algal inoculation</td><td>8.07</td><td>8.19</td><td>8.41</td><td>9.10</td></tr>
<tr><td>800.0 mg/L without algal inoculation</td><td>8.05</td><td>8.15</td><td>8.11</td><td>8.15</td></tr>
<tr><td>1000.0 mg/L with algal inoculation</td><td>8.09</td><td>8.21</td><td>8.39</td><td>9.02</td></tr>
<tr><td>1000.0 mg/L without algal inoculation</td><td>8.09</td><td>8.17</td><td>8.17</td><td>8.10</td></tr>
</table></td></tr>
<tr><td>Aeration of dilution water</td><td>No</td></tr>
<tr><td>Light intensity</td><td>≥120 µE/m²s (≈8000 Lux)</td></tr>
<tr><td>Photoperiod</td><td>constant</td></tr>
</table>

**Table A7_4_1_3-5: Cell concentration data**

| Test-Substance Concentration (nominal) [mg/l] | Cell concentrations (mean values) [cells/ml]sw | | | | Inhibiton of Growth Rate at $t_{72h}$ (%) | pH values after 72 hours |
|---|---|---|---|---|---|---|
| | $t_0$ | $t_{24h}$ | $t_{47.5h}$ | $t_{72h}$ | | |
| 0 | $2.64*10^3$ | $1.53*10^4$ | $1.05*10^5$ | $4.64*10^5$ | - | 8.96 |
| 1.0 | $2.64*10^3$ | $1.30*10^4$ | $9.52*10^4$ | $4.57*10^5$ | 0.2 | 8.97 |
| 10.0 | $2.64*10^3$ | $1.30*10^4$ | $9.72*10^4$ | $4.55*10^5$ | 0.2 | 8.80 |
| 100.0 | $2.64*10^3$ | $1.30*10^4$ | $9.66*10^4$ | $4.65*10^5$ | 0.0 | 8.95 |
| 1000.0 | $2.64*10^3$ | $9.18*10^3$ | $3.64*10^4$ | $1.99*10^5$ | 16.4 | 8.81 |
| **Temperature [°C]** | 23.5 | 23.5 | 23.5 | 23.5 | | |

| Test-Substance Concentration (nominal) [mg/l] | Cell concentrations (mean values) [cells/ml] | | | | | | | | pH values after 72 hours |
|---|---|---|---|---|---|---|---|---|---|
| | measured | | | | Percent of control | | | | |
| | 0 h | 25 h | 48 h | 72 h | 0 h | 25 h | 48 h | 72 h | |
| 0 | $2.69*10^3$ | $1.33*10^4$ | $8.13*10^4$ | $3.94*10^5$ | 100 | 100 | 100 | 100 | 9.16 |
| 100 | $2.69*10^3$ | $1.30*10^3$ | $8.95*10^4$ | $4.46*10^5$ | 100 | 98 | 110 | 113 | 9.37 |
| 200 | $2.69*10^3$ | $1.25*10^3$ | $7.22*10^4$ | $3.57*10^5$ | 100 | 94 | 89 | 91 | 9.18 |
| 400 | $2.69*10^3$ | $1.33*10^3$ | $8.16*10^4$ | $4.37*10^5$ | 100 | 100 | 100 | 111 | 9.27 |
| 800 | $2.69*10^3$ | $1.36*10^3$ | $6.40*10^4$ | $3.74*10^5$ | 100 | 102 | 79 | 95 | 9.10 |
| 1000 | $2.69*10^3$ | $1.08*10^3$ | $5.47*10^4$ | $3.29*10^5$ | 100 | 81 | 67 | 84 | 9.02 |
| **Temperature [°C]** | 23.5 | 23.5 | 23.5 | 23.5 | | | | | |

**Table A7_4_1_3-6: Validity criteria for algal growth inhibition test according to OECD Guideline 201 (2006)**

| | fulfilled | Not fulfilled |
|---|---|---|
| Biomass in control cultures increased exponentially by a factor of at least 16 within 72-hour test period | X | |
| The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2 and 2-3, for 72-hour tests) in the control cultures must not exceed 35%. | X | |
| The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7% | X | |
| The pH of the control medium should not increase by more than 1.5 units during the test. | X | |
| Concentration of test substance maintained within ± 20 % of the nominal or measured initial concentration during test | X | |

**Table A7_4_1_3-7: Additional data / Effects on algal growth and growth rate / Benzoic acid**

| Species | Result | Reference |
|---|---|---|
| Scenedesmus quadracauda | $EC_{50}$ (20 °C, 3 h): > 10 mg/L | Stratton GW, Corke CT, 1982 (A7.4.1.3./03) |
| | 50% photosynthesis inhibition (20°C,3h): 75 mg/L | |
| Chlorella pyrenoidosa | $EC_{50}$ (20 °C, 3 h): > 10 mg/L | |
| | 50% photosynthesis inhibition (20°C,3h): 60 mg/L | |

| | | |
|---|---|---|
| Microcystis aeruginosa | 8 d $EC_0$ 55 mg/L | Verschueren K, 2001 (A7.4.1.3/04) |

## Section A7.4.1.4/01 Inhibition to microbial activity (aquatic)

**Annex Point IIA7.4**

Official use only

| | | |
|---|---|---|
| | | **1 REFERENCE** |
| **1.1** | **Reference** | Klečka GM, Landi LP, Bodner KM, 1985, Evaluation of the OECD Activated Sludge, Respiration Inhibition Test<br>Chemosphere 14, 9, 1239-1251 (published) |
| **1.2** | **Data protection** | No |
| 1.2.1 | Data owner | Not applicable |
| 1.2.2 | Companies with letter of access | - |
| 1.2.3 | Criteria for data protection | No data protection / published data |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** |
| **2.1** | **Guideline study** | Yes, OECD 209, (1984) |
| **2.2** | **GLP** | No. GLP was not compulsory at the time the study was performed. |
| **2.3** | **Deviations** | Not applicable |
| | | **3 METHOD** |
| **3.1** | **Test material** | Benzoic acid (reagent grade) as given in section 2 |
| 3.1.1 | Lot/Batch number | No lot/batch number available |
| 3.1.2 | Specification | As given in section 2 |
| 3.1.3 | Purity | >99% |
| 3.1.4 | Composition of Product | - |
| 3.1.5 | Further relevant properties | - |
| 3.1.6 | Method of analysis | No analytical confirmation of the test substance. |
| **3.2** | **Preparation of TS solution for poorly soluble or volatile test substances** | Not applicable |
| **3.3** | **Reference substance** | Yes, 3,5-Dichlorophenol |
| 3.3.1 | Method of analysis for reference substance | No analytical confirmation of the reference substance |
| **3.4** | **Testing procedure** | *Non-entry field* |

## Section A7.4.1.4/01 Inhibition to microbial activity (aquatic)

**Annex Point IIA7.4**

| | | | |
|---|---|---|---|
| 3.4.1 | Culture medium | **Synthetic sewage** | **g** |
| | | Bacto Peptone | 16.0 |
| | | Bacto Beaf extract | 11.0 |
| | | Urea | 0.3 |
| | | $CaCl_2$ x $2H_2O$ | 0.4 |
| | | $MgSO_4$ x $7H_2O$ | 0.2 |
| | | $K_2HPO_4$ | 2.8 |
| | | NaCl | 0.7 |
| | | Filled to 1 L with deionised water | |
| 3.4.2 | Inoculum / test species | See table A7_4_1_4/01-1 | |
| 3.4.3 | Test system | See table A7_4_1_4/01-2 | |
| 3.4.4 | Test conditions | See table A7_4_1_4/01-3 | |
| 3.4.5 | Duration of the test | 3 hours | |
| 3.4.6 | Test parameter | Inhibition of respiration rate | |
| 3.4.7 | Analytical parameter | Oxygen measurement | |
| 3.4.8 | Sampling | Start and end of test | |
| 3.4.9 | Monitoring of TS concentration | No | |
| 3.4.10 | Controls | 2 controls with activated sludge and sewage without test substance<br>3 positive controls with reference substance and activated sludge and sewage | |
| 3.4.11 | Statistics | Inhibition data were analysed using Thompson's method of moving averages to estimate $LC_{50}$ values.<br>Experimental data were also analysed by probit-transformations model. | |
| o | | **4 RESULTS** | |
| **4.1** | **Preliminary test** | Not performed | |
| 4.1.1 | Concentration | - | |
| 4.1.2 | Effect data | - | |
| **4.2** | **Results test substance** | *Non-entry field* | |
| 4.2.1 | Initial concentrations of test substance | up to 1000 mg/L | |
| 4.2.2 | Actual concentrations of test substance | No measurement of test substance | |
| 4.2.3 | Growth curves | - | |

## Section A7.4.1.4/01 Inhibition to microbial activity (aquatic)

**Annex Point IIA7.4**

| | | |
|---|---|---|
| 4.2.4 | Cell concentration data | - |
| 4.2.5 | Concentration/ response curve | No data given |
| 4.2.6 | Effect data | 3-hour $IC_{50}$ >1000 mg/L |
| 4.2.7 | Other observed effects | No other effects observed |
| **4.3** | **Results of controls** | |
| **4.4** | **Test with reference substance** | Performed |
| 4.4.1 | Concentrations | No data given, the variability inherent in the respiration rate was determined with 15 mg 3,5-Dichlorophenol per litre.<br>At least 3 concentrations of the reference substance |
| 4.4.2 | Results | 3-hour $IC_{50}$ 12.2 mg/L |

**5 APPLICANT'S SUMMARY AND CONCLUSION**

| | | |
|---|---|---|
| **5.1** | **Materials and methods** | The toxicity of 43 chemicals to activated sludge was tested in a respiration inhibition test following OECD 209 (1984) |
| **5.2** | **Results and discussion** | Benzoic acid shows no inhibitory effects.<br>43 chemicals were examined, 23 showed no inhibitory effect. |
| 5.2.1 | $EC_{20}$ | Not determined |
| 5.2.2 | $IC_{50}$ | >1000 mg/L |
| 5.2.3 | $EC_{80}$ | Not determined |
| **5.3** | **Conclusion** | The validity criteria can be considered as fulfilled.<br>The $IC_{50}$ of 3,5-Dichlorophenol was found to be 12.2 mg/L and was within the range of 5-30 mg/L as recommended by the guidelines. |
| 5.3.1 | Reliability | 1 |
| 5.3.2 | Deficiencies | Not applicable |

| **Evaluation by Competent Authorities** | |
|---|---|
| | Use separate "evaluation boxes" to provide transparency as to the comments and views submitted |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | 2008/05/18 |
| **Materials and Methods** | Applicants version is acceptable with the following comment:<br>3.1.6 No analytical confirmation of the test substance. |
| **Results and discussion** | Applicant's version is adopted with the following amendment:<br>The $EC_{50}$ (3 hours) was calculated to be >1000 mg/L. |
| **Conclusion** | The applicant's version is adopted. |

## Section A7.4.1.4/01 Inhibition to microbial activity (aquatic)

**Annex Point IIA7.4**

| | |
|---|---|
| **Reliability** | 2 |
| **Acceptability** | acceptable |
| **Remarks** | Only minor deviations in test performance from OECD 209 guideline occurred. |
| | **COMMENTS FROM ...** *(specify)* |
| **Date** | *Give date of comments submitted* |
| **Materials and Methods** | *Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.*<br>*Discuss if deviating from view of rapporteur member state* |
| **Results and discussion** | *Discuss if deviating from view of rapporteur member state* |
| **Conclusion** | *Discuss if deviating from view of rapporteur member state* |
| **Reliability** | *Discuss if deviating from view of rapporteur member state* |
| **Acceptability** | *Discuss if deviating from view of rapporteur member state* |
| **Remarks** | |

**Table A7_4_1_4-1: Inoculum / Test organism**

| Criteria | Details |
|---|---|
| Nature | Activated sludge |
| Species | - |
| Strain | - |
| Source | Activated sludge from a local municipal sewage plant |
| Sampling site | No data given |
| Laboratory culture | No |
| Method of cultivation | - |
| Preparation of inoculum for exposure | The activated sludge was washed three times with deionised water. The system was aerated with compressed air (approximately 0.5 – 1 litre per minute) |
| Pretreatment | The washed sludge was adjusted to contain 4000 ± 400 mg of mixed liquor suspended solids (dry weight) per litre. |
| Initial cell concentration | The activated sludge was diluted to 3.5 g/L with tap water. |

**Table A7_4_1_4-2: Test system**

| Criteria | Details |
|---|---|
| Culturing apparatus | 500 mL graduated cylinder / 1 l bottle |
| Number of culture flasks/concentration | A series of reaction mixtures for each chemical, in addition at least 3 concentrations of the reference substance. |

| | |
|---|---|
| Aeration device | Compressed air (approximately 0.5 – 1 litre per minute) |
| Measuring equipment | The oxygen concentration was measured with an oxygen electrode (Orion model 97-08) and an Orion model 701 Ion analyzer. |
| Test performed in closed vessels due to significant volatility of TS | No |

**Table A7_4_1_4: Test conditions**

| **Criteria** | **Details** |
|---|---|
| Test temperature | Ambient temperature (21°C) |
| pH | pH 7.5 ± 0.5 |
| Aeration of dilution water | Yes, compressed air (approximately 0.5 – 1 litre per minute) |
| Suspended solids concentration | 4 ± 0.4 g/L |

## Section A7.4.1.4/02 Inhibition to microbial activity (aquatic)

**Annex Point IIA7.4**

Official use only

| | | |
|---|---|---|
| | | **1 REFERENCE** |
| **1.1** | **Reference** | Bringmann G, Kühn R, 1980, Comparison of the Toxicity Thresholds of Water Pollutants to Bacteria, Algae, and Protozoa in the Cell Multiplication Inhibition Test, Institute for Water, Soil and Air Hygiene, Federal Health Office, Berlin, Germany, Water Research, 14, 231-241 (published) |
| **1.2** | **Data protection** | No |
| 1.2.1 | Data owner | Not applicable |
| 1.2.2 | Companies with letter of access | - |
| 1.2.3 | Criteria for data protection | No data protection / published data |
| | | **2 GUIDELINES AND QUALITY ASSURANCE** |
| **2.1** | **Guideline study** | No, Cell Multiplication Inhibition Test |
| **2.2** | **GLP** | No. GLP was not compulsory at the time the study was performed. |
| **2.3** | **Deviations** | Not applicable |
| | | **3 METHOD** |
| **3.1** | **Test material** | Benzoic acid as given in section 2 |
| 3.1.1 | Lot/Batch number | No lot/batch number available |
| 3.1.2 | Specification | As given in section 2 |
| 3.1.3 | Purity | >99% |
| 3.1.4 | Composition of Product | - |
| 3.1.5 | Further relevant properties | - |
| 3.1.6 | Method of analysis | No analytical confirmation of the test substance. |
| **3.2** | **Preparation of TS solution for poorly soluble or volatile test substances** | Not applicable |
| **3.3** | **Reference substance** | 156 chemicals were tested; a lot of them were toxic to *Pseudomonas putida* |
| 3.3.1 | Method of analysis for reference substance | No analytical confirmation of the reference substance |
| **3.4** | **Testing** | *Non-entry field* |

## Section A7.4.1.4/02 Inhibition to microbial activity (aquatic)

**Annex Point IIA7.4**

**procedure**

3.4.1 Culture medium Sterile double distilled water and analytical grade chemicals were used for the preparation of the nutrient medium.

| | g/L |
|---|---|
| $NaNO_3$ | 1.060 |
| $K_2HPO_4$, | 0.600 |
| $KH_2HPO_4$ | 0.300 |
| $MgSO_4$ x 7 $H_2O$ | 0.200 |
| D(+) glucose | 10.000 |
| Difco Bacto agar | 18.000 |
| $FeSO_4$ x 7 $H_2O$ | 0.010 |
| Trace element solution | 1.5 ml |

Trace element solution

| | g/L |
|---|---|
| $Al_2(SO_4)_3$ x 18 $H_2O$ | 0.055 |
| KJ | 0.028 |
| KBr | 0.028 |
| $TiO_2$ | 0.055 |
| $SnCl_2$ x 2 $H_2O$ | 0.028 |
| LiCl | 0.028 |
| $MnCl_2$ x 4 $H_2O$ | 0.389 |
| $H_3BO_3$ | 0.614 |
| $ZnSO_4$ x 7 $H_2O$ | 0.055 |
| $CuSO_4$ x 5 $H_2O$ | 0.055 |
| $NiSO_4$ x 6 $H_2O$ | 0.059 |
| $Co(NO_3)_2$ x 6 $H_2O$ | 0.055 |

Vitamin solution

| | |
|---|---|
| Biotin | 0.2 mg |
| Nicotinic acid | 2.0 mg |
| Thiamine | 1.0 mg |
| p-aminobenzoic acid | 1.0 mg |
| Panthothenic acid | 0.5 mg |
| Pyridoxamine | 5 mg |
| Cyanocobalamin | 2.0 mg |
| Double distilled water | 100 mL |

## Section A7.4.1.4/02 Inhibition to microbial activity (aquatic)

**Annex Point IIA7.4**

| | | |
|---|---|---|
| 3.4.2 | Inoculum / test species | See table A7_4_1_4-1 |
| 3.4.3 | Test system | See table A7_4_1_4-2 |
| 3.4.4 | Test conditions | See table A7_4_1_4-3 |
| 3.4.5 | Duration of the test | 16 hours |
| 3.4.6 | Test parameter | Inhibition of cell multiplication |
| 3.4.7 | Analytical parameter | Determining of the extinction of monochromatic radiation at 436 nm for a 10mm thin layer of the bacterial suspension by photoelectric measurement. Final turbidity value was adjusted by means of sterile saline that the extinction value for a measuring sample that has been subject to onward dilution 1 + 9 with saline will correspond to the extinction value of a Formazin standard suspension TE/F/436 nm = 10.<br>For evaluation of the toxicological findings at the end of the test period, the mean value (A) of the extinction is calculated for all test cultures that are free from toxic influence. |
| 3.4.8 | Sampling | End of test |
| 3.4.9 | Monitoring of TS concentration | No |
| 3.4.10 | Controls | Test culture free from toxic influence and with a defined standardized offer of nutrients |
| 3.4.11 | Statistics | For evaluation of the toxicological findings at the end of the test period, the mean value (A) of the extinction is calculated for all test cultures that are free from toxic influence.<br>For mathematical evaluation by means of a suitable electronic calculator (a) (highest non-toxic pollutant concentration) is plotted against (A) and (b) (lowest toxic pollutant concentration) against (B) as coordinates. After entering (A - 3%), the pollutant concentration at which the inhibitory action (c) begins, may be obtained from the regression line between (a;A) and (b;B) if a negative deviation of the mean extinction by a 3% difference against the mean extinction value for all test cultures having a non-toxic and non-stimulating pollutant concentration is used as an indicator of the beginning of inhibitory action. |
| o | | **4 RESULTS** |
| **4.1** | **Preliminary test** | Not performed |
| 4.1.1 | Concentration | - |
| 4.1.2 | Effect data | - |
| **4.2** | **Results test substance** | *Non-entry field* |
| 4.2.1 | Initial concentrations of test substance | Benzoic acid solution was neutralised of a known content in sterile double-distilled water to be tested, the concentration of the acid solution was selected in such way that the volume added is kept as small as possible. pH was not adjusted were the effect of the pH of the benzoic acid solution is to be included in the test.<br>Four parallel dilution series were prepared. Each of the solutions contains 1 part (v/v) of the benzoic acid solution in $2^0$ of $2^{14}$ parts (v/v) of mixture. |

## Section A7.4.1.4/02 Inhibition to microbial activity (aquatic)

**Annex Point IIA7.4**

| | | |
|---|---|---|
| | | The first flask of the series contains 160 ml of benzoic acid solution at the start. Dilution steps at a dilution ratio by consistently mixing 80 mL double distilled water. Consequently each flask contains 80 ml of culture liquid at start. |
| 4.2.2 | Actual concentrations of test substance | No measurement |
| 4.2.3 | Growth curves | - |
| 4.2.4 | Cell concentration data | No data given |
| 4.2.5 | Concentration/ response curve | No data given |
| 4.2.6 | Effect data | Toxicity threshold of benzoic acid: 480 mg/L |
| 4.2.7 | Other observed effects | No other effects observed |
| **4.3** | **Results of controls** | |
| **4.4** | **Test with reference substance** | Performed |
| 4.4.1 | Concentrations | |
| 4.4.2 | Results | |

### 5 APPLICANT'S SUMMARY AND CONCLUSION

| | | |
|---|---|---|
| **5.1** | **Materials and methods** | The multiplication of bacterial cells of the genus *Pseudomonas* is inhibited by dissolved benzoic acid. After a certain period the increase in the number of cells in a test culture free from toxic influence and with a defined standardized offer of nutrients will exceed that observed in a test culture containing dissolved toxic substances and kept under identical conditions.<br>The concentration of the bacterial suspension was measured turbidimetrically (while diffused light is screened off); it is expressed by the extinction of the primary light of the monochromatic radiation at 436 nm for a layer of 10 mm thickness. The concentration at which the inhibitory action of benzoic acid starts will be present in that step of a dilution series of the pollutant having an extinction value at the end of the test period that is >3% below the mean value of extinction for non-toxic substances. |
| **5.2** | **Results and discussion** | Toxicity threshold of benzoic acid: 480 mg/L |
| 5.2.1 | $EC_{20}$ | Not determined |
| 5.2.2 | $EC_{50}$ | Not determined |
| 5.2.3 | $EC_{80}$ | Not determined |
| **5.3** | **Conclusion** | Benzoic acid is not toxic to *Pseudomonas putida* up to 480 mg/L |
| 5.3.1 | Reliability | 2 |
| 5.3.2 | Deficiencies | Not applicable |

## Section A7.4.1.4/02 Inhibition to microbial activity (aquatic)

**Annex Point IIA7.4**

| Evaluation by Competent Authorities | |
|---|---|
| | Use separate "evaluation boxes" to provide transparency as to the comments and views submitted |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE** |
| **Date** | 2008/05/18 |
| **Materials and Methods** | Applicants version is acceptable with the following amendments:<br>The study provided by the applicant was conducted similar to the approved standard methods according to ISO 10712/DIN 38412 (L8) with some minor deviations:<br><br>**3.3** Several substances with a known toxicity to *Pseudomonas putida* were tested, e.g. 2,4-dichlorophenole, but not the prescribed 3,5-dichlorophenole.<br>**3.4.4** The test was performed at a temperature of 25°C, whereas the ISO/DIN standards require a constant temperature of 23 ± 1°C.<br><br>Criterion validity according to DIN 38412 (L8) could not be verified. The initial turbidity in the controls without test substance measured as TE/F ("Trübungseinheiten Formazan") or FNU ("Formazine Nephelometric Units") have to increase at least 100-fold. This was not checked |
| **Results and discussion** | Applicant's version is adopted with the comment below:<br>A $EC_{50}$ of 480 mg a.s./L was derived. |
| **Conclusion** | Applicant's version accepted. |
| **Reliability** | 2 |
| **Acceptability** | acceptable<br>In spite of deviations in test performance from ISO/DIN standards and a missing documentation. |
| **Remarks** | Although a verification of the study´s validity was not provided, the applicant's version is accepted, because the applied *P. putida* inoculum was delivered by a reliable source. Thus, its growth is assumed to be within the required range. Furthermore, in a second study, Doc. III A7.4.1.4/01, the $EC_{50}$ was determined to lie in a comparable order of magnitude. |
| | **COMMENTS FROM ...** *(specify)* |
| **Date** | *Give date of comments submitted* |
| **Materials and Methods** | *Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.*<br>*Discuss if deviating from view of rapporteur member state* |
| **Results and discussion** | *Discuss if deviating from view of rapporteur member state* |
| **Conclusion** | *Discuss if deviating from view of rapporteur member state* |
| **Reliability** | *Discuss if deviating from view of rapporteur member state* |
| **Acceptability** | *Discuss if deviating from view of rapporteur member state* |
| **Remarks** | |

**Table A7_4_1_4/02-1: Test organism**

| Criteria | Details |
|---|---|
| Nature | Bacteria |
| Species | *Pseudomonas putida* |
| Strain | - |
| Source | - |
| Sampling site | - |
| Laboratory culture | Yes |
| Method of cultivation | *Pseudomonas putida* were kept on the nutrient for stock and preliminary cultures in agar slant tubes. New stock cultures were prepared at intervals of each week. |
| Preparation of inoculum for exposure | Consequently each flask contains 80 ml of culture liquid at start. Inoculation: adding 5 ml each of stock solution I, 5 ml each of stock solution II and 10 mL of the bacterial suspension from the preliminary culture. |

Stock solution I

| | |
|---|---|
| $D_{(+)}$ glucose | 20.000 g |
| $NaNO_3$ | 4.240 |
| $K_2HPO_4$, | 2.400 |
| $KH_2HPO_4$ | 1.200 |
| Trace element solution | 30 ml |

Stock solution I

| | |
|---|---|
| $FeSO_4 \times 7\ H_2O$ | 0.200 |
| $MgSO_4 \times 7\ H_2O$ | 4.000 |

in 1000 mL double distilled water

Saline
0.5 g NaCl in 1000 mL double distilled water

Trace element solution

| | g/L |
|---|---|
| $Al_2(SO_4)_3 \times 18\ H_2O$ | 0.055 |
| KJ | 0.028 |
| KBr | 0.028 |
| $TiO_2$ | 0.055 |
| $SnCl_2 \times 2\ H_2O$ | 0.028 |
| LiCl | 0.028 |
| $MnCl_2 \times 4\ H_2O$ | 0.389 |
| $H_3BO_3$ | 0.614 |
| $ZnSO_4 \times 7\ H_2O$ | 0.055 |
| $CuSO_4 \times 5\ H_2O$ | 0.055 |
| $NiSO_4 \times 6\ H_2O$ | 0.059 |
| $Co(NO_3)_2 \times 6\ H_2O$ | 0.055 |

| | |
|---|---|
| Pretreatment | Incubation at 25°C for 24 hours, washing of with sterile |