



Physiological Sciences  
Location Zeist  
Utrechtseweg 48  
P.O. Box 360  
3700 AJ Zeist  
The Netherlands

**TNO Report**

V5819/04 | final |

**Screening of the effect of p-xylene (CAS# 106-42-3) on the respiration of activated sludge (OECD Guideline No. 209)**

[www.tno.nl](http://www.tno.nl)

P +31 30 694 42 86

F +31 30 694 23 61

[cheminfo@voeding.tno.nl](mailto:cheminfo@voeding.tno.nl)

Date	17 December, 2004
Authors	H. Oldersma C. Hamwijk, M.Sc
Sponsor	CEFIC Aromatics Producers Association Avenue E. Van Nieuwenhuysse 4, bte 2 B – 1160 BRUSSELS Belgium
Study monitor	Mr. Christian Gilliard
TNO project number	010. 30512/01.03
TNO study code	5819/04
Sponsor's study code	
Status	final
Previous versions	
Number of pages	21

All rights reserved.

No part of this publication may be reproduced and/or published by print, photoprint, microfilm or any other means without the previous written consent of TNO.

In case this report was drafted on instructions, the rights and obligations of contracting parties are subject to either the Standard Conditions for Research Instructions given to TNO, or the relevant agreement concluded between the contracting parties. Submitting the report for inspection to parties who have a direct interest is permitted.

© 2004TNO

## Summary

The effect of p-xylene (CAS# 106-42-3) on the respiration rate of activated sludge from an oxidation ditch treating domestic sewage only, was determined in a respiration inhibition test according to the OECD Guideline No. 209: 'Activated Sludge. Respiration Inhibition Test' and the OECD principles of Good Laboratory Practice.

P-xylene is a colourless transparent liquid with a stated solubility of 156 mg.l<sup>-1</sup> in water. As the test substance is volatile and aeration was necessary during the test, loss of the test substance during the incubation period was expected. However, this will also be the case in an activated sludge plant. This test is considered to simulate the practice situation. On request of the sponsor, the test was conducted with the limit concentration (157 mg.l<sup>-1</sup> nominal) only, in duplicate.

Mixtures of activated sludge (5.5 g (d.w.).l<sup>-1</sup>), synthetic sewage feed and test substance were aerated for 3h at 20 ± 2 °C. The decrease in the oxygen concentration in the mixtures was measured during approximately 10 minutes, and the respiration rate for each test substance concentration was calculated.

The concentrations given in this report are nominal concentrations of the test substance p-xylene. A control test with the reference substance 3,5-dichlorophenol yielded an EC50 value of 5.9 mg.l<sup>-1</sup>, between 5 and 30 mg.l<sup>-1</sup>, which is in the range prescribed by the Guideline. The validity criteria of the Guideline were fulfilled.

It was concluded that no inhibition of the respiration rate of activated sludge took place at the limit concentration of p-xylene. Therefore, the NOEC was assessed to be the limit concentration 157 mg.l<sup>-1</sup>, nominally.

# Contents

<b>Summary .....</b>	<b>2</b>
<b>Contents .....</b>	<b>3</b>
<b>Confidentiality statement .....</b>	<b>5</b>
<b>Statement of GLP Compliance .....</b>	<b>6</b>
<b>Quality Assurance Statement.....</b>	<b>7</b>
<b>1 Introduction.....</b>	<b>8</b>
1.1 Guidelines .....	8
1.2 Objective.....	8
1.3 Justification for the method .....	8
1.4 Principle of the method.....	8
1.5 Quality Assurance.....	8
1.6 Relevant dates .....	8
1.7 Testing facility .....	9
1.8 Contributing personnel .....	9
<b>2 Materials and methods .....</b>	<b>10</b>
2.1 Test substance .....	10
2.2 Reference substance.....	10
2.3 Characterization of the activated sludge .....	11
2.4 Dilution water and synthetic sewage feed.....	11
<b>3 Test Method.....</b>	<b>12</b>
3.1 Introduction.....	12
3.2 Preparation of the reference substance solution.....	12
3.3 Preparation of test and control mixtures .....	12
3.4 Test with the reference substance .....	12
3.5 Incubation .....	12
3.6 Oxygen measurements .....	12
3.7 Calculation of the results .....	13
<b>4 Results and discussion .....</b>	<b>14</b>
4.1 Respiration inhibition test with p-xylene .....	14
4.2 Test with the reference substance .....	14
4.3 Validity criteria.....	14
<b>5 Conclusion .....</b>	<b>15</b>
<b>6 References.....</b>	<b>16</b>
<b>7 Retention of records and samples.....</b>	<b>17</b>
<b>8 Deviations from the study plan.....</b>	<b>18</b>

<b>Annex A Dilution water and synthetic sewage feed.....</b>	<b>19</b>
<b>Annex B Results of the respiration inhibition test.....</b>	<b>20</b>
<b>Annex C Endorsement of GLP compliance.....</b>	<b>21</b>

## Confidentiality statement

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

## Statement of GLP Compliance

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

TNO makes no GLP compliance claim for characterisation and verification of the test substance identity and properties; this is the responsibility of the sponsor.

C. Hamwijk, M.Sc  
*Study Director*  
Physiological Sciences

---

Approved by:

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

## Quality Assurance Statement

Report title : <report title>

Report number : <report number>

Report date : <date in letters>

The Study plan was inspected as follows:

Date of inspection  
<date in letters>

Date of report  
<date in letters>

The experimental phase of the study was inspected as follows:

Date of inspection  
<date in letters>

Date of report  
<date in letters>

This report was audited as follows:

Date of audit  
<date in letters>

Date of report  
<date in letters>

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

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

Date:

# 1 Introduction

## 1.1 Guidelines

The activated sludge respiration inhibition test was performed as a limit test with a nominal test substance concentration of 157 mg.l<sup>-1</sup>, essentially according to the OECD Test Guideline No. 209 [2]. The OECD Guideline refers to a concentration range.

## 1.2 Objective

The objective of the study was to determine the effect of p-xylene on the respiration rate of a sample of activated sludge during an incubation period of three hours.

## 1.3 Justification for the method

The purpose of this test was to provide a rapid screening to identify substances that may have an effect on aerobic microbial wastewater treatment plants.

## 1.4 Principle of the method

A mixture of activated sludge, synthetic sewage feed and the highest water soluble concentration of the test substance (limit concentration) or three concentrations of the reference substance were prepared in one litre glass beakers. The mixtures were aerated and after an incubation period of 3 hours at approximately 20 °C a sample of each mixture was placed in a measuring (BOD) flask (volume about 300 ml) and the decrease in the oxygen concentration in each mixture was recorded with an oxygen electrode during a period of approximately 10 minutes.

The respiration rate was calculated from the measurements, and the EC-values were calculated from the concentration-effect curve by an appropriate model [4].

## 1.5 Quality Assurance

The study was carried out in accordance with the OECD principles of Good Laboratory Practice (GLP) [3].

## 1.6 Relevant dates

Study plan signed by Study Director:	17 August 2004
Date of test:	15 September 2004
Date of reference test:	15 September 2004

## 1.7 **Testing facility**

The study was carried out by the Physiological Sciences Department of TNO Nutrition and Food Research. The laboratories of this department are located at:

### **Street address**

Utrechtseweg 48  
3704 HE ZEIST  
The Netherlands

### **Postal address**

P.O. Box 360  
3700 AJ ZEIST  
The Netherlands

## 1.8 **Contributing personnel**

C. Hamwijk, M.Sc  
*Study director*

Drs. T.H.N. Ly  
H. Oldersma  
*Technicians*

Physiological Sciences

## 2 Materials and methods

### 2.1 Test substance

The following test substance was examined:

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

#### *Receipt of test substance*

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

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

### 2.2 Reference substance

The following reference substance was used:

Name	:	3,5-dichlorophenol
Source	:	Aldrich
Physical appearance	:	white powder
Batch No	:	15809KI-392
Purity	:	97%
Stability	:	stable under normal laboratory conditions
Solubility in water	:	soluble
Storage temperature	:	room temperature
Protection from light	:	yes
Expiration date	:	12 May 2008
TNO test substance number	:	030073-001

---

<sup>1</sup> As set by TNO and agreed with the sponsor

### **2.3 Characterization of the activated sludge**

Approximately 15 litres of activated sludge were taken from an oxidation ditch situated at the district of Hazerswoude Dorp, the Netherlands on 13 September 2004. The oxidation ditch is used to treat domestic sewage.

A volume of 6 litres sludge was centrifuged and the supernatant was discarded. The activated sludge was washed three times by centrifugation (10 min at 2000 rpm = max. 1218 g) and re-suspended twice in tap water and twice in dilution water (section 2.4).

The dry weight of the sludge suspension was determined by drying using an infrared drier. The mixed liquor suspended solids (MLSS) content was found to be  $3.8 \text{ g.l}^{-1}$ . The suspension was aerated vigorously, and kept at approximately  $20 \text{ }^{\circ}\text{C}$  in the dark. The pH of the sludge suspension was measured to be 7.2.

Fifty ml of synthetic sewage feed (section 2.4) was added per litre of sludge suspension. The dry weight was checked in the test and resulted in  $5.4$  and  $5.6 \text{ g.l}^{-1}$  mixed liquor suspended solids. Before use the pH was measured to be 8.3.

### **2.4 Dilution water and synthetic sewage feed**

The (BOD) dilution water (Annex A, Table A1) was prepared according to NEN-EN 1899 - 1 [2].

The synthetic sewage feed (Annex A, Table A2) was prepared according to OECD Guideline No. 209 [1].

## 3 Test Method

### 3.1 Introduction

The activated sludge respiration inhibition test was performed as a limit test with a test substance concentration of  $157 \text{ mg.l}^{-1}$ , essentially according to the OECD Guideline No. 209 [1].

### 3.2 Preparation of the reference substance solution

A stock solution of the reference substance was prepared according to OECD Guideline No. 209 [1] by dissolving 0.1253 g of 3,5-dichlorophenol in 5 ml 2M NaOH solution, adjusting its pH to 7.2 with 1M HCl solution, and then filling up to 250 ml with ultrapure water.

### 3.3 Preparation of test and control mixtures

Control mixtures of 16 ml synthetic sewage feed and 284 ml dilution water were prepared in one litre glass beakers. Test mixtures were prepared by combining 16 ml synthetic sewage feed with the  $91 \mu\text{l}$  of the test substance and 284 ml dilution water to a volume of 300 ml in one litre glass beakers.

The nominal (limit) concentration of p-xylene in the final suspensions was  $157 \text{ mg.l}^{-1}$ .

### 3.4 Test with the reference substance

Three concentrations (5, 12 and  $30 \text{ mg.l}^{-1}$ ) of the reference substance 3,5-dichlorophenol were tested.

Five, twelve, and thirty ml of the stock solution of 3,5-dichlorophenol were diluted with dilution water to 284 ml, mixed with 16 ml of synthetic sewage feed and 200 ml sludge suspension, and further treated as described in the following sections.

### 3.5 Incubation

The incubation period was started at intervals of 15 minutes when 200 ml of sludge suspension was added to the 300 ml of test or control mixture. The first and last mixture of the various samples that were incubated, were controls without the test substance. All suspensions were aerated vigorously and incubated in a climate test chamber at approximately  $20 \text{ }^{\circ}\text{C}$  for exactly three hours.

### 3.6 Oxygen measurements

After three hours of incubation, a sample of each mixture was poured into a measuring (BOD) flask (volume  $>200 \text{ ml}$ ) and stirred vigorously on a magnetic stirrer. An oxygen electrode (WTW Oxi2000) was inserted into the sample and the oxygen concentration in the sample was continuously measured. The oxygen concentration was recorded every minute during a period of about 10 minutes.

### 3.7 Calculation of the results

The respiration rate (in mg O<sub>2</sub> per g dry weight active sludge per hour) was calculated by linear regression of the oxygen concentration meter readings in the linear part of the oxygen depletion curve, which is essentially in accordance with the OECD Guideline No. 209. The percentage inhibition was calculated according:

$$\text{percent inhibition} = \left( 1 - \frac{2R_s}{R_{c1} + R_{c2}} \right) \times 100 \quad (1)$$

where

- R<sub>s</sub> = respiration rate (mg O<sub>2</sub>.g<sup>-1</sup>.h<sup>-1</sup>) at tested concentration of test substance
- R<sub>c1</sub> = respiration rate (mg O<sub>2</sub>.g<sup>-1</sup>.h<sup>-1</sup>) of control 1
- R<sub>c2</sub> = respiration rate (mg O<sub>2</sub>.g<sup>-1</sup>.h<sup>-1</sup>) of control 2

The effect of a test substance on the respiration rate of activated sludge can be expressed as an EC50 value; i.e. the concentration of the test substance that reduces the respiration rate by 50%.

The EC50 value of the reference substance was calculated by estimating the parameter 'b' of the function

$$R(\text{conc}) = \frac{R(\text{control})}{1 + (\text{conc} / \text{EC } 50)^b} \quad (2)$$

in which b is a slope parameter of the dose effect function and 'conc' is the nominal test substance concentration. The estimates were maximum likelihood estimates given that the measurement error is normally distributed with a constant variance according to the method described in ref. [4].

## 4 Results and discussion

### 4.1 Respiration inhibition test with p-xylene

The dry weight determination of the sludge suspension used in the final test resulted in an average mixed liquor suspended solids content of 5.5 g.l<sup>-1</sup>.

The oxygen measurements in the mixtures containing the limit concentration of the p-xylene are shown in Annex B, Table B1. The calculated respiration rates, their correlation coefficients and the percentages inhibition of the respiration rate are shown in Table 1 and Annex B, Table B2.

**Table 1** Results of the activated sludge respiration inhibition test with p-xylene and the reference substance.

Test sample	Nominal concentration of test substance (mg.l <sup>-1</sup> )	pH	Respiration rate (mg O <sub>2</sub> .g <sup>-1</sup> .h <sup>-1</sup> )	% inhibition
Initial control	0	8.0	9.6	-
Final control	0	8.1	10.4	-
p-xylene	157	8.1	10.4	-3.9
	157	8.1	10.3	-2.7
3,5-dichlorophenol	5	8.1	5.1	49.5
	12	8.1	4.0	60.6
	30	8.1	1.6	83.7

### 4.2 Test with the reference substance

The oxygen measurements in the mixtures containing the three concentrations of the reference substance are shown in Annex B, Table B1. The calculated mean respiration rates, and the percentages inhibition of the respiration rate are shown in Table 1 and Annex B, Table B2.

The EC50 of 3,5-dichlorophenol was calculated to be 5.9 mg.l<sup>-1</sup>.<sup>[o1][o2][o3]</sup>

### 4.3 Validity criteria

The respiration rates of the two controls of the test were found to be 9.6 and 10.4 mg O<sub>2</sub>.g<sup>-1</sup>.h<sup>-1</sup>, respectively. The difference between the two values amounted to 7.8 % being within the validity criterion (15%) of the OECD Guideline.

The EC50 of 3,5 -dichlorophenol was >5 mg.l<sup>-1</sup> and <30 mg.l<sup>-1</sup> (section 4.2), which is also within the accepted range of the Guideline.

## 5 Conclusion

No inhibition of the respiration rate of activated sludge was found at the limit concentration of p-xylene. The NOEC was, therefore, assessed to be the limit concentration 157 mg.l<sup>-1</sup>, nominally.

## 6 References

- [1] OECD 209  
*Activated Sludge. Respiration Inhibition Test.*  
OECD Guideline for testing of chemicals, Paris (1984).
  
- [2] NEN-EN 1899-1:1998  
*Water - Bepaling van het biochemisch zuurstofverbruik na n dagen (BOD<sub>n</sub>) - Deel 1: Verdunnings- en entmethode met toevoeging van allylthioureum.*  
Nederlands Normalisatie-Instituut, Delft (1998).
  
- [3] *OECD Principles of Good Laboratory Practice (as revised in 1997)*  
Organisation for Economic Co-operation and Development (OECD), Paris,  
ENV/MC/CHEM(98)17.
  
- [4] Kooijman, S.A.L.M.  
*Parametric analyses of mortality rates in bioassays*  
Water Res. 15, 107–109 (1981).

## 7 Retention of records and samples

Remaining test substance will be retained for at least six months after submission of the final report; thereafter, the test substance will be discarded.

All the data generated and all other records and information relevant to the quality and integrity of the study have been filed under the study reference 5819/04 in the archives of the TNO Nutrition and Food Research, Utrechtseweg 48, 3704 HE Zeist, The Netherlands. These records will be retained for a period of at least 15 years after the cover date of this report

## 8 Deviations from the study plan

The incubation of the test mixtures were initiated at a time interval of 15 minutes (instead of 10). Fifteen minutes was a more realistic interval.

At the date of the test the pH of the activated sludge was 8.3. No adjustment took place, because it would interrupt the schedule.

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

## Annex A Dilution water and synthetic sewage feed

**Table A1** *Dilution water.*

Prescription according to NEN-EN 1899 - 1 [2]:

<u>Stock A2</u>	KH <sub>2</sub> PO <sub>4</sub>	2.125	g
	K <sub>2</sub> HPO <sub>4</sub>	5.44	g
	Na <sub>2</sub> HPO <sub>4</sub> ·7H <sub>2</sub> O	8.35	g
	NH <sub>4</sub> Cl	0.42	g
	ultrapure water (pH 7.2)	250	ml
<u>Stock B</u>	MgSO <sub>4</sub> ·7H <sub>2</sub> O	2.25	g
	ultrapure water	100	ml
<u>Stock C</u>	CaCl <sub>2</sub> ·2H <sub>2</sub> O	3.63	g
	ultrapure water	100	ml
<u>Stock D</u>	FeCl <sub>3</sub> ·6H <sub>2</sub> O	0.025	g
	ultrapure water	100	ml

Dilution water preparation by adding 1 ml of stocks 1 to 4 to each litre of ultrapure water and store at 20°C in the dark with aeration.

**Table A2** *Synthetic sewage feed.*

The following amounts of substances to be dissolved in two litres of ultrapure water, according to OECD Guideline No. 209.

peptone	32	g
meat extract	22	g
urea	6	g
NaCl	1.4	g
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.8	g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.4	g
K <sub>2</sub> HPO <sub>4</sub>	5.6	g

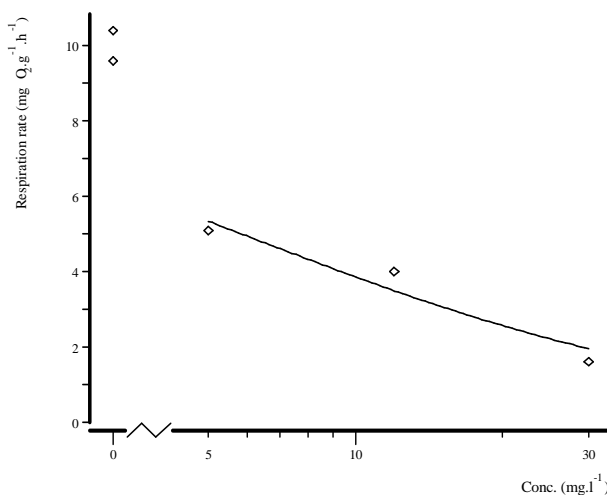
## Annex B Results of the respiration inhibition test

**Table B1** Oxygen measurements ( $\text{mg O}_2 \cdot \text{l}^{-1}$ ) in the respiration inhibition testing. Data were collected at a time interval of 1 minute.

Concentration (mg/L)	mg $\text{O}_2 \cdot \text{l}^{-1}$ at given time interval (minutes)										
	0	1	2	3	4	5	6	7	8	9	10
0	8.00	7.42	7.06	6.73	6.39	6.04	5.67	5.31	4.95	4.59	4.24
ref.subst. 5	8.14	7.81	7.57	7.40	7.21	6.96	6.81	6.65	6.49	6.33	6.14
ref.subst. 12	7.95	7.75	7.53	7.40	7.17	7.10	7.07	7.66	6.77	6.59	6.86
ref.subst. 30	8.27	8.72	8.76	8.71	8.62	8.57	8.53	8.47	8.40	8.35	8.28
test subst.157	7.62	7.32	6.82	6.44	6.08	5.71	5.34	4.94	4.57	4.19	3.80
test subst.157	8.00	7.54	7.17	6.80	6.44	6.07	5.69	5.31	4.92	4.54	4.14
0	7.96	7.45	7.06	6.69	6.32	5.93	5.55	5.17	4.79	4.40	4.01

**Table B2** Oxygen depletion ( $\text{mg O}_2 \cdot \text{l}^{-1}$ ) in the respiration inhibition testing. Valid data are those during the linear part of  $\text{O}_2$ -depletion.

Concentration (mg/L)	respiration $\text{mg O}_2 \cdot \text{l}^{-1}$ at given time interval (minutes)										slope	rate $\text{mg O}_2/\text{g/h}$	inhibition
	0-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10			
0	0.58	0.36	0.33	0.34	0.35	0.37	0.36	0.36	0.36	0.35	-0.353	9.6	3.9%
ref.subst. 5	0.33	0.24	0.17	0.19	0.25	0.15	0.16	0.16	0.16	0.19	-0.186	5.1	49.5%
ref.subst. 12	0.2	0.22	0.13	0.23	0.07	0.03	-0.59	0.1	0.18	-0.27	-0.145	4.0	60.6%
ref.subst. 30	0.45	0.04	0.05	0.09	0.05	0.04	0.06	0.07	0.05	0.07	-0.060	1.6	83.7%
test subst.157	0.3	0.5	0.38	0.36	0.37	0.37	0.4	0.37	0.38	0.39	-0.382	10.4	-3.9%
test subst.157	0.46	0.37	0.37	0.36	0.37	0.38	0.38	0.39	0.38	0.4	-0.378	10.3	-2.7%
0	0.54	0.39	0.37	0.37	0.39	0.38	0.38	0.38	0.39	0.39	-0.382	10.4	-3.9%



**Figure B1** Concentration-effect curve of the effect of 3,5-dichlorophenol on activated sludge respiration rate.

## Annex C Endorsement of GLP compliance



voedsel en waren autoriteit

### ENDORSEMENT OF COMPLIANCE

WITH THE OECD PRINCIPLES OF  
GOOD LABORATORY PRACTICE

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

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

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



The Hague, 19 August 2004

Dr Th. Helder

GLP Compliance Monitoring Department

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