European Union Risk Assessment Report

1-(5,6,7,8-TETRAHYDRO-3,5,5,6,8,8-HEXAMETHYL-2-NAPTHYL)ETHAN-1-ONE

(AHTN)

CAS No: 1506-02-1 or 21145-77-7

EINECS No: 216-133-4 or 244-240-6

RISK ASSESSMENT

FINAL APPROVED VERSION

Cataloguing data can be found at the end of this publication

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Final version, May 2008

The Netherlands

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Foreword

We are pleased to present this Risk Assessment Report which is the result of in-depth work carried out by experts in one Member State, working in co-operation with their counterparts in the other Member States, the Commission Services, Industry and public interest groups.
The Risk Assessment was carried out in accordance with Council Regulation (EEC) 793/93[1] on the evaluation and control of the risks of "existing" substances. "Existing" substances are chemical substances in use within the European Community before September 1981 and listed in the European Inventory of Existing Commercial Chemical Substances. Regulation 793/93 provides a systematic framework for the evaluation of the risks to human health and the environment of these substances if they are produced or imported into the Community in volumes above 10 tonnes per year.
There are four overall stages in the Regulation for reducing the risks: data collection, priority setting, risk assessment and risk reduction. Data provided by Industry are used by Member States and the Commission services to determine the priority of the substances which need to be assessed. For each substance on a priority list, a Member State volunteers to act as "Rapporteur", undertaking the in-depth Risk Assessment and recommending a strategy to limit the risks of exposure to the substance, if necessary.
The methods for carrying out an in-depth Risk Assessment at Community level are laid down in Commission Regulation (EC) 1488/94[2], which is supported by a technical guidance document[3]. Normally, the "Rapporteur" and individual companies producing, importing and/or using the chemicals work closely together to develop a draft Risk Assessment Report, which is then presented at a meeting of Member State technical experts for endorsement. The Risk Assessment Report is then peer-reviewed by the Scientific Committee on Health and Environmental Risks (SCHER) which gives its opinion to the European Commission on the quality of the risk assessment.
If a Risk Assessment Report concludes that measures to reduce the risks of exposure to the substances are needed, beyond any measures which may already be in place, the next step in the process is for the "Rapporteur" to develop a proposal for a strategy to limit those risks.
The Risk Assessment Report is also presented to the Organisation for Economic Co-operation and Development as a contribution to the Chapter 19, Agenda 21 goals for evaluating chemicals, agreed at the United Nations Conference on Environment and Development, held in Rio de Janeiro in 1992 and confirmed in the Johannesburg Declaration on Sustainable Development at the World Summit on Sustainable Development, held in Johannesburg, South Africa in 2002.
This Risk Assessment improves our knowledge about the risks to human health and the environment from exposure to chemicals. We hope you will agree that the results of this in-depth study and intensive co-operation will make a worthwhile contribution to the Community objective of reducing the overall risks from exposure to chemicals.

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1 O.J. No L 084, 05/04/199 p.0001 – 0075

2 O.J. No L 161, 29/06/1994 p. 0003 – 0011

3 Technical Guidance Document, Part I – V, ISBN 92-827-801 [1234]

0 OVERALL RESULTS OF THE RISK ASSESSMENT[4]

CAS Number: 1506-02-1 or 21145-77-7
EINECS Number: 216-204-6 or 244-240-6
IUPAC Name: 6-Acetyl-1,1,2,4,4,7-hexamethyltetraline

Environment

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

Conclusion (ii) applies to all compartments and all scenarios.

Human health

Human health (toxicity)

Workers

Conclusion (iii) There is a need for limiting the risks; risk reduction measures which are already being applied shall be taken into account.

Conclusion (iii) is reached because it cannot be excluded that photosensitising effects may occur in scenarios 1, 2 and 3 (handling).

In the absence of a risk phrase for photosensitisation, a specific Note can be used to warn workers for the photosensitising potential of AHTN. However, at the October 2006 meeting of the TC-C&L the Commission stated that such a Note will not be developed under the current legislation. If a specific Note to warn workers for the photosensitising potential of AHTN will be available, conclusion ii may be applicable.

Consumers

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

4 Conclusion (i) There is a need for further information and/or testing.
Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.
Conclusion (iii) There is a need for limiting the risks; risk reduction measures which are already being applied shall be taken into account.

Humans exposed via the environment

Conclusion (**ii)** There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

Combined exposure

Conclusion (**ii)** There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

Human health (physico-chemical properties)

Conclusion (**ii)** There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

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FIGURES

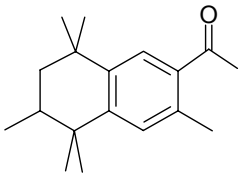
EUSES Calculations can be viewed as part of the report at the website of the European Chemicals Bureau: http://ecb.jrc.it

1 GENERAL SUBSTANCE INFORMATION

1.1 IDENTIFICATION OF THE SUBSTANCE

CAS-No.[5]: 1506-02-1 or 21145-77-7
EINECS-No.: 216-133-4 or 244-240-6
IUPAC name: 6-Acetyl-1,1,2,4,4,7-hexamethyltetraline
Synonyms: 1-(5,6,7,8-Tetrahydro-3,5,5,6,8,8-hexamethyl-2-napthyl)ethan-1-one
2' –Acetonaphtone, 5',6',7',8'-tetrahydro-3',5',5',6',8',8'-hexamethyl
6-Acetyl-1,1,2,4,4,7-hexamethyl-1,2,3,4-tetrahydronaphtalene
6-Acetyl-1,1,2,4,4,7-hexamethyltetraline
7-Aceto-1,1,3,4,4,6-hexamethyltetraline
7-Aceto-1,2,3,4-tetrahydro-1,1,3,4,4,6-hexamethylnaphtalene
7-Acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphtalene
AHMT
AHTN
Ethanone, 1-(5,6,7,8-tetrahydro-3,5,5,6,8,8-hexamethyl-2-naphtalenyl)-
Fixolide (CAS No: 21145-77-7)
Tentarome
Tetralide
Tonalid
Molecular formula: $C_{18}H_{26}O$

Structural formula:



Molecular weight: 258.41

1.2 PURITY/IMPURITIES, ADDITIVES

Purity: ≥ 98% w/w
Isomers: The molecular structure of AHTN has one stereogenic center so there are two enantiomers. The enatiomer ratio in technical AHTN is 1:1.

Impurities: 6-Acetyl-3-isopropyl-1,1,3,5-tetramethylindane, ca. 0.35% w/w
1,1,2,3,3,6-Hexamethylindan-5-yl methyl ketone, ca. 0.18% w/w (CAS-No 15323-35-0)
7-Acetyl-1,1,3,4,4,6-hexamethyltetraline, ca. 0.08% w/w
Additives: none
Reference: PFW (2001)

[5] The presence of two different CAS No for AHTN was caused by the registration of a not completely identified molecular structure for AHTN by one company and a fully identified and therefore different molecular structure by a second company. Afterwards the molecular structure was elucidated and both entries refer to the same substance. The presence of two CAS No from two companies resulted in two EINECS No.

1.3 PHYSICO-CHEMICAL PROPERTIES

In **Table 1.1** the physico-chemical properties are summarised.

Table 1.1 Physico-chemical properties of AHTN

Property	Result	Comment	References
Physical state	Solid, granules		
Melting point	> 54 °C		Janssen, 2004a
Boiling point	326 °C at 1 atm		Janssen, 2004b
Density	Bulk density: 600 kg/m^3	92/69/EEC A.3	Janssen, 2004c
	Density: 600 kg/ m^3 < D_4^{20} < 960 kg/m^3		
Vapour pressure	0.0682 Pa at 25 °C	gas saturation method, OECD TG 104, ^{14}C-labelled material	MacGillivray, 1996
Henry's Law Constant	37.1 Pa.m^3/mol determined at 25°C #	equilibrium partitioning in closed system and SPME	Artola-Garciana, 2002
Surface tension		solid, not applicable	--
Water solubility	1.25 mg/l at 25 °C #	flask method, OECD TG 105, ^{14}C-labelled material	Edwards, 1996
	(1.31 mg/l at pH 5; 1.22 mg/l at pH 7 and 9)		
	0.4 mg/l	calculation	SRC WsKow, version 1.27
	0.91 (± 0.04) mg/l	column elution method	Artola-Garciana, 2002
Solubility in other solvents		used in aquatic toxicity tests: dimethylformamide and Tween 80, triethylene glycol	-
Partition coefficient n-octanol/water (log value)	5.7	reversed-phase HPLC, OECD TG 117	Rudio, 1993a
	6.35	calculation	SRC, version 1.57
	6.25	calculation	Biobyte ClogP 4.01
	5.4 #	slow stirring method	Artola-Garciana, 2002
Flash point		not applicable, melting point > 54 °C	
Flammability	non flammable	Dir. 84/449/EEC A.10	Van Ek, 2004a
Autoflammability temperature	no spontaneous ignition	Dir. 92/69/EEC A.16	Van Rijsbergen (2002)
Explosive properties	not explosive	Expert judgement	Van Ek, 2004b
Oxidizing properties	not oxidizing	Bretherick's Handbook of Reactive Chemical Hazards, Ed. 1995 by P.G. Urben	Van Ek, 2004c
Hydrolysis	can not hydrolyse	OECD 111	Van Ek, 2004d
Granulometry	10% < 140.6 µm		Baker, 2001
Particle size distribution	25% < 270.6 µm	Laser diffraction analysis	
	50% < 479.1 µm		
	75% < 686.7 µm		
	90% < 810.0 µm		

: value selected for environmental risk assessment

Conclusion:

The data is mainly obtained by contract laboratories or in-company laboratories and reported in test reports to the Research Institute of Fragrance Materials RIFM. The Henry's Law Constant, water solubility and log K_{ow} were also reported in a PhD thesis (Artola-Garciana, 2002).

All data are considered sufficiently reliable to fulfil the Annex VIIA requirements. No R- and S-sentences are applicable based on the physico-chemical properties.

1.4 CLASSIFICATION

1.4.1 Current classification

- Present classification according to Annex I: none
- Provisional classification by producer: R22-50/53, S61

1.4.2 Proposed classification[6]

- Proposed classification:

  Symbols: Xn, N

  R-phrases: R22-50/53

  S-phrases: S(2-)46-60-61

6 The TC-C&L concluded that AHTN is a photosensitiser, an effect for which however no criteria for classification are present in Annex VI of Directive 67/548. To communicate the photosensitising potential of AHTN to users, the TC-C&L concluded to add an additional safety phrases and a Note, rather than applying R43. The Commission stated that such a note will not be developed under the current legislation.

2 GENERAL INFORMATION ON EXPOSURE

2.1 PRODUCTION

AHTN is produced on one site in Europe, with a production volume 1000 - 5000 ton/y. Circa 62% of the production volume is exported outside Europe. This is explained later in more detail, see Figure 2.2.

2.1.1 Production process

AHTN is produced by a two step synthesis. The first step is a cyclo-alkylation. All raw materials are pumped directly into a closed vessel where the reaction starts. The reaction temperature is kept slightly below room temperature. The intermediate is isolated by distillation and stored. In the next reaction step the raw material is pumped into the closed reaction vessel and acetylation takes place at room temperature. The product mixture is washed twice with water. The washed product is distilled and the distilled product is stored in liquid form at 60 °C outside the building and from there it is finally crystallised on a cooled conveyor belt. From the conveyor belt the crystallised product is packed in fibre drums.

2.1.2 Production capacity

Use volumes in Europe are according to RIFM (Research Institute for Fragrance Materials) and IFRA (International Fragrance Industry Association) based on surveys carried out between 1993 and 2006. The figures refer to the countries belonging to EU-15 plus the two associated countries Norway and Switzerland. This group will be noted as EU-15+2. Based on a recent survey for 2004, the use volume has declined with 30% between the year 2000 and 2004 (IFRA 2006). For the calculations the data for the year 2000 were used.

Table 2.1 Use volume in Europe

Year	ton/year	reference
1992	885	RIVM 1997
1995	585	RIVM 1997
1998	385	Letter EFFA to BUWAL, Switzerland of July 8, 1999
2000	358	Letter IFRA to PFW 22 November 2001
2003	indicative: 265	Letter EFFA/IFRA April 2005
2004	247	Letter IFRA to PFW August 1, 2006

2.2 USES

2.2.1 Introduction

The crystallised product is used as an ingredient in fragrance oils (by IFRA definition described as fragrance compounds [IFRA Code of Practice for the Fragrance Industry]; sometimes in literature also referred to as fragrances, fragrance composition, perfume oil or

perfume compositions). AHTN is the second largest volume product of the fragrance materials known collectively as polycyclic musks. Fragrance oils are complex mixtures, prepared by blending many fragrance ingredients in varying concentrations. Most of these ingredients are liquids, in which AHTN has to be dissolved.

Applications of the fragrance oils are in consumer products such as perfumes, cosmetics, soaps, shampoos, detergents, fabric conditioners, household cleaning products, air fresheners etc. The distribution over the various categories is shown in **Figure 2.1**.

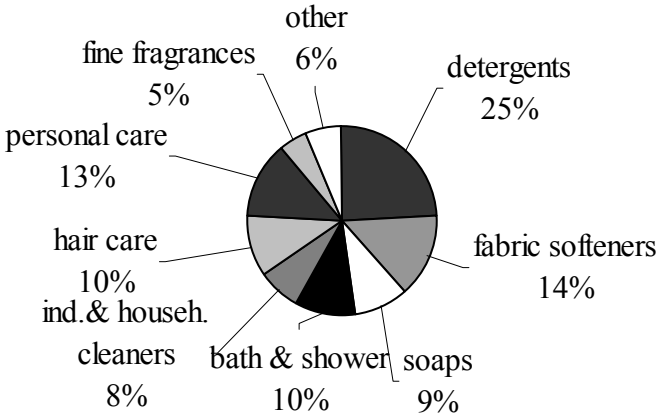


Figure 2.1 The use of fragrance oils in the EU-15 [figure taken from Balk et al. 2001, ACS Symposium Series 791, p. 171]

The use in compounding and formulation can be classified as 'non-dispersive, industrial controlled use', whereas the use by consumers can be classified as 'wide-dispersive use'.

Therefore the use is classified as follows:

- Main category: wide dispersive use
- Industrial category: category 5: personal/domestic use and/or
  category 6: public domain;
- Use category: category 9: cleaning/washing agents and additives and/or;
  category 15: cosmetics and/or
  category 36: odour agents.

2.2.2 Scenarios

2.2.2.1 Compounding

Crystalline AHTN is transported in 50 kg fibre drums lined with plastic to fragrance oil compounders. In some cases AHTN is transported in tanker trucks as a warm liquid. Two to three thousand fragrance ingredients may be mixed to prepare several thousand different fragrance oils. About one fifth of all fragrance oils contain AHTN and, when present, at an

average concentration of 2%. Fragrance oils are produced in batch volumes varying between 1 and 20,000 kg.

In Europe there are approximately 26 larger and medium sized compounding sites that receive AHTN. The volumes used for the preparation of fragrance oils at 6 large compounding sites that were visited are given in **Table 2.2**Table 2.2.

Table 2.2 Use volumes of AHTN per major compounding site (2000) (Reported during site visits)

Site	Use volume
site 1	4500 kg
site 2	6000 kg
site 3	31,140 kg
site 4	40,600 kg
site 5	10,400 kg
site 6	94,000 kg
TOTAL 6 sites	186,640 kg

This is 52% of the total use volume of 358 ton for the year 2000 reported for the EU-15+2 by IFRA. It was reported that 20% of the use volume is used directly for formulation by the major producers of detergents and cosmetics, leaving 286 ton for compounding. The volume handled by the 6 major compounding sites that were visited is 65% of the volume used for compounding.

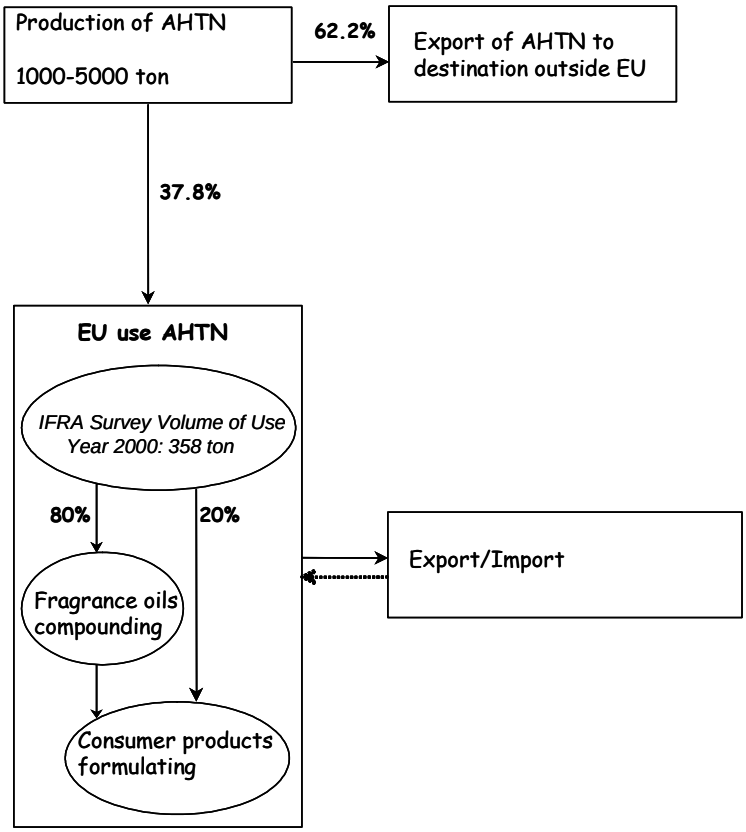


Figure 2.2 Material Flow of AHTN based on Production/Sales History, Industry data 2000

The mass flow for AHTN in Europe is illustrated in Figure 2.2. For the exposure calculations for the life-cycle part “end product formulation” and “private use” the volume reported by IFRA for 2000 will be used. This might be an overestimation of the real use as export of fragrance oils and formulated products to non-EU countries is included. Industry sources estimate that 20-30% of their production is exported outside the EU-15+2 as finished fragrance oils or in consumer products (letter IFF 1998).

2.2.2.2 Formulation

The fragrance oils are used in the formulation of a large variety of consumer products. A fraction of the production is directly used into bulk formulation of consumer products, such as the preparation of detergents by the larger producers. The fraction directly used is estimated at 20%.

2.2.2.3 Private use

Regional variation

For the exposure calculations for the life-cycle part 'private use' the volume reported by IFRA for 2000 of 358 ton will be used. AHTN is applied in consumer products, mainly in cleaning agents, but also in cosmetics. An analysis was made of the regional differences of the use throughout the EU-15 member states. Data on the consumption of detergents and cosmetics in each EU member state are presented in Figure 2.3 and Figure 2.4. The first figure shows that the use of detergents per inhabitant is lower in some northern European countries than in southern Europe, with a maximum difference between Italy and Finland of a factor of 3.3. However, the highest per capita use (Italy, 12.6 kg per year) is above the EU average (10.1 kg) only by a factor of 1.25 (AISE 2001). The use of cosmetics (expressed in monetary units) is lowest in some southern countries. Yet the highest consumption in the EU, in France (€ 174), is above the EU average (€ 147) by a factor of 1.18 only. Figure 2.4 shows that for rinse-off cosmetics only, the maximum (Denmark, € 88) is above the EU average (€ 73) by a factor of 1.21 (COLIPA 2004).

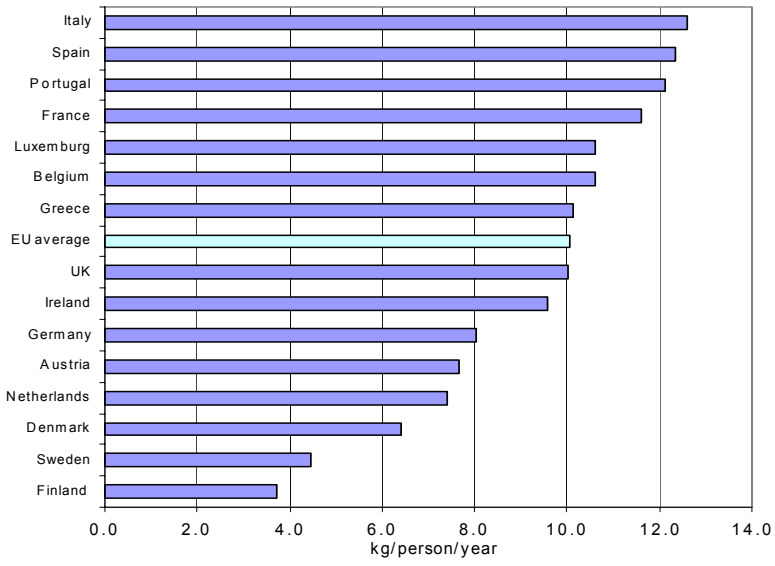


Figure 2.3 Per capita detergent consumption by country, 1998. EU-15 average is 10.1 kg (AISE, 2001).

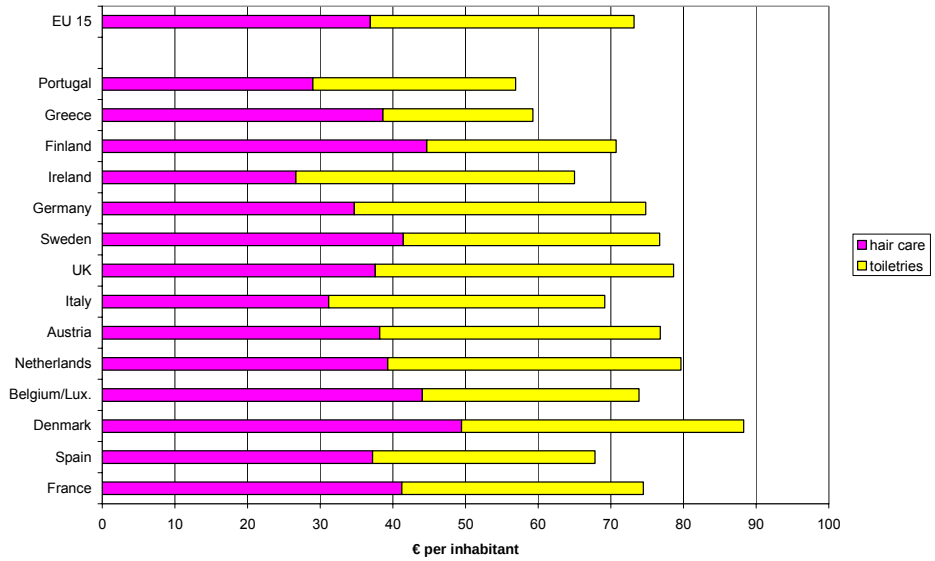


Figure 2.4 Per capita consumption of cosmetics with potential disposal to the sewer in the EU-15 in € per inhabitant, at retail prices for 2003. Weighted mean is € 73 per inhabitant (COLIPA 2004)

The use of polycyclic musk ingredients for fragrances in cosmetic and household cleaning products for the European market has significantly decreased during the second half of the nineties. Due to the publicity on these musk ingredients major producers of personal care and detergent products with European wide brands asked their fragrance suppliers to abstain from using polycyclic musks. As a result fragrances for such European wide brands were in many instances modified and no longer contain polycyclic musks. This trend was followed by many producers of locally marketed cosmetics and household cleaning products in the Northern European countries such as Germany, The Netherlands, Switzerland, Austria and Scandinavia. In other parts of Europe locally operating producers possibly followed this trend to a lesser extent.

Due to these market developments cosmetics and household cleaning products are probably less often fragranced with polycyclic musks in northern Europe than in southern Europe. In the extreme case that the entire European volume of use of AHTN would be consumed only in southern Europe (i.e. France, Spain, Portugal, Greece, Italy) and the UK/Ireland with about two-thirds of the EU-15's population, the outcome of the risk assessment, (based on an evenly distributed use of cosmetics and detergents) would have to be corrected by a factor of 3/2 (=1.5). However, it should be noted that the factor 1.5 is rather hypothetical. It is very unlikely that a gradual replacement in North Europe coincides with an increased use volume *per capita* in Southern Europe. This assumption would imply that the EU-15 consumer's market is divided into different sections, whereas the major producers indicated that this would not be the case.

In conclusion, there are two factors that may cause an uneven distribution of the use volume of AHTN *per capita* in Europe. A 'cultural' factor of different use volumes of detergents may cause a higher use of detergents per capita by factor of 1.25 in southern EU countries (Italy, Spain, Portugal, France, 166 million inhabitants), whereas an average use volume is found in Belgium/Luxembourg, Greece, UK and Ireland, with 84.6 million inhabitants. In the Northern countries (Germany, Austria, Netherlands, Denmark, Sweden and Finland) with 125.5 million inhabitants, the detergent use is below average by a factor of 0.7.

The second factor is the market development factor, where since 1995 polycyclic musks are gradually being replaced by other fragrance ingredients. As a maximum this would result in a higher use in the southern countries by factor of 1.5 as compared to the average *per capita* use of AHTN. As both factors are independent, the combination gives a factor of 1.25 · 1.5 = 1.88 above the average use in a 'worst case scenario' for the year 2000.

Environmental monitoring data between 1996 and 2000 in northern countries show a downward trend in the concentrations in the environmental compartments by a factor of circa 3 (e.g. in Hessen, HLUG 2001). Data after 2000 suggest leveling off of the concentrations as well as for the use volume. Therefore initially the risk assessment is based on the use volume for the year 2000.

For 2000 an evenly distributed use would mean 358 ton/370 million inhabitants (0.97 g *per capita* per year) and to cover the uneven use in a realistic worst case scenario this would be 1.88 · 0.97 = 1.81 g *per capita* per year (Southern EU). In the northern countries the minimum use volume would be the maximum/3.3 = 1.81/3.3 = 0.55 g *per capita* per year, whereas there the highest level would theoretically be 1.0 · EU-average = 0.97 g *per capita* per year.

According to the TGD (EC 2003), the regional use is 10% of the total use. This is used by 20 million inhabitants in the region, resulting in a *per capita* use of 1.79 g per year. Thus the TGD regional approach equals the worst case scenario based on an extreme interpretation of the data for 2000. The various scenarios are summarised in **Table 2.3**.

Table 2.3 Scenarios for private use

Year, ton	Scenario	Derivation	Consumption in g per capita per year	Consumption in mg per capita per day
2000, 358 t	TGD regional (10%)	10% of total use	1.79	4.9
	southern Europe	1.25 · 1.5 · average	1.81	5.0
	northern Europe	maximum (1.81)/3.3	0.55	1.5
	EU-15 average	average	0.97	2.7

2.3 TRENDS

Trends in the use of AHTN, both in time and possibly per region were discussed in the previous section.

2.4 LEGISLATIVE CONTROLS

No legislative controls are in place at the time of reporting.

3 ENVIRONMENT

3.1 ENVIRONMENTAL EXPOSURE

3.1.1 General discussion

Releases are discussed for each life stage: production, compounding, end-product formulation and the consumer use stage. The calculations are based on data for the year 2000. It should be remarked that between 2000 and 2004 the use volume has declined by 30%, see section 2.1.2.

3.1.2 Environmental releases

3.1.2.1 Release from production

Production of AHTN takes place on one site in the EU-15+2. Site-specific information on the emission was reported to the rapporteur. The following waste streams are identified:

1. Process water containing organic waste

The flow is < 5 m^3 per hour. The average residue level of AHTN in the water is 50 µg/l (6 samples, s.d. 16). With 259 days per year, this implies a discharge of 1.554 kg AHTN per year to the STP. This water is discharged to a municipal STP treating $14 \cdot 10^6$ m^3 per year. The concentration in the influent will be 0.111 µg/l.

The dilution factor of the effluent into surface water is 1.

2. Water containing aluminium chloride

The amount of Al^{3+}-containing water related to the production of AHTN is 5680 m^3 (5652 m^3 in 2000, 5709 m^3 in 2001). The load of AHTN in the Al^{3+}-containing water is given as 1.8 kg per year. The average concentration of AHTN in 3 samples was expressed as 257 µg/kg (circa 257 µg/l).

This solution is transported for use in sewage treatment plants for phosphate removal. As an example, the concentration is estimated for an STP where biological phosphate removal is relatively low and thus the dosing of aluminium is relatively high. The average daily water flow is 5881 m^3 or $2.1 \cdot 10^6$ m^3 per year. The solution was dosed at 35 m^3 per year in the aeration tank. This implies a dilution of 1 : 61,330.

Thus the concentration of AHTN in the influent is: 257 (µg/l) / 61330 = 0.004 µg/l.

3. Residue oil including the waste fraction of the AHTN production

This fraction is either recycled in the process or removed by a certified contractor for incineration.

4. Low chlorine content waste

This fraction is incinerated. Therefore the concentrations related to production are mainly determined by the background (regional) concentrations.

3.1.2.2 Release from formulation

3.1.2.2.1 Fragrance compounding

Emissions of AHTN in fragrance compounding facilities depend on the standard operating procedures of these facilities. Several emission routes can be distinguished:

- Mixing vessels, containers and pumps may be cleaned with an organic solvent, which is collected and disposed of by incineration or recycled. Emission to waste water does not occur in this case.
- Mixing vessels and containers may be washed with steam and/or water and AHTN present in the remaining fragrance oils is discharged to the waste water.
- Spills may be cleaned with water and AHTN present in the spilled material is discharged to the waste water.

The total volume of AHTN used in the EU-15+2 is taken as 358 ton per year (100%, reported IFRA volume of use in the EU-15+2 for the year 2000). Fragrance compounding is conducted by special fragrance compounding departments of large and medium scale companies (70%), by small enterprises (10%) and by large formulators of detergents (20%).

Local releases are calculated for the 6 large compounding sites that were visited as presented in **Table 2.2**Table 2.2. Site-specific emission data are submitted for these sites. The volume used in these sites was 187 ton, covering 65% of the EU-15+2 volume used in compounding of AHTN. Therefore these sites are considered representative for the all medium/large compounding activities. The PECs are estimated for the 6 sites visited, for a generic large/medium scale site (site 7) and for a small enterprise (site 8).

Site 1

A site visit report was prepared (Balk 2001a). The volume of AHTN used in compounding was 4500 kg in 2000. The number of working days is 240 days per year. The rinsing/washing water is collected and treated on site in a pre-treatment facility consisting of sedimentation, oil-separation and filtration. The yearly sludge production of 80 tons (90-95% water) is disposed of in a controlled way.

A conservative estimate for the loss during compounding to the trade effluent, based on proportionality of the COD in the effluent, is 0.02%. Therefore the loss through the trade effluent would be $0.0002 \cdot 4500 = 900$ g per year, or, in 240 days, 4 g per day.

The yearly trade effluent volume was 1400 m^3 in 2000. This effluent is discharged to the municipal STP (67,000 i.e., dry weather flow 18,789 m^3/d). After dilution the concentration of AHTN in the influent to the STP is 0.2 μg/l.

The STP discharges into a river with a flow of $4.3 \cdot 10^6$ m^3/d so the dilution factor for the effluent into the river is 229.

Site 2

A site visit report presents the details for this site (Balk 2001b). The tonnage of AHTN for fragrance compounding is 6 ton per year. There are 250 working days per year. The water of the washing machines is pre-treated on site. The process includes sedimentation, oil

separation and pH control. The emission to waste water leaving the site was estimated from the COD of the effluent of the oil skimmer, based on proportionality of the use volume of AHTN to the total volume of compounds. The loss is conservatively estimated at 0.05% of the daily use. This implies an emission of the site of 3 kg AHTN per year or 12 g/day.

The effluent of the pre-treatment plant goes to a municipal STP, with 250,000 p.e. and a mean flow of 40,605 m^3 per day. The concentration in the STP influent is 0.3 μg/l.

The canal receiving the effluent starts at the point of discharge so there is no additional dilution with surface water. After ca 15 km this stream joins a larger surface water body.

Site 3

The data for this site are described in a site visit report (Balk 2001c). The tonnage of AHTN for fragrance compounding is 31.1 ton per year. There are 250 working days per year. The amount of AHTN lost per year by washings of drums, containers and filtration pumps was estimated by weightings of the residue in containers and TOC analyses of the washing water from an average batch size of 1200 kg. The loss of ingredients was estimated at 0.16%. This implies that 50 kg AHTN is emitted with the washing water per year, or 0.20 kg per working day.

This process water is collected and treated on site in an oil skimmer. With a general efficiency for this type of operation of 90% removal, 0.02 kg will be released in the daily waste stream to the municipal STP. The STP is an activated sludge plant with a flow of 11,000 m3 per day or it treats the equivalent of 21,500 inhabitant equivalents. The concentration in the influent of the STP is 1.8 μg/l.

The actual dilution factor for the effluent into the river is 1100 (based on low-flow conditions). The TGD sets a maximum of 1000 to the dilution factor for a site-specific assessment.

Site 4

A site visit report was prepared (Balk 2001d). The tonnage per year for fragrance compounding is 40.6 ton/yr. With 250 working days per year, this is 162 kg per day. The fraction released to water is estimated by weighing the residue in mixing vessels varying in size from 3 to 1200 kg and from the scrap factor for the batches from 4 to 17 m^3 assessment amount. (A scrap factor is the amount that is produced in surplus to the ordered amount to take into account volumes needed for sampling, losses in various mixing vessels, volatilisation, etc. during the process. Therefore it is an overestimation of the release to water). This is converted to a loss per day as a fraction of the total daily production, or 0.12%. So the release to waste water is 195 g AHTN per day. The mixing room produces 80 m^3 process water per day. This is collected with other process water from the site and first treated mechanically: oil skimming, pH control, flocculation and sedimentation, and than treated in a biological treatment plant on site.

The efficiency of the oil skimmer with respect to AHTN is not known, but should be very high. In the oil phase, 90% of the organics consisted of toluene that will cause AHTN to partition to the oil phase and thus be effectively removed. As a very conservative value, the removal in the oil skimmer is set at 50%. The biological treatment plant on site (2700 m^3 per day, activated sludge) treats predominantly industrial waste water and 10-20% domestic waste

water. The concentration in the influent to the industrial WWTP is set at 195 g/d·0.5 / 2700 m^3 = 36.1 µg/l.

The actual dilution factor for the effluent of the WWTP into the river is 4481 (based on low-flow conditions). The TGD sets a maximum of 1000 to the dilution factor for a site-specific assessment.

The sludge of the industrial wastewater treatment plant is incinerated.

Site 5

The details for this site are presented in a site visit report (Balk 2001e). The tonnage for fragrance compounding is 10.4 ton/yr. With 250 working days per year, this amounts to a use of 41.6 kg per day. The overall scrap factor for this site is 0.2%, giving a release to water less than 83 g/d.

The water from the mixing rooms (5 m^3 per day) is pre-treated in a settling tank and oil skimmer. As a worst case approach in this site the removal during mechanical treatment is set at 0%. This water is discharged to a municipal STP, treating both domestic and industrial waste water. This is an activated sludge plant with a flow of $2.1 \cdot 10^6$ m^3 per day. The influent concentration is 0.04 µg/l.

The dilution factor for the effluent into the river is 5 (based on low-flow conditions).

Site 6

The tonnage for fragrance compounding is 94 ton per year. Compounding tanks are rinsed with solvent and the rinsings are recycled or disposed off by incineration. There are no emissions to water (Balk 2002a).

Site 7 – Generic scenario for a large/medium compounding site

Information from the producer shows that approximately 20% of the total use volume in the EU-15+2 is directly used in formulation, 70% is sold to large and medium compounders and 10% to small compounders. In Europe there are approximately 26 larger and medium sized compounding companies that receive AHTN. The six sites visited account for 75% of the volume used by the medium/large compounders[7]. A generic scenario was developed for the large and medium scale companies that were not visited. This scenario is applied for compounding activities at remaining larger and medium size companies. It is assumed that at all these sites a high level of emission control and good housekeeping is applied in addition to some type of wastewater treatment on the site.

The emissions for the 6 sites presented above are based on site-specific information, see **Table 3.1**. Based on this data set, the realistic worst-case emission factor before treatment is set at 0.2% and the emission factor after treatment is set at 0.06%.

The remaining 20 compounding sites that were not visited used 64 ton. This means the average use is 3.2 ton per year. Compensating for differences in size and production volume, a factor of 5 is applied to the average use volume to find the use volume for the generic

[7] total compounded volume = 0.8*358 = 286 ton
L+Mcomp. = 70% of 358 ton = 251 ton; six sites 187 ton; 187/ 251 = 75% ; Remaining sites = 251 – 187 = 64 ton; 64 / 286 = 22% of total compounded volume

compounding scenario: 3.2 * 5 = 16 ton per year. The emission factor for these sites is based on the highest emission factor after treatment observed at the six visited plants. This emission is divided over 250 working days.

Table 3.1 Emission factors of six visited plants (sites 1 to 6)

Site	Emission factor before treatment,%	emission factor after treatment,%	specification of treatment
1	-	0.02 **	sedimentation, oil separation, filtration
2	-	0.05 **	oil skimmer
3	0.16	0.016 – 0.048	oil skimmer
4	<0.12 *	0.06	oil skimmer, sedimentation, biol. oxidation
5	0.08 – 0.2 *	0.008 – 0.002	oil skimmer
6	-	0.00	no wastewater incinerator

* emission factors before treatment are based on empirically verified average scrap factors for tanks and other equipment.

** the emission after treatment is based on measured COD and an assumed proportionality

Thus the generic emission for large and medium size compounders is described by:

$$16 \text{ ton} / 250 \text{ d} \cdot 0.06\% = 0.0384 \text{ kg/d}$$

The release to the aquatic environment for a generic site is approached by the realistic worst case scenario of the TGD, where the waste water is discharged to the sewer of a small city with a sewage treatment plant for 10,000 inhabitants, with a water flow of 2,000 m^3/d. The effluent of the STP is discharged to a relatively small size river with a dilution factor of 10.

Therefore the influent concentration is:

$$0.0384 \text{ kg. } d^{-1} / 2000 \text{ m}^3\text{. } d^{-1} = 0.0192 \text{ mg/l}$$

Site 8 - Generic scenario for a small compounding site

A representative of the group of small size enterprises was not visited. Small enterprises use less than 0.1% of the total use volume. It can be assumed that the emissions are controlled less strictly and that no in-house treatment is applied. For production activities in the chemical industry the TGD gives an emission factor to waste water of 2%. Emission scenario documents for IC-5 and IC-6 give losses to water of surfactants during production < 0.3% for batch and < 0.1% for continuous processes and for the formulation of washing powders 0.01% and liquids 0.09%. These TGD emission scenarios are not specific for the type of formulation that is conducted at compounding of fragrances. As the compounding processes in small enterprises and large companies are completely similar there is a good reason to apply the empirically verified scrap factors. At Site 5 the overall scrap factor including sampling and oversupply to customers is 0.2%. Thus, the highest emission factor to waste water before treatment is << 0.2%, and 0.2% seems a realistic worst case for small size enterprises.

AHTN is a very generally used compound and therefore it will be used daily (number of days 250). The maximum use volume of a small compounding site is 0.1% of the total use volume,

or 0.358 ton per year. Since this is a relatively low volume, as a conservative approach the number of days has been reduced to 125 days.

Thus the generic emission by all small size clients is described by:

$$0.358 \text{ ton} / 125 \text{ d} \cdot 0.2\% = 0.00573 \text{ kg/d}$$

The emission is released to the standard STP with a water flow of 2,000 m^3/d and the effluent is discharged with a dilution factor of 10. The influent concentration is:

$$0.00573 \text{ kg. } d^{-1} / 2000 \text{ } m^3 . \text{ } d^{-1} = 0.0029 \text{ mg/l}$$

An overview of all relevant data from this section used for calculation of emissions for compounding sites 1-8 is given in **Table 3.2**

Table 3.2 Summary of relevant emission data for compounding sites 1 to 8, based on the year 2000

Compounding site	Volume of AHTN undiluted, kg/year	# of working days per year	Emission factor after treatment,%	Conc. in influent to STP, µg/l
1	4,500	240	0.02	0.2
2	6,000	250	0.05	0.3
3	31,140	250	0.016 – 0.048	1.8
4	40,600	250	0.06	36.1 (WWTP)
5	10,400	250	0.008 – 0.002	0.04
6	94,000	250	0.00	0
7 – Generic scenario for a large/medium site	16,000	250	0.06 *	19.2 #
8 – Generic scenario for a small site	358	125	0.2 **	2.9 #

* Higherst release rate after treatment from the sites visited (1-6)

** Highest empirically derived overall scrap factor for large/medium compounding site 5

\# TGD realistic worst case calculations

3.1.2.2.2 End product formulation

Fragrance compounding is followed by the formulation of fragrance compounds (mixtures including AHTN) in end products (cosmetics, detergents, fabric softeners etc). Extensive information was obtained from a site visit to a small formulator of cleaning agents (Balk 2002b), and additional information from questionnaires and interviews from larger companies (Communications to RIVM 2002). In the TGD an emission scenario document (ESD) "Assessment of the environmental release of soaps, fabric washing, dish cleaning and surface cleaning substances" is available. This scenario document[8] comprises Personal/domestic use (no.5) and Public domain (no 6) and use category Cleaning/washing agent (no. 9) and cosmetics (no.15).

[8] The emission scenario document does not include air fresheners and/or odour agents.

The total volume of AHTN in end product formulation in Europe for 2000 is assumed to be 358 tonnes (See section 2.1). According to the emission scenario document the emission factor "washing liquid"[9] for waste water is 0.0009 and air is 0.00002 (TGD **Table 2.1**). For the small formulator, the percentage lost to water was very conservatively estimated at 0.2%, whereas in larger companies it was estimated to be lower by a factor of 5 to 10. The number of sites in the EU-15+2 is estimated on the basis of the number of members of the branch organisations involved in the production of end products (soaps/detergents and cosmetics industry in the EU-15+2), which is likely to be over 2000. As a conservative estimate, 1000 sites in the EU-15+2 are assumed.

Large formulator scenario

One of the large formulators was supplied with 19 ton, directly from the producer. It is assumed that this volume is used on one site. The formulation on this particular site takes place during 345 working days and the specific emission factor to water is 0.017%. Therefore the daily emission to water is 19 ton · 0.00017 / 345 d = 0.009 kg/d. This is released to the municipal STP. By lack of data, the standard STP characteristics are assumed, resulting in an influent concentration of 0.0047 mg/l.

Formulators, generic scenario

No specific information is available for deliveries by compounders to formulators. The use volume by these formulators is 358 ton minus the 20% sold directly to the formulators, thus 286 ton/year. Assuming 1000 formulation sites in the EU-15+2, the use of AHTN on an average formulator site is 286 ton / 1000 = 0.286 ton per year. For the assessment of a 'reasonable worst case, this use volume is multiplied by a factor of 5, thus 0.286 · 5 = 1430 kg/year (or 0.4% of total use). With the conservative emission factor to waste water of 0.2%, and 250 working days per year (Balk 2002b) for a small formulator, the loss to the STP is 1430 kg · 0.002 / 250 d = 11.4 g/day. Release into the standard STP results in an influent concentration of 0.006 mg/l.

3.1.2.3 Release from private use

After use of the fragranced consumer products (cosmetics, detergents, fabric softeners etc.) most of the materials will be emitted with the waste water of households to the sewer. Depending on the use for body care, shampoo, textile or floor cleaning, a larger or smaller fraction of the use volume will evaporate. In general, cosmetics will be emitted to waste water to a lesser extent than detergents. As a first approach for the estimation of the PECs, it is assumed that the total volume of AHTN used in compounding fragrances in Europe for 2000, i.e. 358 tonnes is released to waste water going to a STP. **Table 3.3** presents the release per inhabitant per day as derived in **Table 2.3**. Since the high and low estimates differ only by a factor of 3, the estimations are first carried out according to the default TGD regional (10%) scenario.

[9] Other release factors (Table 2 of ESD in TGD) are available for regular washing powders and compact powder. The column of "washing liquid" was selected because fragrances are complex mixtures wich are nearly always liquids. The category washing liquid also represents a worst case approach concerning the% emission to water (a factor of 9 higher than the other two categories).

Table 3.3 Private use

Year, volume	Scenario	Consumption in mg per capita per day
2000: 358 tonnes	**TGD regional (10%)**	**4.9**
	southern Europe	5.0
	EU-15 average	2.7

In Switzerland the average *per capita* use was calculated from use data as 3.2 mg per day or 1.17 g per year (cited in Buerge et al. 2003). Analysis of the concentrations in sewage sludge samples of 16 STPs and the sludge production allowed to determine the *per capita* input of AHTN in the areas. The mean input was 139 mg cap $^{-1}$.year^{-1} or 0.38 mg . cap $^{-1}$.day^{-1} (Kupper et al. 2003a, calculated from their table 10). The data of Bester (2004) in Germany showed a daily use of 0.3 mg for the year 2002. The mass balance calculations in Yang and Metcalfe (2006) for an STP in Ontario, Canada show an average *per capita* input of AHTN and HHCB of 0.64 mg per day. These values are all below the estimates given above.

3.1.2.4 Release from disposal

AHTN is used in consumer products like cosmetics and cleaning agents. The use of these consumer products is mostly associated with water that will be discharged to the sewer system. Therefore the disposal phase is already included in the use phase. The disposal of residues in empty containers is expected to be a minor volume; moreover it is expected to be disposed of as solid waste in a controlled way.

3.1.2.5 Summary of releases

In summary, AHTN may be released during the production phase, during compounding and formulation and during/after use by consumers. For the risk assessment, as a conservative approach, it is assumed that the total volume used in fragrance compounding is discharged to the sewer.

3.1.3 Environmental fate

3.1.3.1 Degradation in the environment

3.1.3.1.1 Photodegradation

A study is available on the rate constants for the gas phase reactions of OH radicals of 4 fragrance molecules with a structure similar to AHTN, including HHCB (Aschmann et al. 2001). The experimental results show that the estimated rate constants for this reaction are within a factor of 1.3 to 1.5 for these 4 substances. This implies that the estimated rate constant for AHTN is expected to be relatively accurate as well. The estimated rate constant for the gas-phase reaction with OH radicals $k = 1.7 \cdot 10^{-11}$ cm^3 molecule^{-1} s^{-1}. Assuming a daylight period of 12 h and $1.5 \cdot 10^6$ OH cm^{-3}, the estimated atmospheric half-life is 7.3 hours or 0.6 d (Syracuse estimation programme AOP). These data suggest that the atmospheric

lifetime of AHTN is sufficiently short that it will not undergo long-range transport to any significant extent.

The photo-induced degradation of AHTN was studied using a solid-phase micro-extraction fibre. For the UV radiation low-pressure mercury lamps (8-10 W, 245 nm) were used. The irradiation was conducted with AHTN adsorbed on the fibre, as well as solved in water. 100 µm fibres coated with polydimethylsiloxane (PDMS) were used. A 1 and 20 µg/l solution in water was heated to 100 °C with the fibre in the headspace over the sample (HSSPME) for 30 minutes. Next the fibre was thermally desorbed in the GC injection port. The results showed that irradiation in water showed similar degradation kinetics but only slightly faster than on fibre. Photodegradation of the parent AHTN showed a half-life of less than 5 minutes. After 30 minutes more than 95% was degraded. The tentatively identified degradation products show that AHTN is not attacked on the hexamethyltetralin group but on the acetyl group, either by formation of a five-ring with aldehyde group or by removal of the oxygen moiety or by addition of an extra oxygen (Sanchez-Prado et al. 2004).

The photochemical degradation of AHTN in water was studied using a Mercury *high pressure lamp* (OMNILAB TQ 150) at 20 °C. After extraction with hexane, quantification followed by GC/MS and UV-detection. The degradation followed a first order reaction kinetics with t½ = 1.25 minutes. The reaction did not take place in the dark and the rate was strongly reduced in the absence of oxygen (t½ = 20 min.). Some unidentified reaction products were only temporarily observed. At the end of the experiment no stable metabolites were detectable even if the medium was concentrated by a factor of 1000. This may be explained in various ways, e.g., the formation of volatile transformation products, the formation of polymers (not chromatographically detectable), or mineralisation (Willenborg and Butte 1998). Suggested temporary transformation products were modified only on the acetyl side, whereas the hexamethyltetralin moiety was not attacked. Although this experiment shows the potential for photochemical degradation, the experimental conditions are not relevant from an environmental point of view.

In a laboratory set-up AHTN at 1 µg/l was incubated in lake water from the Zürichsee (CH) or in distilled water at 20 ± 1 ºC. Aliquots of 25 ml were illuminated with actinic lamps (mercury-vapor fluorescent lamps emitting UV-radiation between 300 and 460 nm with a maximum at 365 nm, comparable to that of 24h-averaged sunlight at 50 ºN in July under clear sky conditions. Degradation of AHTN followed first-order kinetics with half-lives of ≈ 4 h (photolysis rate constants 4.6 and 4.4 d^{-1} in lake water and in distilled water, respectively). The molar absorptivity of AHTN around 300 nm in distilled water at pH 7 was circa 1000 cm^{-1} M^{-1}, giving a calculated quantum yield for AHTN of 0.12. The minimal differences between the photolysis rate constants determined in lake water and distilled water indicate that AHTN is degraded primarily via direct photolysis and that indirect photochemical degradation by reactive oxygen species is of minor importance. Control experiments in the dark indicated that AHTN was not eliminated by other processes. The photodegradation may explain the decreased concentrations in the epilimnion of the lake in summer. The average photolysis rate for a typical winter situation, integrated over the whole depth of the Zürichsee (mean depth 50 m, attenuation 0.01 – 0.02 per cm at 315 nm) ranges from 1.0 to 2.0 * 10^{-3} d^{-1}. In summer, considering only the epilimnion, the estimate is about 2 orders of magnitude higher then in winter for the whole lake: 0.10 – 0.19 d^{-1} (Buerge et al. 2003).

3.1.3.1.2 Aquatic degradation (incl. sediment)

Mineralisation

The biodegradability of AHTN was assessed in various tests in which the bioavailability and the probability of adaptation of the micro-organisms involved were increased in several ways. Bioavailability was enhanced by use of solvents, dispersants or emulsifiers, whereas the probability of adaptation was increased by pre-exposure of the inoculum to the test substance or by repetitive additions. The tests show the absence of mineralisation under the conditions of the standard tests for ready biodegradation. In the two-phase closed bottle test, 21% oxidation was observed after 3 weeks. After repetitive additions of AHTN, a marginal oxygen consumption of 12% was observed. The tests are summarised in **Table 3.4**.

Table 3.4 Summary of tests for biodegradation (mineralisation)

AHTN	Results
Test	**OECD 302C: modified MITI II**, respirometric method
	(Rudio 1993b)
Inoculum	Adapted Industrial Sludge 100 mg/l from AHTN production facility
Test substance	AHTN, nominal 30 mg/l
Dispersion	1) fine grinding + ultrasonic dispersion
	2) melting at 60°C + ultrasonic dispersion
	3) solvent (methylene chloride) + evaporation
Test duration	28 days
Controls	Reference substance aniline
	No toxicity control
Results	% oxidation: zero, no additional BOD in comparison with blank.
Test	**Modification of OECD 301B**, Sealed vessel TIC test acc. to Birch and Fletcher, 1991 (King 1994)
Inoculum	Effluent from SCAS (Semi-Continuous Activated Sludge) test after 8 weeks adaptation
Test substance	AHTN, 1.34 mg C/l; carbon 83.72% w/w
Dispersion	In Tween 80
Test duration	28 days
Controls	Reference substance benzyl alcohol
	No toxicity control
Detection	TIC (Total Inorganic Carbon)
Results	% CO_2 release: zero
Test	**Two-phase closed bottle test, NEN 6515, 1989** Oxygen uptake
	(Boersma and Hagens 1991)
Inoculum	Activated sludge from communal STP, 30 mg/l
Test substance	AHTN, nominal 22 mg C/l, repetitive additions after 4 and 5 weeks
Dispersion	no
Test duration	7 weeks
Controls	Reference substances Sodium acetate and diethylene glycol; Toxicity control
Results	21% oxidation after 3 weeks
	12% oxidation after 7 weeks

AHTN	Results
Test	**Modified Sturm test OECD 301B**, CO_2- evolution (Jenkins 1991)
Inoculum	sewage effluent, 1 drop/l
Test substance	AHTN, nominal 10 and 20 mg/l
Dispersion	no
Test duration	28 days
Controls	Reference substance sodium benzoate; Toxicity control
Results	% CO_2 release: zero

Primary degradation

Lee et al. (2001) reported on a Continuous Activated Sludge test performed at 10 µg/l with ^{14}C-labelled AHTN and realistic STP operation conditions (addition of waste water, sludge retention time 10 d, Hydraulic retention time 6 h). The disappearance of parent material and the formation of metabolites were determined with thin layer chromatography (Rad-TLC). The test design allowed drawing up a complete mass balance:

- The total removal of the parent AHTN from the water phase was 87.5%. The effluent contained 46.5% of the dosed radioactivity, consisting of parent AHTN (12.5%) and polar metabolites.
- The wasted solids included 50.3% of the radioactivity, including 44.3% of the parent AHTN, 4.8% as polar metabolites and 1.3% incorporated into biomass.
- The fraction volatilised was 3.3% of the dosed radioactivity, consisting entirely of the parent AHTN.

In conclusion, the study shows that in the total removal of the parent AHTN of 87.5%, half (42.5%) was caused by biotransformation and half by sorption (44.3%), whereas volatilisation played a minor role (3.3%) (Lee et al. 2001, Federle et al. 2002).

A die-away study was performed on ^{14}C-AHTN in activated sludge. AHTN was dosed at 5 and 50 µg/l to freshly collected activated sludge to examine the formation of metabolites. The parent and transformation products were extracted and separated. After 3 days, a variety of more polar metabolites were detected, as classified by their Rf-values. The half-life of the parent AHTN was 12-24 h. The related first-order reaction rate was 0.029 – 0.057 h^{-1}. AHTN was largely biotransformed to polar metabolites within 20 days. For the parent AHTN (log Kow of 5.4) the retention was given as Rf 0.59, whereas the peaks for the increasingly more polar metabolites were observed at Rf 0.34, 0.24, 0.05-0.09, 0.0) In **Table 3.5** mass balances are tabulated as read from the graphs (Lee et al. 2001)[10].

A similar die-away test was conducted in river water (Schaefer and Koper 2006). AHTN was tested at a concentration of 5 µg/l in a sample of river water with activated sludge added at a

[10] With AHTN mainly bound to sludge, this reaction rate constant of 0.029 to 0.057 h^{-1} would imply that during the solids retention time (SRT) of circa 9 days a significant part (>99%) would be degraded: With $Ct = Co \cdot e^{-k \cdot SRT}$, SRT = 9 d and k = 0.029 to 0.057 hr^{-1}, Ct/Co = 0.002 to $4.5 \cdot 10^{-6}$ (after 9 days, 0.2% or less remains of the initial concentration)

level of 10 mg AS/l. Mass balances were made for the components in the test medium, see **Table 3.6**.

Table 3.5 Mass balance for die-away experiment with radio-labelled AHTN in activated sludge (Lee et al. 2001, read from graphs)

Time	day 0	day 3	day 20	day 28
^{14}C-AHTN, 50 µg/l , activated sludge 2.5 g SS/l**				
% recovered in extracts with solvent				
As parent *	95	55	20	22
As metabolites *		34	57	59
% volatilised *		1	4	5

* data read from graphs
** SS: Suspended sludge

Table 3.6 Mass balance for die-away experiment with radio labelled AHTN in river water (Schaefer and Koper 2006)

Time	5 min	day 7	day 14	Day 21	Day 28	Abiotic Day 28
^{14}C-AHTN , 5 µg/l, river water + 10 mg AS/l*						
% recovered in extracts with solvent						
As parent	84.0	54.6	39.5	32.2	28.9	44.2
As metabolite	0	14.9	19.3	21.2	20.3	0
% recovered in aqueous	0.93	0.46	0.83	0.84	0.79	0.15
% not extractable from solids	0.63	0.99	0.80	1.0	0.84	5.3
% volatilised	n.a.	15.9	26.4	33.8	40.4	42.6
Total recovery %	85.6	86.8	86.8	89.1	91.2	92.2

* AS: Activated sludge

The parent substance steadily disappeared with an overall half-life of 200 hours in the river die-away test. A range of metabolites was formed at different rates and with increasing polarity in time. The total recovery for the individual samples ranged between 83 and 93%. The total radioactivity of metabolites rose to 20% of the initial activity from day 14 onwards. The TLC showed that the peak of the parent was at 120 – 140 mm or retention factor (Rf) 0.63 to 0.69. The disappearance of the parent was associated with the appearance of three more polar peaks. The first peak at Rf 0.44 – 0.51 was at 4.0% on day 7 and rose to 4.9% and 4.7% on days 14 and 28, respectively. The second peak at Rf 0.28 – 0.34 was detected at 6.1 % on day 7 and increased to 9.8 on day 28. The third peak at Rf <0.00 to 0.06) was 4.8% at day 7 and increased to 6.8 and 5.8% on day 21 and 28, respectively (Schaefer and Koper 2006). When the results are corrected for the amount volatilised as well as for the non-recovered fraction, the primary biodegradation on day 28 amounts to 42%. In another approach to evaluate the impact of the biological activity, the results of the abiotic control are compared to the results on day 28. Also in this way it is calculated that the difference due to biological activity, is 41%. According to the kinetic analysis in Schaefer and Koper (2006) the overall half-life of the parent is 9 d.

Free (dissolved) and total concentrations of AHTN in duplicate activated sludge samples were followed in time during 2 days. The samples were not additionally spiked. Loss due to

volatilisation was also determined. The initial free and total concentrations were 1.15 and 5.25 µg/l, respectively. The 'true biodegradation rate constant' based on the free concentration was 0.023 ± 0.010 h^{-1}. The rate constant based on total concentrations was 0.0075 h^{-1} (Artola 2002).

3.1.3.1.3 Degradation in soil

A variety of 64 soil samples (sand, clay, peat and loam) from various locations in The Netherlands was screened for the presence of micro-organisms able to transform AHTN into metabolites with a more polar behaviour as indicated by the lower retention factor (Rf)-value on TLC plates. This screening resulted in several pure cultures of the fungi *Aureobasidium pullulans* and *Phanerochaete chrysosporium* being incubated with AHTN and AHTN-alcohol. The ethyl acetate extracts of the cultures were then analysed by GC-MS (PFW 1996).

Approximately 40% of the 64 soil samples showed a positive degrading potential towards one or both of the polycyclic musks AHTN and HHCB (28% for AHTN). In cultures of the fungus *A. pollulans* (ATCC 66657) about 80% of AHTN disappeared in 3 weeks. GC-MS analysis of the ethyl acetate extracts indicated reduction of the acetyl-group. In cultures of the white rot fungus *P. chrysosporium* (ATCC 32629) AHTN disappeared within 3 days. GC-MS analysis of the metabolites of AHTN in ethyl acetate extracts showed the temporary presence of small amounts of AHTN + O and AHTN + 2 O. It is suggested that later stages of degradation were too polar to be extracted by ethyl acetate (PFW 1997).

The dissipation of fragrance materials in sludge-amended soils was studied in a 1-year die-away experiment with four different soils, with and without spiking of the test materials. The four different soils were characterised as: sandy agricultural soil from Georgetown, DE, organic matter content (OM) 1.55%, silty Midwestern agricultural soil, ILL, OM 2.63%, clayey soil from Newark, DE, OM 7.01%, highly weathered oxide rich soil from Aiken, SC, OM 0.61%. Anaerobically digested and dewatered sludge was obtained from two activated sludge plants: Georgetown DE (100% domestic, 10% solids) and Wilmington DE (70% domestic, 17% solids). The concentration of AHTN in the digested sludge of Georgetown was 51 and 17.7 mg/kg dwt in the years 2000 and 2002, respectively. For Wilmington the concentrations were 17 and 8.1 mg/kg dwt, respectively. The test was carried out in trays containing 24 liter mixed with 1 liter of sludge, simulating sludge applications of 7000 wet gallons per acre and a 15 cm plow depth (or 0.6 to 1.1 kg sludge per m2). Each of the four soils was incubated with digested sludge, unspiked as well as spiked with a mixture of fragrance ingredients. The spiking of sludge was performed by the wall coating method and the soil and sludge were mixed during 30 minutes in a cement mixer to ensure uniform mixing. Four combinations were duplicated, so a total of 20 trays were set up outdoors. Leachate samples were collected at the end of each rain event. Soil samples were extracted by accelerated solvent extraction, the leachate was extracted using C-18 speed disks. All extracts were analysed by GC/MS. The initial concentrations in spiked soil were 6 and 13 mg/kg soil, whereas the levels in unspiked soil, simulating real practice were between 0.1 and 0.27 mg/kg. In the spiked sludge experiments, the concentrations in soil rapidly decreased during the first month and then decreased steadily in time. In the non-spiked soils this phenomenon was not observed. After 3 months, the concentrations were 65 to 80% of the initial concentrations. During the next three months period the soil was frozen and the concentrations of all test materials remained stable. After one year the concentrations of AHTN in unspiked soils ranged from 42 to 61% of the initial concentration. In spiked soils the concentrations were slightly more variable. Loss processes may include volatilisation,

leaching and biotransformation. Leachate was collected during 3 to 5 months. The leached amount was 0.04 to 0.18% of the initial amount in the spiked soils. A relation with the organic material content was absent. In the leachate from the unspiked soils AHTN was not detected (DiFrancesco et al. 2004).

The degradation of AHTN was studied in three soil types: sand, clay and humic. Dehydrated sludge was ground and sieved over 2 mm. Soil stored during more than 6 months in the freezer was thawed, dried, sieved over 2 mm and dried during 3 weeks. The water content was increased to 50% of the water capacity of the soil. 5 g of sludge (sampled in January) was weighed into a 80 ml tube and spiked with AHTN. After evaporation of the solvent (acetone) 10 g of soil was added. The nominal concentration in the soil-mixture was 10 mg/kg. The actual concentrations were around 14 mg/kg. The soil-sludge combination was mixed by turning and lightly shaking of the closed tube. The samples were incubated in the dark at 20 + 1 ºC in a humid environment. At different time intervals the total content of the tube was extracted and analysed. The AHTN levels in the soil-sludge mixture showed no trend in time. The residues after 3 weeks were 68%, 74% and 73% and after 18 weeks they were 72%, 74% and 76% of the added amount in the sand, clay and humic soil, respectively. There was a high variability in the results. The report states that circa 20 to 30% had disappeared irrespective of time. In view of the low activity, additional tests were carried out. After 24 weeks freshly collected soil was sampled and 5 g was added to the remaining tubes and mixed. The AHTN residues seemed to remain around the same levels as before. It was concluded that the fresh soil did not supply the inoculum needed for the degradation. In another experiment the sandy and clay soils were incubated as before, but with freshly collected sludge and a sterile control was included. In this test no degradation was found after 8 weeks for AHTN as well as for HHCB but also here the variability in the results was high. It was suggested that the fresh sludge (sampled in June) was less active than the previous sample to explain the observed lack of biodegradation (Müller et al. 2003). Some general remarks can be made regarding this test. The test was started with soil that had been frozen and dried to air, so the microbial activity was far from optimal. The test was carried out in a small volume of 15 g soil in a centrifuging tube. It is questionable whether the mixing by 'turning and light shaking' ensured a homogeneous mixing of (1) the stock solution with the sludge and (2) the sludge with the soil and (3) the soil mixture and the freshly collected soil in the extended experiment. All tests showed a high variability in the results over the weeks. The total lack of degradation in the non-sterile replicate of the experiment including the sterile control showed that the results of the first tests could not be repeated. A positive control to check the quality of the inoculum as required in the tests for ready biodegradation, was not included here. Another remark refers to the actual concentration levels present in the system, which were relatively high as compared to the levels predicted in soil (PECsoil of AHTN around 0.03 mg/kg dwt). These substances are highly sorptive and only a small fraction would have been in the water phase and available for biodegradation by micro-organisms.

In the state of Baden-Württemberg, Germany, 13 study locations were selected comprising a sewage sludge field with known history of sludge applications and a reference field. Over the years, different quantities of sludge had been applied in different periods. Total quantities of sludge applied to the fields ranged from 3.2 ton/ha to 31.5 ton/ha. A total load of 85 and 510 ton/ha was brought on the two experimental plots during 18 years, between 1972 and 1989. Soil concentrations were expressed as the sum of AHTN and HHCB. The concentrations measured in 2002 in the experimental fields were 1.1 and 5.3 µg/kg dwt. The concentrations fields amended with sludge on a regular basis between 1993 and 2001 were below 1 µg/kg dwt except on one field (2.1 µg/kg) where the concentration in the reference field was also elevated. Also the concentrations in the reference plots of the experimental areas were

elevated: 0.54 µg/kg dwt (LfU-BW 2003). Using the data in the report on the quantities of sludge applied and the number of years after the last sludge application, and assuming a relatively low load of 15 mg AHTN + HHCB per kg dwt sludge, a conservative estimate was made of the estimated accumulated concentrations in soil. In the experimental plot with the highest sludge load, a total of 31.5 ton/ha was dosed over the years. This was estimated to give a concentration of 2550 µg/kg dwt, whereas the residue measured 13 years after the last application was 5.3 µg/kg, which is a maximal of 0.2% of the applied dose. (With likely higher sludge concentrations in the past, the residue will be lower.) Similarly, in the six fields amended with sludge on a regular basis the residues were <0.3% three years after the last application, 1.8% after 2 y, 0.5% after 7 y, 1.3% after 1 y, 1.9% after 3 y and <0.4% after 4 years, respectively.

Biosolids from an STP in Ontario, Canada with 96 µg AHTN per kg dw were applied on an agricultural field in October 2003. Soil samples were collected to a depth of 10 cm before and after application. No musks were detected pre-application. The concentration on day 1 was 2.6 µg/kg, after 2 week 2.0 µg/kg. After 4 weeks the level had dropped below the limit of quantitation (0.2-2 µg/kg wet weight) and after 6 months AHTN was not detected anymore (Yang and Metcalfe 2006).

3.1.3.1.4 Summary of environmental degradation

Under atmospheric conditions the half-life is estimated at 7.3 hours (Syracuse AOP). The half-life in a laboratory set-up with lakewater was 4h (summer conditions, 50 ºN, Buerge et al. 2003).

In a primary degradation process, AHTN is rapidly transformed to polar metabolites. These substances still contain the same amount of organic carbon and only a small fraction of the theoretical oxygen demand has been incorporated. Thus this metabolism is in agreement with the observed low degree of mineralisation.

The half-life of the parent compound AHTN in activated sludge was less then 1 day and within 20 days AHTN was largely transformed to metabolites (Lee et al. 2001, Federle et al. 2002, Artola 2002). In river water the overall half-life was circa 9 days (Schaefer and Koper 2006).

In soil studies the residual AHTN present after 9 months ranged from below 42 to 61% of the initial concentrations (DiFrancesco et al. 2004). Comparison of the results in the available tests with those for HHCB showed that the half-life of AHTN in sludge (Lee et al. 2001, Artola 2002) and in soil (DiFrancesco et al. 2004) was similar to that for HHCB or twice as long as for HHCB.

For the environmental risk assessment, AHTN may be considered as inherently biodegradable', not fulfilling criteria' (terminology of the EU-TGD, EC 2003). For surface water, sediment and soil, the biodegradation rate constants are based both on the data for AHTN and on the results for HHCB. As a conservative approach, the rates for AHTN are taken as twice the rates for HHCB: 150 d in surface water (20 ºC) and 365 d in the soil and sediment compartments (12 ºC).

3.1.3.2 Distribution

3.1.3.2.1 Adsorption

In a critical review on synthetic musks in sludge, sediment and suspended matter, partition coefficients were summarised for these solid matter matrices (Fooken 2004). Together with the K_{oc} and Kd determined from empirical data in more recent studies there is a broad database on the sorptive behaviour of AHTN, see **Table 3.4**. By definition, the partitioning coefficicient Kd is the ratio between the concentration in the solid matrix versus the concentration in water. When degradation as well as partitioning may influence this ratio, for example in sludge and effluent, the resulting Kd is not a 'true Kd', but it is an 'apparent Kd'.This is indicated in **Table 3.4**. It is clear that the distinction between the true and the apparent Kd does not explain the wide range in the variation of Kd in sludge.

Activated sludge and soil

The sorption to activated sludge was estimated from isotherm experiments producing the Freundlich isotherm constants for the test substances. Liquid Scintillation Counting (LCS) analyses were used as non-specific analytical techniques. The study was carried out with 2.5 g Suspended Solids/l (19% OC) and 10, 50, 150 and 300 µg test substance/l. The test system was equilibrated for 16 hours. The total recovery was 81 to 91%. The sorption isotherm was linear. The sorption coefficient Kd was 11,586 l/kg. The sorption coefficient related to organic carbon was 63,658 l/kg (log K_{oc} = 4.80) (MacGillivray 1996).

K_{oc} was determined in activated sludge. Samples with different sludge concentrations were prepared and after extraction by nd-SPME and liquid/liquid extraction for free (dissolved) and total concentrations, respectively, the concentrations were determined by GC analysis. Log K_{oc} was 3.8 (±0.2) (Artola 2002).

Sludge-water partitioning coefficients for adsorption and desorption were studied by entering AHTN either to the water or to the sludge. Sludge was spiked with AHTN in 4L-glass jars and rolled at 10 rpm for 1 hour. The sludge was equilibrated at 5ºC in the darkness during 3 weeks and sterilised by 0.01 M HgCl. Log Kd (adsorption) = 2.94, whereas log Kd (desorption) = 3.16 (Zhang et al. 2003).

Kd was determined as the ratio between the concentration of AHTN in sludge and in effluent sampled at the same time. A total of 15 different STPs in Italy, Spain and Greece was sampled four times over a period of three months in 2004 (Blok et al. 2005). The relation between the 60 effluent and sludge concentrations can be expressed in a regression line with a slope of 45º resulting in log Kd = 3.95 ([11]). Individual values varied between 3.47 and 4.36. It was questioned whether Kd was actually independent of the concentration levels. If the partition coefficient depends in some way on the concentration of AHTN, this relation will deviate from the straight 45º line. The deviation from this line was significant ($p<0.0002$) with best fitting line

$$\log C_{effluent} = -0.665 + 0.601 \cdot \log C_{sludge} \text{ , so } C_{effluent} = -0.216 \cdot C_{sludge}^{0.601}.$$

[11] With Kd independent of the concentration levels, the sorption isotherm on a non-logaritmic scale is linear. Then the log-log transformed graph is a straight line with a slope parameter of 1, thus forming an angle of 45º with the X-axis (if y = a*x, logy = log x + C). Kd is calculated as the ratio of the back-transformed values in this line.

The deviation from a constant partition coefficient implied that at low (overall) AHTN concentration the effluent concentration was relatively high (small Kd) and at high overall AHTN concentration the effluent concentration was relatively small (high Kd). Apparently sludges have a higher adsorptive potential at higher concentrations (Van der Hoeven 2005). The TOC was measured at only 9 sampling points. The organic carbon fraction (TOC) varied between 0.24 and 0.29. In these 9 samples the relation between Kd and TOC was not significant (p=0.27). Transformation of log Kd for the average OC fraction of 0.26 gives log K_{oc} = 4.5 (Blok et al. 2005), see Figure 3.1.

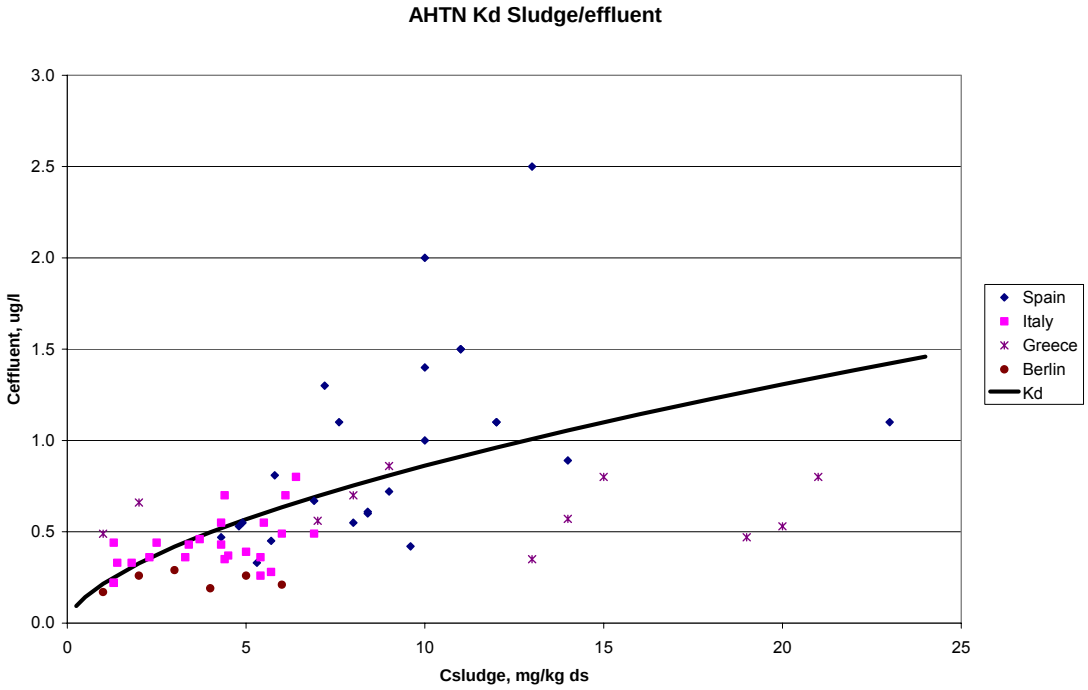


Figure 3.1 Kd (defined as Csludge/Ceffluent) based on the data of Blok et al. 2005. Kd is not linear with the concentration in sludge and effluent

Kd was determined based on concentrations measured in effluents and sludge reported by Müller et al. (2002) for 21 STPs sampled in Germany. From their results we derived the ratio of sludge and effluent sampled on the same day. Log Kd is 4.6, ranging from 4.1 to 5.0. The same authors also determined K_{oc} using the HPLC method: log K_{oc} = 3.41. Kd values in three soil types with different organic matter content ranged from 150 to 660 l/kg. Desorption tests showed that the adsorption is not reversible: up to only 30 to 35% of the material is desorbed. Log K_{oc} values ranged from 3.7 to 4.13 (Müller et al. 2002).

For the determination of Kd, batches of primary and secondary sludge were sampled from a large STP in Germany and spiked to a nominal concentration of 70 to 80 µg AHTN/l. The sludge was stirred under argon for a variable time and the concentrations in water and sludge were determined to give the Kd. Kd in primary sludge (35% TOC per total suspended solids) was 5300 ± 1900 l/kg, in secondary sludge (34% TOC) it was 2400 ± 960 l/kg (Ternes et al. 2004).

Suspended matter and sediment

Samples of suspended particulate matter (SPM) and surface water were taken from the Elbe river in Magdeburg, Germany from June 1996 to May 1997 weekly in the first 4 months and biweekly thereafter (Winkler et al., 1998). Field derived partition coefficients varied in time: from 2533 l/kg to 20773 l/kg, average was 9204 l/kg. Normalisation for K_{oc} reduced the variation to some extent: the mean field derived log K_{oc} for the Elbe was 4.9 (range 4.5 – 5.2). Apparently the sorption varies over time with the composition and the quantity of the suspended materials.

Partition coefficients may also be derived from concentrations in suspended matter and surface water samples taken in rivers and brooks in Hessen, Germany, in 1999 and 2000, and from data on effluents and sludge (HLUG 2001). The partition coefficients varied in time and place, from 480 to 5580 l/kg, the mean value was 2900 l/kg in surface water. Normalisation for organic material was not possible.

The partition coefficient between suspended matter or sediment and water was determined in water from small streams with a relatively high input of waste water in Baden-Württemberg, Germany. The total organic carbon content in the suspended matter ranged from 9.7 and 13.1% and in the sediment from 1.0 to 2.9% (LfU-BW 2001).

The partition coefficient was reported for field samples of suspended solids and streamwater in Pennsylvania. Log Kd was determined in three samples: 2.7, 4.6 and 5 (l/kg) (Standley et al. 2000).

Calculated

A Koc value for AHTN can be estimated from the K_{ow} value of 5.4 using the QSAR outlined in chapter 4 of the Technical Guidance document. The equation recommended for predominantly hydrophobics is log Koc = 0.81xlog Kow + 0.10. Using this equation a log Koc value of 4.47 can be estimated.

The theoretical partition coefficients derived from EUSES are compared to experimentally derived data in **Table 3.7**. It is concluded that the empirical values vary considerably but the predictions by EUSES are within that range. Therefore the calculations were carried out with the predictions made by EUSES on the basis of log K_{ow}.

Table 3.7 Partition coefficients ('true' partition coefficients, or else determined from concentration ratios not excluding other processes like, e.g., degradation)

Partition coefficient	Estimated by EUSES	Empirical
log K_{oc} in activated sludge	4.47 *)	'true' 4.80 (MacGillivray 1996) 'true' 3.8, 'apparent' 3.1 (Artola 2002) 'apparent' 4.5 (Blok 2005) 'true' 3.7 (based on Ternes et al. 2004)
activated sludge - water	Kd: 11,000	'true' Kd: 11,590 l/kg (MacGillivray 1996) 'apparent' Kd: 8700 l/kg (Blok 2005) 'true' Kd: 2400 ± 960 l/kg (Ternes et al 2004) 'apparent' Kd: 39,600 l/kg (based on data Müller et al. 2002) 'true' Kd in primary sludge: 5,300 l/kg (Ternes et al 2004) 'true' log Kd (ads): 2.94; log Kd (des): 3.16 (Zhang et al. 2003)

Partition coefficient	Estimated by EUSES	Empirical
soil - water	Kd: 596 l/kg $K_{soil\ water}$: 895 m^3/m^3	Kd: 150 – 660 l/kg (Müller et al. 2002)
log K_{oc} in soil		3.7 – 4.13 (Müller et al. 2002)
suspended matter -water	Kd: 2981 l/kg $K_{susp\ water}$; 746 m^3/m^3	'apparent' Kd: 9204 l/kg (with high variability, from data of Winkler 1998, 1999) 'apparent' Kd: 2900 l/kg (with high variability, HLUG 2001)'apparent' Kd: 3100 – 9550 l/kg (Fooken 2004) 'apparent' Kd: 600 – 4400 l/kg (LfU-BW 2001)
log K_{oc} in suspended matter		'apparent' 4.4 (Fooken 2004) 'apparent' 3.76 – 4.65 (LfU-BW 2001)
sediment – water	Kd: 1491 l/kg $K_{sed.water}$: 746 m^3/m^3	Kd: 144 l/kg (Fooken 2004) Kd: 33 – 270 l/kg (LfU-BW 2001)
log K_{oc} in sediment		4.0 (Fooken 2004) 3.04 – 4.23 (LfU-BW 2001)

*) based on log K_{ow}, used in the further calculations in EUSES

3.1.3.2.2 Precipitation

No information available.

3.1.3.2.3 Volatilisation

Using a vapour pressure of 0.0682 Pa and a water solubility of 1.25 mg/l a Henry's Law Constant of 14.1 Pa.m3/mol is calculated. The Henry's Law Constant was empirically determined by Artola (2002) at 37.1 $Pa.m^3/mol$. The latter was used in the PEC calculations.

Losses from the waterbody of the Zürichsee in Switzerland were estimated by an approximation using the fraction of AHTN solved in water (Fw), the transfer rate in water (Vw) and the depth of the water body: Kwater-air = (Fw · Vw) / DEPTH. In winter during circulation, Kwater-air values thus range from ≈ 1.5 to $3.0 \cdot 10^{-3}$ d^{-1} with the lower limit expected to be closer to real conditions. In summer, considering only the upper layer (epilimnion), the rate constant is estimated ≈ 1.4 to $2.9 \cdot 10^{-2}$ d^{-1} with the upper limit expected to be more realistic. In winter AHTN is eliminated primarily by outflowing water and due to losses to the atmosphere. Sorption and photolysis are minor processes. In summer, all processes in the epilimnion are faster by at least a factor of 10 than those for the whole lake in winter. For AHTN, direct photolysis is an additional order of magnitude more important and it represents the predominant elimination process in summer (Buerge et al. 2003).

Volatilisation rates were obtained by purging head-space of a spiked soil-sludge mixture (24:1) with nitrogen and trapping AHTN vapours with polyurethane foam. The initial volatility rate strongly related with the vapour pressure for a range of fragrance ingredients, including AHTN. Details are not available (Zhang et al. 2003).

3.1.3.3 Fate and behaviour in wastewater treatment plants

For a discussion of the behaviour of AHTN in a sewage treatment plant, various approaches are evaluated: the model SimpleTreat in EUSES, predictions based on the mass balance in a CAS (continuous activated sludge) test, and on the removal in actual sewage treatment plants.

3.1.3.3.1 Predictions in EUSES

According to the SimpleTreat model, AHTN entering an STP partitions between the sludge, water and air. The partitioning is predicted on the basis of K_{oc}, water solubility and vapour pressure. Then the fraction in the water phase is degraded according to the rate constant assigned to inherently degradable substances (TGD: 0.1 h^{-1} or 0 h^{-1}). In EUSES the volume of domestic waste water is set at 200l/d *per capita*, the amount of surplus solids from the STP is 79 g/d *per capita*, and the concentration of suspended solids in the effluent is 30 mg/l. With log K_{ow} = 5.4 and k_{biodeg} = 0, the fate of AHTN predicted by EUSES is presented in **Table 3.8**.

Table 3.8 Distribution of AHTN in an STP (%) predicted by EUSES and observed in a CAS test

	EUSES predictions % of input to STP	**CAS test, mass balance (Lee et al. 2001) (parent,% of dosed)**
Air	9.2	3.3
Water	20.6	12.5
Sludge	70.2	44.3
Degraded	0 (rate = 0 h^{-1})	42.5 (1st-order rate constant 0.029 – 0.057 h^{-1})

3.1.3.3.2 Removal percentages

The levels of fragrance materials were measured in the influent and effluent of two STPs in the USA. During three days, daily composite flow-proportional samples were taken from the influent, primary effluent and final effluent of an activated sludge plant and a trickling filter STP. The total concentration of fragrance materials in the influent showed a considerable diurnal variation around the mean (± 60%), whereas the effluent concentration was very stable. The measurements in influents and effluents showed that on average 89.5% of AHTN is removed after treatment in an activated sludge plant (Simonich et al. 2000).

A mass balance was made for AHTN in a simulation test, a Continuous Activated Sludge system where ^{14}C-labelled AHTN was dosed at 10 µg/l. Of the parent AHTN, 87.5% was removed. Next to transformation of 42.5% of the parent AHTN into metabolites, 44.3% of the AHTN was removed by sorption and 3.3% by volatilisation. One tenth of the metabolites was sorbed (Lee et al. 2001).

In Austria the behaviour of AHTN was studied in a small municipal STP in 2002 (Clara et al. 2004). The STP includes an activated sludge process, nitrate removal by intermittent aeration and simultaneous sludge stabilization and has a relatively high Solid Retention Time (SRT). The process was compared to a pilot Membrane Bioreactor plant operated in parallel with the same wastewater. Sludge separation was performed by a membrane module. The partitioning of polycyclic musks by effluent and sludge was estimated from the picture (no data are given). The removal ranged from 82 to 92%. The highest removal was achieved at the highest

temperature of 27 ºC. At 22 ºC a difference of SRT between 275 days and 11 days gave the same removal (Clara et al. 2004).

In a rural area in the most eastern part of Austria, composite samples were taken seven times over a 24 hour period at different stages of wastewater treatment and post treatment of an STP, as well as in the groundwater. These samples were taken at 2-month intervals during 2000 and 2001. The system has no primary settling but low loaded aeration with a long hydraulic retention time of 10 days and full nitrification and denitrification and iron precipitation of phosphorous. After settling the effluent is treated in a polishing lagoon, in a gravel filter and is lead into an infiltration pond to the groundwater. In the STP the reduction of the AHTN concentration was 85%: from 0.95 μg/l in the influent to 0.14 μg/l in the effluent. After the polishing lagoon the concentration is 5% and after the infiltration pond only 2% of the influent concentration. In the groundwater no further decrease of the concentration was observed (Kreuzinger et al. 2004).

The mean removal in a Swiss STP, 2001, serving 210 inhabitants (60 m^3 per day) in a sequencing batch reactor was 83% (Berset et al. 2004). The difference between the minimum and maximum concentrations in the influent and effluent as well as the sludge was within a factor of 2 (Berset et al. 2004).

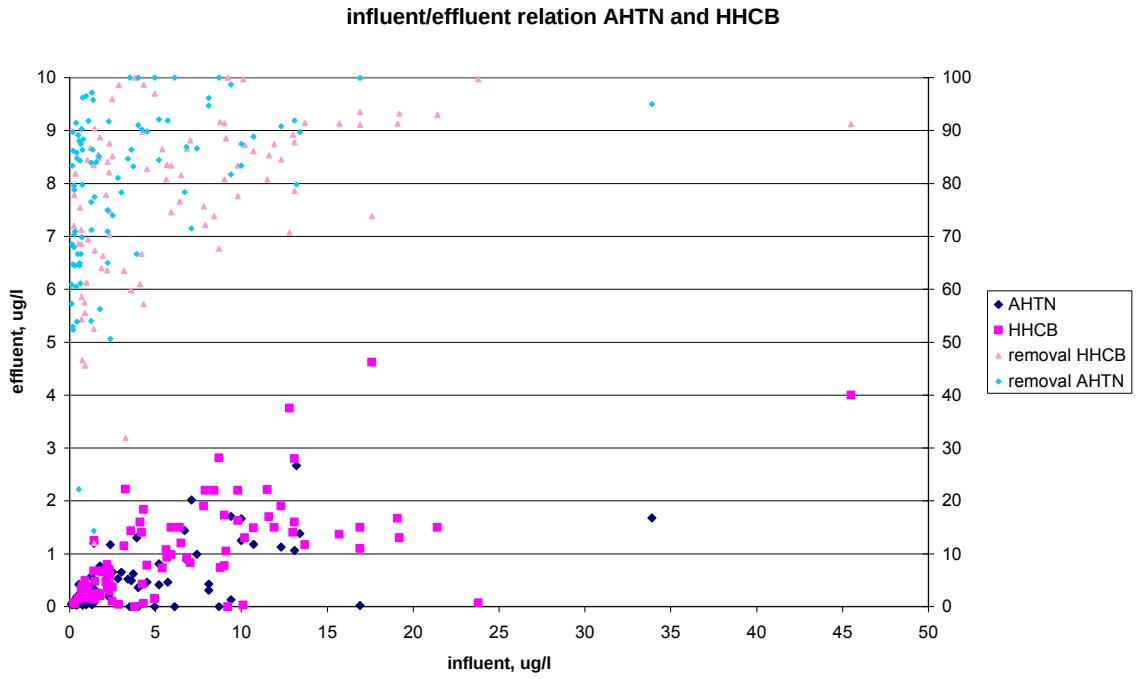
Samples were taken in 21 activated sludge plants in Germany in summer 2000 and in January 2001. The concentrations were measured in influents and effluents. The mean removal percentage was 79%, varying between 52 and 97% (Müller et al. 2002). The higher removal percentages were related to the higher influent concentrations (>1 μg/l), the lower removal percentages were observed for the lowest influent levels (<0.25 μg/l).

Samples were taken in 4 activated sludge plants in the Netherlands. The total influent and total effluent concentrations were used to estimate the removal (Artola 2002). The resulting removal was low as compared to the other studies, but this may be explained by the relatively low influent levels.

In UK samples were taken of influents and effluents in 6 different types of STP in 2001. The removal ranged from 79 to 90%. Here the influent concentrations were between 2.2 and 8.1 μg/l (Kanda et al. 2003).

The removal of AHTN was studied in the STP in Dortmund, Germany (Bester 2004). The plant received 184,000 m^3 water per day from 350,000 inhabitants and industries. The system includes primary settling, activated sludge treatment, secondary settling and anaerobic digestion. The Hydraulic Retention Time was 8 hours; the Solids Retention Time was 8-10 days in the aerator and 20 days in the digester. Time proportional composite samples were taken during 5 days in April 2002. The mean concentration in the influent was 0.58 μg/l, in the effluent it was 0.21 μg/l, a removal of 64%. The concentration in digested sludge was 1.5 mg/kg. An attempt was made for a mass balance over the STP but it varied between -23% and +57% (Bester 2004) and thus it seems that the basic data are not sufficiently accurate for this calculation (e.g., incomplete recovery in analytical procedure, time proportional sampling rather than flow-proportional, non-representative sampling scheme of the digested sludge and estimate of sludge production).

A



B

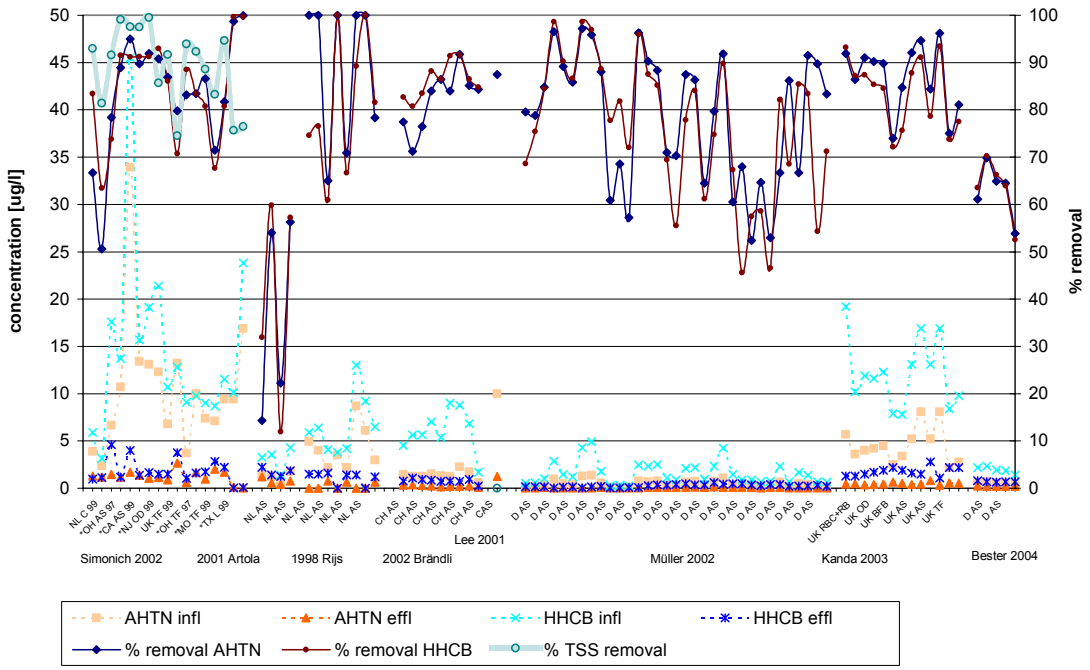


Figure 3.2 Removal of AHTN and HHCB in various STPs.

A. The picture shows the concentrations in 91 paired samples of influent and effluent (left Y-axis) and the percentage removal in 40 different STPs (right Y-axis)
B. The picture shows the concentrations in the same paired samples (left Y-axis, dotted lines) measured in the STPs indicated on the X-axis. The removal% is given on the right Y-axis.
NL, UK, D, CH etc: European countries and (with *) USA states;
C: Caroussel, AS: Activated sludge plant, OD: oxidation ditch, TF: trickling filter, RBC: Rotating Biological Contactor, BFB: Biological Filter Bed, L: Lagoon, CAS: Continuous Activated Sludge (test) System.
Median, 90th perc. and maximum effluent concentrations for AHTN (n=86): 0.2, 1.3 and 2.7 µg/l
Sources: Simonich et al. 2002, Artola et al. 2002, Rijs 1998, Brändli 2002, Berset et al. 2004, Eschke et al. 1994, Lee et al. 2001, Müller et al 2002, Kanda et al. 2003, Bester 2004.

A pilot plant for ozonisation received effluent from an STP to test the removal of AHTN . The effluent had pH 7.2, COD 30 mg/l, BOD 2.8 mg/l and 4.5 mg suspended solids/l. The water was treated by ozone, 5, 10 and 15 mg/l during 18 minutes. With the lowest ozone dose the concentration in the effluent was reduced from 0.10 μg/l to below the limit of quantitation (< 0.05 μg/l) (Ternes et al. 2003).

An analysis of the results of a number of these studies carried out in the USA, UK, The Netherlands, Germany and Switzerland (n=86 in 40 different STPs) generally showed removal percentages of 80 – 90%, see Figure 3.2. However, also higher and considerably lower removal percentages were observed, see the lines in the upper parts of graph B in Figure 3.2. In the study of Simonich et al. (2002) the removal percentage correlated well with the removal of Total Suspended Solids (TSS) in the system (graph B). The TSS removal is a characteristic of the STP which is determined by the treatment process, the plant design and the settling properties of the sludge and is not related to AHTN. The high correlation of the removal of AHTN and HHCB in the STPs supports this thesis.

It is concluded that a fixed removal percentage (according to a fixed first order rate constant dependant on the concentration in water) is not the best model for the removal of AHTN in an STP. The observation of the non-linearity of Kd (see Figure 3.1) supports this observation. The removal percentage is lower at lower influent concentrations and increases at higher levels, see also Figure 3.2. A key in the explanation of this phenomenon may be that biodegradation is not taken into account in the modelling.

3.1.3.3.3 Mass balance in STP

In a CAS test carried out at environmentally relevant influent concentrations (Lee et al. 2001, see section 3.1.3.1.2) AHTN was dosed in the influent at 10 μg/l. A complete mass balance was established including the distribution to the various compartments and the loss due to primary degradation. The results are included in **Table 3.8**. This mass balance showed 42.5% degradation.

The removal of AHTN was studied in the STP in Dortmund, Germany. An attempt was made for a mass balance over the STP but it varied between -23% and +57% (Bester 2004) and thus it seems that the basic data are not sufficiently accurate for this calculation (e.g., incomplete recovery in analytical procedure, time proportional sampling rather than flow-proportional, non-representative sampling scheme of the digested sludge and estimate of sludge production).

In two large STPs in Düsseldorf and Cologne in Germany, both serving > 10^6 inhabitant equivalents (i.e.), mass balances were made including sampling of influent, effluents and surplus sludge as well as digested sludge. The total elimination was more then 83% in both plants, whereas the percentage that was biodegraded was >42 % in one plant. For the second plant the data were not sufficiently detailed to establish the degraded fraction (Fahlenkamp et al. 2005).

A mass balance over a week was established for primary and biological water treatment and the sludge line in a conventional activated sludge sewage treatment plant in Switzerland serving a population of 23,000 inhabitants. The degradation of AHTN in the activated sludge tank was 43 $\pm$ 8%, whereas more than 50% was sorbed to sludge. In the anaerobic digester the

polycyclic musks decreased by 50% suggesting that they are degraded anaerobically (Kupper et al. 2006).

In conclusion, the available studies are not conclusive on the quantitation of the biodegradation of AHTN in an STP.

As no suitable data are available to predict biodegradation in the STP, the EUSES model is used for local industrial scenarios (**Table 3.8**) whereas the estimation of PEClocal for consumer use is based on the concentrations measured in effluent and sludge in recent monitoring programmes (see section 3.1.4.1.3).

3.1.3.4 Accumulation and metabolism

3.1.3.4.1 Accumulation and metabolism in fish

The bioconcentration in fish was studied in various experiments. A GLP-study was carried out according to OECD Guideline 305E. Bluegill sunfish (*Lepomis macrochirus*) were exposed in a flow-through system to two concentrations of radio-labelled AHTN (78.8% radiochemical purity, impurities due to radio-labelling) (Van Dijk 1996). A solubiliser (DMF, Tween 80) was used to prepare a solution (concentration 0.001% (w/v)). Identification of the parent compound in water and fish was performed by TLC/HPLC. Nominal exposure concentrations were 1 and 10 µg/l. The fish were exposed for 28 days; the elimination period was also 28 days. The bioconcentration factor (BCF) was derived from actual concentrations of parent compound in exposure water C_w and the plateau level in fish C_f (days 21 and 28): $BCF = C_f / C_w$. The elimination rate constant (k_2) was estimated from the elimination curve (first order kinetics). The uptake rate constant (k_1) was calculated by: $k_1 = BCF \cdot k_2$.

The results are presented in **Table 3.9**. The concentration of AHTN in the fish reached plateau levels after 3-7 days of exposure. An uptake rate constant (k_1) could not be directly calculated from the increase of concentrations in fish due to rapid attainment of the final plateau level. Elimination followed first order kinetics with a half-life of 0.8 - 2.1 days, allowing calculation of the rate constant for elimination (k_2). Based on concentrations of parent material, the BCF for the whole fish was 597.

Metabolites

In this bioconcentration test with AHTN at least two polar radioactive fractions were found in the water, accounting for 6 to 8% of the total radioactivity in water. In the organic extracts of fish edibles and non-edibles the main polar metabolite fraction accounted for about half the total radioactivity. The main metabolite fractions in water and in tissue proved to be identical based on TLC and with HPLC retention times. Smaller fractions of metabolites with intermediate polarity occurred both in tissue and in water but were not clearly identical (Van Dijk 1996).

For the same experimental results, a mass balance was made for the dynamic flow-through system containing water plus fish. For the mass balance of the dynamic system, the amounts of AHTN and metabolites in the fish and leaving the fish body (expressed in mg/kg bw and day) should be compared to the amounts measured in water that passes through the system with a certain flow rate (expressed in µg. $l^{-1}.d^{-1}$) These two compartments, fish and water, are related by the so-called “fish to water loading rate” (of 0.12 g. $l^{-1}.d^{-1}$) as mentioned in the

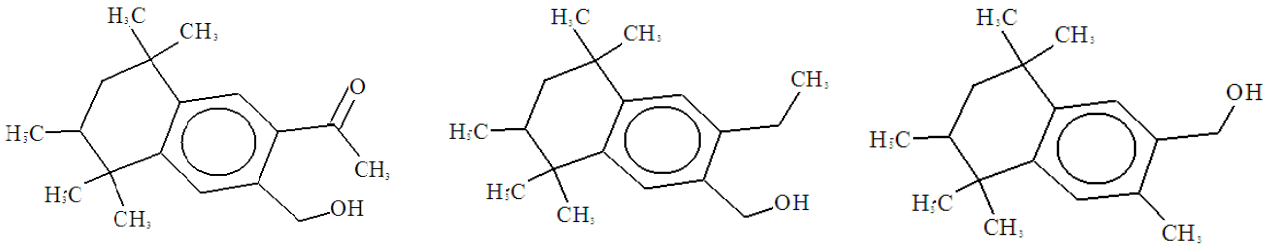
study reports. This expression is in fact the reciprocal of the water flow per unit of fish weight. During the exposure period of 28 days a steady state was reached between the uptake and the depuration to give a plateau level for radio-activity in the body. A mass balance can be made for day 28 where the depuration rate is compared to the level in the tissue at day 28. The depuration rate can be estimated from the curve between day 28 and 32. This loss of total radio-activity from fish is then compared to the measured radioactivity in metabolites in the water leaving the system, to see if the depuration can be explained quantitatively by the metabolite. After termination of the dosing of AHTN on day 28, the radioactivity in the fish tissue decreased with a rate of 0.3 mg/kg/d from a level of 1.3 mg/kg (23% per day) and with 3 mg/kg/d from a level of 13 mg/kg (23%) at the two dose levels. Multiplication of this 'loss' by the average fish-to-water loading rate (0.12 g/l/d) gives the nominal concentration of 'lost' radioactivity in water leaving the system: 0.036 µg/l in the low dose level and 0.48 µg/l in the high dose level. These concentrations were compared to the average measured concentrations of metabolites in the water: 0.059 for the low dose level and 0.8 µg/l for the high dose levels. Here the measured concentrations of polar metabolite in water exceeded the estimated loss of radioactivity from the tissue.

Water soluble metabolites became apparent in the water on day 3 of the exposure period. During the period between day 3 and day 28 the daily amount of water-soluble metabolite was 38 – 50% of the average level of radioactivity present in the fish tissue. Thus it can be concluded that during exposure and during depuration the parent compound is metabolised and the metabolite is excreted with a turnover rate of about 38 – 50% per day (Van de Plassche and Balk, 1997, Balk and Ford, 1999a).

Table 3.9 Bioconcentration of AHTN in bluegill sunfish (*Lepomis macrochirus*) in a flow-through system (Van Dijk 1996).

	AHTN		
Initial fish weight [g]	0.35		
Low dose [µg/l]	0.99 ± 0.12		
High dose [µg/l]	9.81 ± 0.85		
	edibles	non-edibles	whole fish
Plateau level low dose [mg/kg]	0.49	2.12	1.30
Depuration residue low [mg/kg]	0.029	0.0096	0.049
Plateau level high dose [mg/kg]	5.17	20.60	12.99
Depuration residue high [mg/kg]	0.431	1.265	1.118
Elimination rate constant k_2 [d^{-1}]	low: 0.337, r^2=0.99 ; high: 0.577, r^2=0.96		
Uptake rate constant k_1 [l/kg/d]	low: 442 high: 765		
Bioconcentration factor (whole fish, wet weight) [l/kg]	parent substance: 597 total radio-activity: 1320		
Partitioning of radio-activity in:			
- water	AHTN 91-93%; metabolites 2-8%		
- fish edibles	AHTN 41-49%; metabolites 33-48%		
- fish non-edibles	AHTN 31-42%; metabolites 44-60%		

In a recent study several transformation products of AHTN were identified in three fish samples caught in Norwegian harbours at a concentration range of around 10 ng/kg wwt (Valdersnes et al. 2006). The structures are presented in Figure 3.3.



Derivative 2: 3-acetyl-5,5,7,8,8-pentamethyl-5,6,7,8-tetrahydronaphtalene-2-carbaldehyde

Derivative 4: (3-ethyl-5,5,7,8,8-pentamethyl-5,6,7,8-tetrahydronaphtalene-2-yl)methanol

Derivative 6: 3-acetyl-5,6,7,8-tetrahydro-5,5,7,8,8-pentamethyl-2-naphtalenecarbaldehyde

Figure 3.3 Structures of some transformation products identified in Norwegian fish (Valdersnes et al. 2006)

An accumulation study was performed with zebrafish (*Brachydanio rerio*), aged 4-6 months with a length of 2.5-3 cm and a weight of 0.15-0.4 g. The exposure concentration of AHTN was 10.5 µg/L in a flow-through study according to former OECD guidline 305E. Tap water with a pH around 8 was used. Methanol (0.05%) was used as co-solvent. The concentrations in fish and water were analysed by GC-MS/MS. No information is given on the number of fish, the test volume, the number of replicates, the test temperature, and feeding regime. The exposure during the uptake phase was 14 days. The concentration in fish reached its plateau level after less than 5 days. The elimination period lasted 26 days with a depuration half-life of less than three days. The uptake rate (k_1) was 570 d^{-1} and the clearance rate was 1.2 d^{-1}. The bioconcentration factor was 600 L/kg (fresh weight) or 33700 based on the lipid content of the fish (Butte and Ewald 1999).

In another study with the same species, juvenile transgenic zebrafish of 4 to 5 weeks were exposed in 200 ml tap water at 26-27 ºC for 96 h to concentrations of 25.8 and 258 µg/l. Fish were fed once daily with live *Artemia salinas* and half of the medium was renewed daily. After 96 h the concentrations in water without fish were 25-40% below nominal but in the presence of fish the concentrations were 1.5 and 13 µg/l (circa 5% of nominal). This decrease in concentrations was observed after 24 h as well as after the next intervals. Also the internal concentrations observed just before the renewals were roughly the same during the 4-day exposure period: 15.8 and 143 mg/kg wwt in the low and high concentrations, respectively (Schreurs et al. 2004). The decrease in exposure concentrations can at least partly be explained by sorption by the fish and subsequent metabolism. Assuming an exponential concentration decrease between the renewal periods the average concentrations in the low and high exposure concentrations would be in the order of 5 and 60 µg/L. With the corresponding internal concentrations, this would imply BCF values of 2300-3200 L/kg.

The latter value is substantially higher than the BCF value from the BCF study with the same species performed according to OECD guideline 305E. The study by Schreurs et al. (2004) was not intended as a bioaccumulation study. According to the test guidelines for fish bioaccumulation testing (OECD TG 305, EU C.13), the exposure of the fish is solely through the aquatic phase where a constant concentration is maintained and care is taken to minimise

oral exposure by removing excessive food and faeces from the test containers within 30 minutes to an hour after feeding. In the test by Schreurs et al. (2004) it is not clear whether feeding with live brine shrimp took place before or after renewal. Consequently, part of the decrease in aqueous concentration might be caused by sorption of test substance to the brine shrimp. Since the medium was only partly renewed, also half of the *Artemia* that had not yet been consumed as well as faeces continued to be present. In this way, more of the test substance would sorb causing an increasing oral exposure next to exposure through the aquatic phase.

Further, the number of fish is relatively small. Only 5 or 6 fish per group were tested, whereas 50 fish per group and at least two groups are used according to the OECD305. The recommended weight of the fish is 0.2 to 0.4 g, which corresponds to zebrafish of several months old. In the study by Schreurs et al. the tested fish were only 4-5 weeks old. Although the weight of the fish is not given it is likely that the maximum loading of fish of 0.8 g/L is exceeded due to the small water volume of 200 mL. According to the OECD guideline the test concentration may not deviate more than 20% from the mean measured concentration in the uptake phase. It is evident that the decrease in concentrations caused the exposure concentration to fluctuate widely. Further, the highest concentration must be at most $1/100^{th}$ of the acute 96-h EC50, or the 96-h EC50 divided by a proper acute-to-chronic ratio. In the study by Schreurs, the highest concentration is on average somewhat higher than the NOEC for the same species reported in section 3.2, with inititial concentrations clearly exceeding this value.

Given the uncertainties in especially exposure concentration and contribution of additional oral exposure, bioconcentration factors derived from the study by Schreurs are prone to deviations. The bioconcentration study with the same species performed according to the OECD guideline showed similar results as the bioconcentration study with bluegill sunfish. For this reason, it is concluded that the bioconcentration factor of AHTN in different fish species is in the order of 600 L/kg.

As an indication of the bioconcentration under natural conditions, ratios were calculated for the concentration in fish and in surface water, see **Table 3.10**. The resulting field ratios seem to be lower than the ones determined in the laboratory. An explanation may be that wildlife fish tend to have a lower lipid content than laboratory specimen.

Fromme et al. (2001b) determined the bioconcentration factor in 165 eel samples in the Berlin area. The BCF(eel) ranged from 250 to 1791. Based on the same data set Heberer (2002) uses the median surface water concentration of 0.47 μg/l , median content (2.833 mg/kg lipids or 0.668 mg/kg wwt) to estimate the BCF_{lipids} (eel) = 6028 and BCF_{fw} (eel) = 1421.

Gatermann et al. (2002) published concentrations in fish measured in an effluent pond (1997). They suggest that bioconcentration factor C_f/C_w is species dependant and thus they postulate a species dependant metabolism. However, a closer look at their data reveals that this dependency is completely explained by the lipid content of the fish. A different metabolism may be postulated only for eel, which accumulates AHTN to lower concentrations than expected on the basis of its lipid content.

Table 3.10 Comparison of field derived ratios of C_{fish} and C_{water} to experimentally determined bioconcentration factors for fish

BCF	Chemical analysis	Source
Bluegill sunfish (28+28)d-BCFwwt = 597*	^{14}C, LC/HPLC	Balk and Ford 1999a
Zebrafish (14+26)d-BCFwwt = 600	GC/MS	Butte and Ewald 1999
Zebrafish (4d exp)-BCF_{wwt} = 600 [a]; 2300-3200 [b]	GC/MS	Schreurs et al 2004
Environmental samples: Eel BCFwwt = 200 to 650 non-eel BCFwwt = 50 to 145	GC/MS	Balk and Ford 1999a from data in Eschke et al. 1995, Rijs and Schäfer 1998, Rimkus 1997
Environmental samples: Eel BCFwwt = 1069 (range 250 – 1791)	GC/MS	Fromme et al 2001b
Eel BCF_{wwt} = 1421		Heberer 2002 from data of Fromme et al. 2001b
Environmental samples [c] : Rudd BCFwwt = 40 Tench BCFwwt = 280 Crucian carp BCFwwt = 670 Eel BCFwwt = 400 Zebra mussel BCFwwt = 570	GC/MS	Gatermann et al. 2002

[a] Based on initial concentrations, see text

[b] Based on estimated average concentrations

[c] Species differences related to fat content

* Used in EUSES calculations

For substances with log K_{ow} < 6 the BCF can be estimated using the following QSAR according to the TGD (EC 2003): log BCF(wet weight)=0.85 · log K_{ow} - 0.70. With log K_{ow} = 5.4, the estimated BCF is 7762 l/kg. This theoretical value exceeds the measured BCF value (597) by more than one order of magnitude. A more recently developed relation included in the SRC estimation programme BCFWin (SRC, 1999), taking also the molecular structure into account, predicts a lower bioconcentration factor: 415. This prediction seems highly accurate for AHTN.

In conclusion, for the environmental risk assessment the experimental BCF value of 597 l/kg, derived from the study of Van Dijk (1996) will be used.

3.1.3.4.2 Accumulation in benthic organisms

The bioconcentration of AHTN in two benthic organisms was described by Artola (2002) and Artola et al. (2003). Fourth instar midge larvae (*Chironomus riparius*) and the worm *Lumbriculus variegatus* were exposed in a 9 litre-flow-through system with a flow of circa 2 litre per hour at a loading of 600 mg midge larvae and 2600 mg of worm. The organisms were

not fed during the 12d-exposure period. Samples of water and organisms were extracted in cyclohexane and analysed by GC/MS. The lipid content of *L. variegatus* was determined gravimetrically. In parallel to the bioconcentration experiment, a similar experiment was run with the addition of 5 mg/l of the cytochrome P-450 inhibitor piperonyl butoxide (PBO).

The aqueous concentrations in the test with *C. riparius* were stable at 5.8 ± 0.6 µg/l. The concentrations in the organisms increased to a maximum level between day 1 and 4 and then the level decreased to a new steady state. With log BCF given as 1.7 and 2.05, BCF = 50 – 112. These values are lower than predicted based on a correlation for chlorobenzenes (Roghair et al, 1992: BCF 5470) by a factor of 100. With the addition of PBO, BCF was 7943, and Artola (2002) concludes that the low BCF values are likely caused by a relatively fast biotransformation of AHTN in *C. riparius*. The uptake curve showed that the enzyme activity responsible for the transformation of AHTN may be induced after a short lag time.

The concentration in water in the experiment with *L. variegatus* was 3.6 ± 1.6 µg/l. The uptake of AHTN in worms reached a plateau level after 3 days. After 8 days a new plateau seemed to be reached, although with an extremely large variance caused by some outliers. Including these outliers, log BCF was 3.84 which is at the same level as the predicted BCF based on K_{ow} and lipid content (McCarty et al. 1991), indicating that in this organism biotransformation does not take place.

3.1.3.4.3 Accumulation in earthworms

The bioconcentration in earthworms is assumed to be proportional to the soil pore water concentration. Bioconcentration can be described as a hydrophobic partitioning between the pore water and the phases inside the organism and can be modeled according to the following equation as described by Jager (1998).

$$BCFworm = (0.84 + 0.012 \cdot K_{ow}) / RHOearthworm$$

For RHOearthworm (bulk density of worm) by default a value of 1 (kgwwt l^{-1}) can be assumed.

This leads to a BCFworm of 3015 $l \cdot kg^{-1}$.

3.1.3.4.4 Accumulation in plants

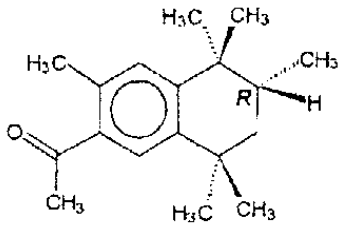
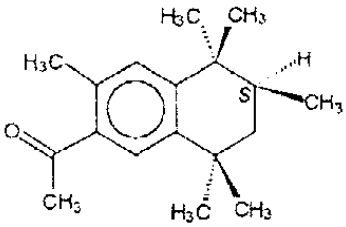
Transfer coefficients were determined in lettuce and carrots growing on sludge amended soil samples. Dried sludge was spiked with AHTN to a level of 300 mg/kg dwt and mixed with three different soils, sand, loam and a humic soil, to reach a soil concentration of 30 mg/kg soil dwt. The test design required such high concentrations in soil to allow precise measurements. After a week young salat seedlings were planted and carrots seeds were sown in the treated soil. The plants were harvested and analysed after 12 weeks. Parallel to the laboratory tests, similar tests were also carried out in the field. With the extremely high soil concentrations the growth of the plants was inhibited. The concentrations detected in salat leaves in the replicate tests were highly variable and seemed to be independent of the type of soil. For carrots the soil type did seem to influence the levels in the leaves. The soil-plant transfer factor (ratio Cplant/Csoil) was small for the salat leaves as well as for the carrot leaves (0.003 – 0.026). For carrot root, the transfer ratios were 0.082 and 0.37 in two different soils. The authors conclude that in spite of the high dosage no relevant accumulation in leaves

is observed and that even for the carrot root the ratio is still below the 'critical level of 1'. They suggest that the higher ratio in carrot roots is related to a direct contact between the musks in the soil and the oil deposits in the carrot roots that allows a direct partitioning. The low concentrations in the above ground parts of the carrot plant show that there is no transport within the plant (Müller et al. 2002). Thus it is concluded under normal conditions that transfer of AHTN from the soil to plants is not relevant.

3.1.3.5 Isomers of AHTN

Technical AHTN has a purity of ≥98%. The molecular structure of AHTN has one stereogenic center so it occurs in two enantiomers, the 3R and 3S isomer, see Figure 3.4. Technical AHTN has an enantiomeric ratio (ER) of 1. Enantiomers are not separated on capillary columns commonly used in environmental analysis. Separation of enantiomers is only possible with very special enantioselective (chiral) capillary columns. For the specific identification of the respective peaks in the chromatogram as the 3R and the 3S enantiomers, authentic reference samples or retention elution data are necessary.

Figure 3.4 Enantiomers of technical AHTN

AHTN Technical		
Two enantiomers		
	3R	*3S*

Concentrations in the environment (summarised in various tables in the next sections) are therefore reported for AHTN irrespective of its ER. These determinations were carried out according to standard analytical principles for complex mixture analysis using common capillary columns. After extraction, AHTN is identified and quantified by the combination of common capillary gas chromatography and mass spectrometry, GC-MS using SIM mode. Quantification and identification is based on the use of an AHTN Technical sample as a reference.

In the calculations, the appropriate dilutions and weights of the investigated sample and the weight and purity of the reference sample are taken into account.

Analysis of enantiomers

Seperation of enantiomers and determination of the ER is only possible with very special enantioselective (chiral) GC or HPLC columns. Identification of 3R and 3S was not conducted in the here presented studies in which the ER of AHTN in environmental samples was determined. The studies do confirm that technical AHTN has an ER of 1.

Franke et al. (1999) quantified the ER in 5 (shell) fish species and SPMD samples and found the largest deviations in tench (ER up to 2). The ER in the aquatic medium deviated only slightly from 1 as determined by SPMD (Franke et al. 1999, p. 800).

In another study tench also showed a moderate selective metabolisation potential for an enantiomer of AHTN. In other species the ratios were close to 1 and calculations support that no enantioselective metabolism had occurred (Gatermann et al. 2002b, p. 451-452).

Berset et al. (2003) report on the enantiomeric ratios of AHTN in sewage water, activated sludge and effluents of sewage treatment plants. They observed that the ratios do not significantly deviate from technical AHTN (Berset et al. 2003a, page 17). In Berset et al. (2004), however, it is stated that the ER in 2 sludge samples differ significantly from technical AHTN (1.17 and 0.91 versus 1.01), whereas the ER in sludge from two other systems did not deviate from 1.

Bester (2002) determined the enantiomeric ratios (ER) of AHTN in two activated sludge samples. He found no deviation of the ER in sludge from the standard.

Conclusion
Available studies indicate that the enantiomeric ratio in environmental samples is the same as in technical AHTN, used as reference. Selective transformation of one of the 2 enantiomers was observed in one fish species (an ER between 1.6 and 2.0). In 4 other fish species and in zebramussels minor to no enantioselective transformation was observed.

Toxicity and ecotoxicity studies have been carried out with technical AHTN. As the enantiomeric ratios in the environment are generally the same as in this material, no recalculation or correction is needed for the risk assessment. The values can be directly compared.

3.1.4 Aquatic compartment (incl. sediment)

3.1.4.1 Calculation of predicted environmental concentrations (PEC_{local})

For AHTN a large database is available with concentrations measured in the environmental compartments in a large number of countries within the EU-15+2 area. In addition, the fate and behaviour of the substance is also well known. However, the quantification of the material actually being discharged to the sewer is rather complicated. This implies that modelling starting from the volume used in compounding should be given less weight than the results from monitoring campaigns.

Modelling by EUSES serves different purposes. When no data are available, it helps to identify the compartments at risk. However, with the large database available, EUSES may be used as a tool to better understand and evaluate the environmental exposure of AHTN. For that purpose the predictions should be refined as well as possible. Therefore a refinement of the predicted concentrations was introduced using the 90th-percentiles of the available data.

3.1.4.1.1 Calculation of PEC_{local} for production

The influent concentration was estimated in section 3.1.2.1. The fraction of input to the STP to air is 0.09, to water 0.21 and to sludge 0.70 (see section 3.1.3.3). Further calculations were performed to the TGD using EUSES. For PECregional, see section 3.1.4.1.4.

Table 3.11 Calculation of PEC_{STP}, $PEClocal_{water}$ and $PEC_{sediment}$ for production for the year 2000

Site	Influent STP, µg/l	Effluent STP, µg/l	Csludge, µg/kg dwt	Dilution factor to surface water	$Clocal_{water}$ µg/l	PEC regional = xxx µg/l → $PEClocal_{water}$	$PEClocal_{sediment}$ (equil. part.) mg/kg wwt
Production	1) 0.111 2) 0.004	1) 0.02 2) 0.0009	1) 0.17 2) 0.006	1) 1 2) 10 (default)	1) 0.02 2) 0.0001	+ 0.002 → 0.021 + 0.002 → 0.002	1) 0.014 2) 0.001

3.1.4.1.2 Calculation of PEC_{local} for formulation

The influent concentration was estimated in section 3.1.2.2.1 and 3.1.2.2.2. The concentration in effluent of the local STP is estimated based on partitioning beween air, water and sludge from SimpleTreat (EUSES). Further calculations were performed according to the TGD.

Table 3.12 Calculation of PEC_{STP}, $PEClocal_{water}$ and $PEC_{sediment}$ for compounding and end product formulation for the year 2000

Site	Influent STP, µg/l	Effluent STP, µg/l	Csludge, mg/kg dwt	Dilution factor to surface water	$Clocal_{water}$ µg/l	PEC regional = xxx µg/l → $PEClocal_{water}$	$PEClocal_{sediment}$ (equil. part.) mg/kg wwt
Compounding Site 1	0.2	0.04	0.31	229	0.0002	+ 0.008 → 0.008	0.005
Compounding Site 2	0.3	0.06	0.46	1	0.057	+ 0.002 → 0.059	0.038
Compounding Site 3	1.8	0.39	2.85	1100 default 1000	0.0004	+ 0.002 → 0.002	0.0014
Compounding Site 4	36.1	7.8 (WWTP)	<56.8>	4481 default 1000	0.008	+ 0.002 → 0.009	0.006
Compounding Site 5	0.04	0.01	0.06	5	0.0019	+ 0.008 → 0.010	0.006
Compounding Site 6	-				0	+ 0.008 → 0.008	0.005
Compounding Site 7 (Large-medium generic)	19.2	4.13	30.2	default 10	0.41	+ 0.008 → 0.42	0.273
Compounding Site 8 (Small generic)	2.9	0.62	4.51	default 10	0.062	+ 0.008 → 0.069	0.045
Formulation Large company	4.7	1.01	7.37	default 10	0.10	+ 0.008 → 0.109	0.070
Formulation Generic scenario	5.7	1.2	9.0	default 10	0.12	+ 0.008 → 0.131	0.085

3.1.4.1.3 Calculation of PEC_{local} for private use

According to EUSES the predicted distribution of AHTN over air, sludge and effluent in the generic STP is 0.092, 0.702 and 0.206, respectively. The volume of domestic waste water is 200 l/d *per capita*, the amount of surplus solids from the STP is 79 g/d *per capita*, and the concentration of suspended solids in the effluent is 30 mg/l. The concentrations in influent, effluent and sludge are calculated with the standard settings in EUSES (TGD regional 10%). The results are given in **Table 3.13**. The results predicted by EUSES are compared to the median and 90th-percentiles of the concentrations measured in effluents and sludge in the northern and southern regions of the EU.

An extensive monitoring campaign was carried out in southern Europe to generate details on the concentrations in STPs in those regions. Samples were taken in three countries, Greece, Spain and Italy (15 STPs, 60 samples) in 2004. More details are given in sections 3.1.4.3.2 and 3.1.4.3.3. The results showed that the concentrations (overall 90th-percentiles) are below the EUSES predictions by a factor of 15, see **Table 3.13**. For the northern European countries, in particular Germany, a large database is available with concentrations measured recently in the STPs (84 samples in total). These concentrations are below the EUSES predictions by a factor of 30 at least. The ratio between the levels in the two regions is proportional to the ratio of the assumed daily consumer use (5 and 2.7 mg per inhabitant).

Table 3.13 Concentrations predicted by EUSES and recently measured concentrations

Scenario	Predicted Cinfluent µg/l	Predicted Ceffluent* µg/l	Predicted Csludge mg/kg dwt	Ceffluent measured * µg/l	Csludge measured mg/kg dwt
TGD regional (10%)	98.1	20.2	174	southern Europe median: 0.55 90-perc.: 1.3, ratio**: 15.5 northern Europe median: 0.10 90-perc.: 0.54, ratio**: 37.4	southern Europe median: 5.45 90-perc.: 11.0, ratio**: 15.8 northern Europe median: 2.7 90-perc.: 5.9, ratio**: 29.5

*) total effluent concentration (including the amount adsorbed to suspended solids)
**) the ratio is calculated ast Cpredicted over Cmeasured

The large deviation of the observations from the predictions may be explained by a number of factors as illustrated in the diagram in Figure 3.5. As already indicated in section 2.2.2.1, part of the volume used in compounding is exported as fragrance oil (estimated at 15 to 30% of the volume used in compounding) or in fragranced consumer products (10-20% of the remaining 70 to 85% that was not exported). Import of these materials is considered to be of minor importance. AHTN is used for detergents and cosmetics. Part of the products will be in applications of products that are not discharged with water to the sewer (estimated at 10 to 30% of all material in consumer products). During use a fraction of the AHTN in detergents and cleaning agents will stick to fibres and surfaces and will eventually evaporate. The remaining fraction, an estimated 40% of the initial 100% used in compounding, will be discharged to the sewer. In the STP the material will biodegrade and partition to air, sludge and effluent. The fraction finally going to effluent is estimated at 5% of the initial 100% used in compounding (40% to STP * 60% not degraded * 20% to effluent), and similarly the fraction to sludge is 17% (40% to STP * 60% not degraded * 70% to sludge). The figures in

the diagram are based on expert judgement; they are rounded to serve as an illustration of possible pathways other than to the sewer. They are not used for the further calculations.

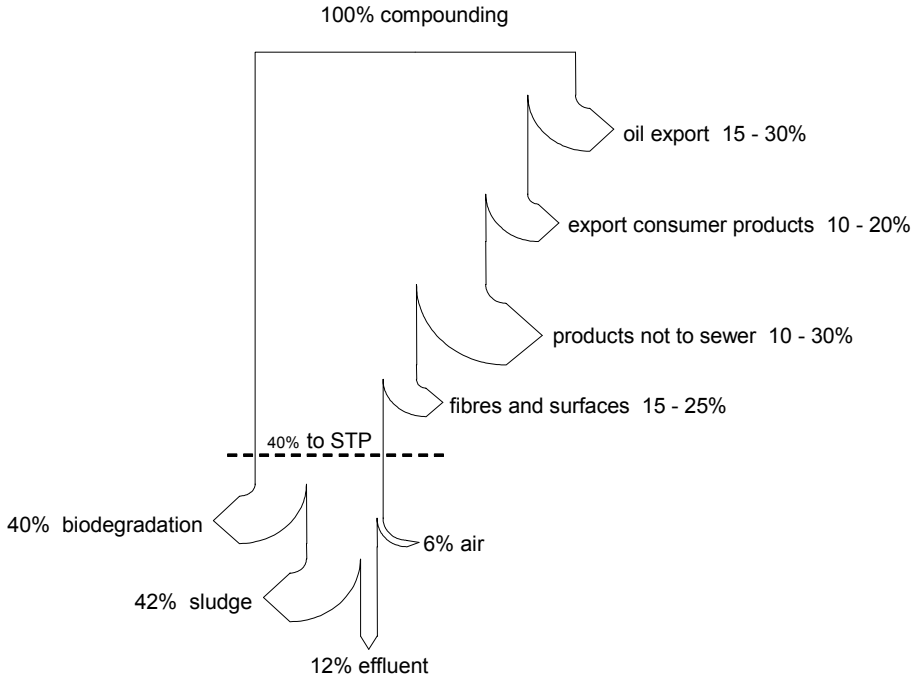


Figure 3.5 Pathways to the environmental compartments, tentative quantification of losses for AHTN. The percentages quantifying the 'arrows out' refer to the amount present at that level, see further explanation in the text.

In contrast, with the approach in EUSES the total use volume is released to the sewer (release factor = 1). Next the use volume is multiplied by a factor of 4 to account for both temporal and spatial variability in use within a region. In the model the rate of biodegradation is zero and the fractions to sludge and effluent are 70% and 21% of the initial volume. The combination of these deviations: (1) a factor of 4 for the variability, and (2) the amounts in effluents (21% vs 5%), in sludge (70% vs 17%) and biodegraded (0% vs 40%), explains the difference between the predictions by the TGD regional (10%) scenario and the observed concentrations in the southern EU-15 countries. This theory is supported by the observation of Buerge et al. 2003 who stated that the effluent load to the environment per capita is 0.32 ± 0.1 mg p.p.p.d., corresponding to 10% of the daily use cited as 3.2 mg p.p.p.d (in Switzerland).

Modelling by EUSES serves different purposes. When no data are available, it helps to identify the compartments at risk. However, for AHTN a large database is available and then EUSES may be used as a tool to better understand and evaluate the environmental exposure of AHTN. For that purpose the predictions should be refined as well as possible. Therefore a refinement of the predicted concentrations was introduced using the 90^{th}-percentiles of the available data for effluent and sludge. This refinement was used for the estimation of the concentrations in the compartments following the environmental pathways of the effluent and sludge, see **Table 3.14**.

Table 3.14 Local concentrations predicted by EUSES and based on measurements (90th-percentiles) in effluent and sludge.

Scenario	Influent STP, µg/l	Effluent STP, µg/l	Csludge, mg/kg dwt	$Clocal_{water}$ µg/l	PECregional [1)]	$PEClocal_{water}$ µg/l	$PEClocal_{sediment}$ mg/kg wwt
TGD regional (10%)	98.1	20.2	174	1.93	0.0844	2.02	1.31
southern EU-15 measurements		90th-percentile: 1.3	90th-percentile: 11.0				
ratio TGD regional (10%) / southern EU-15		*ratio* 15.5	*ratio* 15.8				
southern EU-15 predictions	6.38	EUSES: 1.31	EUSES: 11.3	0.124 *	0.00784	0.132 *	0.086 *
northern EU-15 measurements		90th-percentile: 0.54	90th-percentile: 5.9				
ratio TGD regional (10%) / northern EU-15		*ratio* 37.4	*ratio* 29.5				
northern EU-15 predictions	2.94	EUSES: 0.606	EUSES: 5.23	0.0517 *	0.00173	0.053 *	0.0347 *

1) PECregional, see explanation in 3.1.4.1.4

* Prediction based on concentrations measured in effluent and sludge (90th-percentiles)

3.1.4.1.4 Regional concentrations

For calculating the PECs at the regional scale of 200 · 200 km^2, only the emissions due to private use are taken into account. At such scale emissions from compounding sites are negligible as compared to those from private use. According to the 10% rule, the regional emission is 10% of the EU(-15+2) volume or 35.8 ton. Assuming a split up of 20% discharge directly to surface water and 80% to a STP (EU-TGD, EC 2003), EUSES calculates a PECregional of 0.0882 µg/l for surface water (total) and 0.0999 mg/kg wwt for sediment.

However, as shown in **Table 3.13**, the initial prediction of $Csludge_{local}$ and $Ceffluent_{local}$ were highly conservative for a number of reasons and therefore, for the further calculations they are replaced by measured values. This implies that also the estimates of the regional concentrations will need to be reduced proportionally. In EUSES this can be achieved by adapting the fraction of the tonnage released to wastewater which is originally set at 1 [12]. The implication of the diagram in Figure 3.5 is that only a relatively small fraction will be released to wastewater whereas at the same time an equal fraction will be released to air (the fractions 'cosmetics not to sewer' and 'fibres and substrates'). The effluent and sludge concentrations in southern European countries are predicted well when the release factor to water and air is set at 0.065 (=1/15; this value is derived from the ratios in **Table 3.14** 1/15.5 and 1/15.8), whereas an acceptable prediction for northern European countries is achieved with a release factor of 0.03 (=1/33,see **Table 3.14**: ratios 1/37.4 and 1/29.5). In this way PECregional is set

[12] Under the entry in EUSES: Release estimation/Intermediate Results/Use pattern1/Private Use/Release Fractions

for these scenarios. The calculations of $PEClocal_{water}$, $PEC_{sediment}$, PEC_{soil}, etc. are carried out using the 90th-percentile values of the actually measured effluent and sludge concentrations.

In the generic approach 80% of the population is connected to a sewage treatment plant. When different scenarios are used for northern and southern Europe, the connection rates should be differentiated as well. According to EUROSTAT data, the current connection rate in Germany (origin of effluent and sludge data, representative of Northern EU-15+2 country) is > 95%, whereas according to experts in the three southern EU countries Greece, Spain and Italy, the lowest connection rate is 60%. These connection rates were used in the calculation of PECregional. The results of the calculations are shown in **Table 3.15**.

Table 3.15 Regional concentrations for the various scenarios

Consumption, mg /d *per capita*	**PECregional $_{water}$, µg/l (dissolved)**	**PECregional $_{sediment}$, mg/kg wwt**
2000 – TGD regional (10%)	0.0844	0.0999
southern EU-15	0.00785	0.00929
northern EU-15	0.00173	0.00205

3.1.4.2 Measured levels

The presence of polycyclic musks in the environment has been investigated in several European countries. They were detected in sewage treatment plants, surface waters, sediment and in fish and shell fish. The results are summarised in **Table 3.16**, **Table 3.17** and **Table 3.18**. The results are often summarised as a minimum and a maximum, with occasionally a mean, median or 90th-percentile value. For the risk assessment, the 90th-percentile is the preferred value, whereas for an understanding of processes and trends, the median value is more relevant.

Most used extraction techniques are by solid phase micro-extraction of the aqueous phase and solvent extraction of the solid phase. Identification and quantification is mainly by GC/MS. Total concentrations are presented unless explicitly stated that free (dissolved) concentrations are given.

3.1.4.2.1 Concentrations in sewage treatment plants

Total influent and effluent concentrations were determined for three STPs of the German Ruhrverband during one week. Effluent samples were 24-hr. time-proportional samples. The median concentration in the effluent was 1.8 µg/l (Eschke et al. 1994, 1995).

Flow-proportional influent and effluent samples and samples of primary, activated and digested sludge were taken from three STPs in The Netherlands in 1997-1998. The median concentration of AHTN in raw influents was 4 µg/l, whereas the concentration in effluent was below the detection level (Rijs and Schäfer 1998).

"Free dissolved concentrations" were estimated in effluents of four STPs in The Netherlands (1995-96) from amounts accumulated on Empore disks. The mean of the concentrations, which according to the author, are accurate within a factor of 2, was 0.28 µg/l (Verbruggen et al. 1999). In 1997 concentrations determined with the same technique were obtained from

Leonards et al. (2000) showing influent concentrations of 0.3 and 0.4 µg/l and effluent concentrations of 0.3 and 0.6 µg/l.

Time proportional daily composite samples were collected over a 3-day period of influent, primary effluent and final effluent of an activated sludge and a trickling filter wastewater treatment plant in Ohio (USA) in 1997. The mean concentration in the influent was 10.7 and 10.0 µg/l, and 1.2 and 1.7 µg/l in the effluent of the activated sludge plant and in the trickling filter system, respectively. Whereas influent concentrations showed considerable diurnal fluctuation (a factor of 4), the effluent concentration remained relatively constant throughout the day (Simonich et al. 2000). In a more extensive project on fragrance ingredients the concentrations of AHTN were measured in the influent, primary effluent and effluent of 17 STPs in the USA, the –UK and The Netherlands. The treatment plants were representative of a diversity of systems: with and without primary gravitational settling, activated sludge, carousel, oxidation ditch, trickling filter, rotating biological contactor and lagoon types were included. Industrial contribution to the STPs was in all cases less than 20%. Apart from the two STPs in Ohio (1997) mentioned above, samples were taken from 1998 to 2000. Hourly samples were collected over several days with an autosampler. Flow proportional daily composite samples were produced (Simonich et al. 2002). Concentrations in influent in 2 STPs in the Netherlands ranged from 3 to 6 µg/l, with effluents at 1 µg/l. In the 3 STPs in the UK the influents were 3.7 to 12.8 µg/l with effluents from 1 to 4.6 µg/l. In the 12 STPs in the USA the influents ranged from 7 to 34 µg/l with effluents between 1.2 and 4 µg/l. There was a clear relation between removal of TSS (total suspended solids) and concentration in the effluent (see also Figure 3.5). The purification process in lagoons deviates from the other treatment plants. Concentrations in the effluent of lagoons were clearly very low: < 0.1 µg/l (Data Simonich pers. comm. 2002).

In Switzerland (1995) effluent samples (grab or mixed) were taken from 17 STPs in March 1998. The median concentration was 1.4 µg/l (BUWAL, 1998).

In Switzerland in the Kanton of Basel effluent samples were taken in 6 STPs in March 1997. The median concentration as determined by SPME was 2 µg/l (Noser et al. 2000).

In a screening programme of the Berlin surface waters in 1996, samples were taken from three STP discharge points. The mean concentration of AHTN (SPME) was 4.5 µg/l (Heberer et al. 1999). In a more extensive study of the same area, 30 proportional daily composite samples were collected from five municipal treatment facilities in 1996. These samples were prepared by simultaneous steam distillation/solvent extraction. The median concentration was 2.2 µg/l. The effluent concentrations in one STP throughout the year varied by a factor of 2, with the highest value (4.4 µg/l) observed in winter (Fromme et al. 2000, 2001a,b). Fromme et al. (2000) indicate that their measurements probably show a worst case scenario, because of the high degree of urbanisation, little producing industry and for this sampling series, they were taken in a dry weather period. Moreover, the water system of Berlin has a very high fraction of effluent input (> 90%) and low natural surface water flow rates. (In addition, he states that the total drinking water use in Germany is only 127 liter per i.e.).

In a research programme in the state Hessen, Germany, in 9 STPs one effluent sample was taken in 1999 and one in 2000 (HLUG 2001). The median concentration for the two years was 0.4 µg/l. The median concentration in 2000 was lower than in 1999.

In Germany, the concentrations were measured in the effluent of 3 STPs along tributaries to the River Elbe in the region of Leipzig in 1999. The concentrations ranged from 0.53 to 0.61

µg/l (ARGE 2000). In Sachsen-Anhalt the effluent concentrations of 5 STPs ranged from 1.2 to 2.6 µg/l (cited in ARGE 2000).

The concentrations were measured in the STP in Dortmund, Germany (Bester 2004). The plant received 184,000 m^3 water per day from 350,000 inhabitants and industries. Time proportional composite samples were taken during 5 days in April 2002. The mean concentration in the influent was 0.58 µg/l, in the effluent it was 0.21 µg/l, a removal of 64%. Large STPs in Köln and Düsseldorf (1,000,000 i.e.) were sampled in 2003 resulting in similar levels (Fahlenkamp et al. 2004).

As part of an investigation in the Lippe River, tributary to the River Rhine, in North-Rhine/Westphalia, Germany, the concentrations of influent and effluent of an STP in a city of 182,000 inhabitants were measured. They were at the level of 0.3 µg/l (Dsikowitzky et al. 2002a).

In North-Rhine/Westphalia, Germany an overall study was completed on the presence of organic pollutants in sewage sludge. A one-year analysis in 156 municipal STPs was sampled (representing 80% of the state's annual sewage sludge volume); in 17 STPs the analyses included the polycyclic musks. The median concentration of AHTN was 2.3 mg/kg dwt, the 90^{th}-percentile was 4.9 mg/kg dwt. A correlation between the AHTN levels in sludge and characteristics of the region served by the STP as well as the treatment method was low. However, it was clear that aerobic sludge stabilisation resulted in lower levels on sludge than when the sludge was not treated in this way (Friedrich et al. 2004). When sludge is stabilised, the dry solids content is decreasing and as a result the concentration of a persistent substance on the dry solids would increase. Thus if the AHTN level on the sludge decreased (to 40%), AHTN was degraded during the aerobic stabilisation as well.

Samples were taken from 21 STPs in Germany in summer 2000 and in January 2001. The concentrations were measured in influents, effluents and activated and digested sludge. The median concentration in the influent was 0.40 µg/l (range from 0.11 to 1.37 µg/l), whereas the median concentration in the effluents was 0.06 µg/l (0.03 to 0.15 µg/l). The influent concentrations seem to be higher in summer than in winter (factor 1.6), whereas the effluent concentrations were lower in summer (factor 0.7). The variation, expressed as the ratio of the 90^{th}-percentile/median in the influent concentrations was 2.4. For the effluents this ratio was smaller: 1.5 (Müller et al. 2002).

Samples were taken in all compartments of four STPs (activated sludge plants) in The Netherlands in 2001 and both free (dissolved) and total concentrations were determined. The free concentration was constant in all matrices (influent, primary settler, primary sludge, aeration tank, effluent, waste sludge): 0.3 – 0.5 µg/l, whereas the total concentrations ranged between 1 and 90 µg/l (Artola 2002). The median (total) influent concentration was 1.3 µg/l, the median (total) effluent concentration was 0.7 µg/l. In this study the diurnal variation found by two-hourly sampling was circa 19% for the total influent concentration (sum of AHTN and HHCB) but it was not even 10% for the free concentrations in the influent (Artola 2002).

In Switzerland a larger monitoring network was initiated where the various compartments in a wastewater treatment plant were sampled. Flow proportional samples were taken daily during a week in April 2002. After extraction with hexane and clean-up by GPC, the quantification of the substances was by GC-MS. Mean concentrations in influent ranged from 0.6 to 1.5 µg/l, in effluent from 0.1 to 0.3 µg/l (Brändli 2002). In the same programme in Switzerland daily fluctuations in the wastewater concentrations were measured in a small STP (210 i.e.) in a week in April 2001. The mean influent concentration was 1.5 µg/l and ranged from 1.2 to

2.3 μg/l, whereas the effluent concentration ranged from 0.2 to 0.4 μg/l, mean 0.3 ug/l. Overall the variation within a week is ± 50% (Kupper et al. 2003b).

In Switzerland 24h-flow-proportional samples were obtained from 5 STPs in the region of Zürich in 2001. The samples were taken of the effluent of activated sludge plants after nitrification, phosphate precipitation and sand filtration. The capacity of the plants ranged from 5000 to 36,000 i.e. The concentrations ranged from 0.31 to 0.76 μg/l (Buerge et al. 2003).

Samples were taken in a rural area in Austria at 2-month intervals between 2000 and 2001. The composite samples were taken seven times over a 24 hour period at different stages of wastewater treatment and post treatment of a STP, as well as in the groundwater. The system has no primary settling but low loaded aeration with a long hydraulic retention time of 10 days and full nitrification and denitrification and iron precipitation of phosphorous. The mean concentration was 0.95 μg/l in the influent and 0.14 μg/l in the effluent (Kreuzinger et al. 2004).

In Galicia, NW Spain, the behaviour of AHTN was studied in a STP serving 100,000 inhabitants. The plant consists of three sections including pre-treament with coarse screens and grit and fat removal, primary treatment by sedimentation and secondary treatment in an activated sludge process. In 2001 - 2002 three campaigns were carried out. Samples were taken every hour during 24 hours and mixed. The samples were extracted by SPE and analysed by GC/MS as well as by a complementary technique where an SPME fibre was exposed to the headspace over the sample and analysed by GC/MS. The concentration in the raw influent was in the range of 0.9 to 1.7 μg/l, after the filters and primary settling it was 0.6 to 0.97 μg/l and after secondary settling is was 0.15 to 0.20 μg/l. The average removal efficiency was 88% (78 – 91%) (Carballa et al. 2004). In an earlier report on this SPME fiber technique the AHTN concentration in effluent was 0.12 μg/l (García-Jares et al. 2002).

In Girona, Spain, the concentration in the effluent of a STP serving 32,000 to 60,000 enhabitants (winter and summer) was measured between 1999 and 2001. The concentration was 0.68 μg/l. Further treatment (infiltration/percolation) reduced the concentration to 0.09 μg/l. The samples were extracted on a closed loop stripping on an activated carbon filter that was extracte by CS_2 followed by GC/MS (Romero et al. 2003).

During the development of a new analytical technique using closed-loop stripping analysis followed by GC/MS, samples of influent and effluents in STPs in Germany, Austria, Belgium and Spain and surface waters in Germany, Spain and France were analysed. Samples were taken in May 2003. The concentrations in Germany ranged from 0.1 to 0.2 μg/l in the 4 influents, and from 0.03 to 0.06 in the effluents (removal 50 – 70%). The effluent concentration in the sample of Austria was of the same level. The influent samples from Belgium and Spain (each one sample) were slightly higher (factor of 2-3), whereas the effluents were between 0.1 and 0.3 μg/l, showing a lower removal. AHTN was not detected in drinking water (Mitjans and Ventura 2004).

A large campaign was carried out in Italy, Spain, Greece and Berlin, Germany to sample the effluents and activated sludge in 2004. In both Italy and Spain, samples were taken in 6 STPs, whereas in Greece three STPs were sampled. A total of 4 samples were collected with intervals of 2 to 4 weeks in Italy, Spain and Greece. The selected plants treated mainly municipal sewage for more then 10,000 inhabitants. The process and design characteristics were collected from the operators. Sampling was conducted under normal dry weather conditions between May and October 2005. The activated sludge was centrifuged and freeze

dried. The effluent was left to settle for 0.5 hour and the supernatant was extracted by SPE (Speed Disk). After elution with solvent and concentration of volume, the extract were analysed. Sludge samples (freezed dried) were solvent extracted with dichloromethane (DCM) by Accelerated Solvent Extraction equipment (ASE) and cleaned-up over a silicagel chromatographic column. The analyses were performed by GC/MS in SIM mode. Control samples, standard additions and analytical controls were included to check the loss of substances and the recovery of the system (Blok et al. 2005). A detailed statistical analysis was made of the results (Van der Hoeven 2005). The median concentration in effluent in Italy was 0.41 µg/l, in Spain 0.85 µg/l, and in Greece 0.57 µg/l. As an indication of the variation in these countries and in time, the ratio between the median and the 90th-percentile concentration was established. The ratio was 2.4. The median concentrations in sludge were 4.4 mg/kg dwt in Italy, 8.7 mg/kg dwt in Spain and 4.3 mg/kg dwt in Greece. For sludge the ratio between median and 90th-percentile was 2.0. The overall 90th-percentile for these southern European countries was 1.3 µg/l in the effluent and 11 mg/kg dwt in sludge (Blok et al. 2005).

In the same campaign the concentrations were measured in Berlin, Germany in 3 STPs. The samples were collected twice with an interval of 6 months. Two of the three STPs had been included in the study carried out by Heberer in 2000. In 2004 the mean concentration in sludge was 2.9 mg/kg dwt, which was at the same level as in the year 2000. However, the mean concentration in effluent was also low, 0.23 µg/l. In 1996/1997 these concentrations had been relatively high and no reasonable explanation was found at that time (Heberer et al. 1999, Fromme et al. 2001a). The observations in 2004 are in line with other recent observations in Germany (see **Table 3.16**).

In the UK samples were taken of influent and effluent in 6 STPs (Kanda et al. 2003). The STPs included a variety of processes and systems: a rotating biological contactor and reed beds, an oxidation ditch, biological filter beds, activated sludge processes, and trickling filters. A system with a submerged aerated filter was not taken into consideration as was not fully matured at the time samples were taken. The influent concentrations ranged from 2.2 and 8.1 µg/l and the effluent concentratons ranged from 0.31 to 0.81 µg/l (Kanda et al. 2003). It is remarkable that these overall minimum and maximum concentrations in the effluent were observed on two consecutive days in one of the 6 STPs.

In The Netherlands flow proportional samples were taken of the effluent of 5 STPs along the River Meuse and its tributaries. The samples were collected in three periods in 2002/2003 with intervals of two months and screened for a large number of substances. AHTN was detected in 13 of the 15 collected samples. The concentrations ranged from 0.01 to 3.1 µg/l (Berbee et al. 2004). The concentrations were extremely variable in time (a factor of 400) as well as in place and the AHTN/HHCB ratios were not always consistent (in contrast to the majority of results from other studies). The analytical procedure followed enables a correct identification of the substance. However, the authors (Berbee et al. 2004) acknowledge that the quantification was not reliable as it is not known whether the response in a GC/MS for the substance is the same as for the standard used (1-chlorodecane). Also the used calculation programmes gave considerably different results due to differences in background levels. Therefore the results are only indicative for the presence of AHTN.

In 5 STPs in Sweden composite effluent samples were taken by means of SPE extraction in June-December 1999. All STPs received mixed household/industry effluents and serve populations ranging from 2600 to 80,000 inhabitants. The wastewater treatment includes an activated sludge process supplemented by varying additions to facilitate sludge settling. The concentrations ranged from 0.042 to 0.104 µg/l. The same authors report on samples taken in 3 STPs in Nova Scotia and New Brunswick, Canada in January 2002. The STPs treat mixed

houshold/industry effluents of cities with 20,000 to 350,000 inhabitants. The concentrations ranged from 0.11 to 0.52 µg/l (Ricking et al. 2003b).

An on site sampling technique was developed in south-western USA where 60 l water samples were extracted on solid phase. Samples were from two dedicated effluent receiving streams (only effluent and run-off). Concentrations varied in time, between 0.027 and 0.92 µg/l (Osemwengie and Steinberg, 2001). In 2001 concentrations were measured in the confluence of effluent streams from 3 Nevada STPs, twice per month. The concentrations ranged from 0.02 to 0.05 µg/l (Osemwengie and Gersenberger 2004).

Table 3.16 Concentrations of AHTN in influents, effluents and surface water (µg/l). Mean, median, maximum and 90th-percentile of the data

Location	n	Influent	n	Effluent	n	Surface water	Reference
Germany, Ruhr <1994	7	settled infl. [mean 2.2][1]	21	median 1.8 90-perc. 3.0	30	median 0.2 90-perc. 0.3	Eschke '94, '95
Germany, North Sea 1990, 1995 Germany, Elbe 1995					12 1	median 0.00019 90-perc. 0.0009 0.07	Bester '98 Bester '98
Germany, Elbe 1995					2	0.09	Lagois '96
Germany, Elbe 1996-97					25	median 0.05 90-perc. 0.07	Winkler '98
Germany, Elbe 1998-1999 Elbe Czech border to North Sea 1996-1997 Tributaries region Leipzig 1999 Germany, Sachsen-Anhalt, 5 STPs <2000			3 5	mean 0.6 max. 0.6 1.2 – 2.6	 41 11 	 median 0.09 0.03 – 0.25 median 0.25 0.14 – 0.33 	ARGE 2000 cited in ARGE 2000
Germany, Berlin 1996			3	mean 4.5 max. 5.8	27	median 0.5 90-perc. 2.4[2]	Heberer '99
Germany, Berlin 1996 (5 STPs)			30	median 2.2 90-perc. 3.4 max. 4.4	34 40 28 	*low effluent input* median 0.02 90-perc. 0.03 max. 0.06 *moderate effluent input* median 0.05 90-perc. 0.14 max. 0.27 *high effluent input* median 0.47 90-perc. 0.91	Fromme '01a

Location	n	Influent	n	Effluent	n	Surface water	Reference
						max. 1.10	
Germany, Hessen 1999-2000			2 · 9	median 0.4 90-perc. 0.6	2 · 20	median 0.05 90-perc. 0.17	HLUG 2001
Germany, Dortmund 2002	5	mean 0.58 0.43 – 0.71	5	mean 0.21 0.20 – 0.24			Bester 2004
Germany, Düsseldorf, Köln 2003	2	0.7 – 1.1	2	0.2 – 0.25			Fahlenkamp 2004
Germany, Lippe 1999	1	0.3	1	0.3	76	median 0.03 90-perc. 0.052	Dsikowitzky 2002
Germany, 2002-2001	2 · 21	summer median 0.49 90th perc. 1.16 winter median 0.31 90th perc. 0.73 0.11 – 1.37	2 · 21	summer median 0.06 90th perc. 0.08 winter median 0.08 90th perc. 0.10 0.03 – 0.15			Müller 2002
The Netherlands River Rhine, '94-'96 River Meuse, '94-'96					32 35	median 0.05 90-perc. 0.10 median 0.07 90-perc. 0.11	Breukel '96
The Netherlands 1997-98	9	raw influents median 4.0 max 8.7	8	median < d.l. max. 0.77			Rijs '98
The Netherlands 1995-96			4	mean 0.28 (4 max. 0.42	14	median 0.04 (4 90-perc. 0.14	Verbruggen '99
The Netherlands 1997	2	0.3 – 0.4 (4	2	0.3 – 0.6 (4	5	0.027 – 0.354 (4	Leonards 2000, Van Stee 2000
The Netherlands 2001	4	median 1.3 max 1.8	4	median 0.7 max. 1.2			Artola 2002
The Netherlands 2002-03			15	median 0.74(5) 90-perc. 2.44(5)			Berbee 2004
The Netherlands River Rhine 2001					8	median 0.003 (4, 5) 90-perc. 0.005	Geerdink 2004
Switzerland, Glatt ≤ 1995					1	0.075	Müller '96
Switzerland 1998			17	median 1.4 90-perc. 2.0	20	median 0.025 90-perc. 0.045	BUWAL '98
Switzerland 1997			6	median 2.0 max. 2.8	8	median 0.05 max. 0.2	Noser 2000

Location	n	Influent	n	Effluent	n	Surface water	Reference
Switzerland 2002 	7 7 1	mean 1.4 1.2 – 2.0 mean 1.5 0.6 (after prim. settling)	7 7 1	mean 0.2 0.1 – 0.4 mean 0.25 0.09			Brändli 2002
Switzerland 2001			5	0.31 – 0.76	28	contam. rivers median 0.06 max 0.19 lakes median 0.004 max 0.015	Buerge 2003
Austria 2000-2001	7	mean 0.95 0.09 – 1.8	7	mean 0.14 0.10 – 0.19			Kreuzinger 2004
Ohio, U.S.A. 1997	2 · 3	mean 10.5	2 · 3	mean 1.3			Simonich 2000
Spain 2001-2002	3	0.9 – 1.7	3	0.15 – 0.20			Carballa 2004
UK, 6 STPs 2001	13	mean 4.2 2.2 – 8.1	13	mean 0.5 0.31 – 0.81			Kanda 2003
Germany 2003 Austria Belgium Spain France	4 1 1	0.1 – 0.2 0.19 0.61	4 1 1 2	0.03 – 0.06 0.041 0.12 0.11 – 0.29	2 1 1	< 0.007 0.06 < 0.007	Mitjans 2004
Germany 2004 Spain 2004 Italy 2004 Greece 2004 Overall SEU-15			2 · 3 4 · 6 4 · 6 4 · 3 60	mean 0.23 0.17 – 0.29 median 0.85 90-perc 1.73 0.33 – 2.5 median 0.41 90-perc 0.70 0.22 – 0.80 median 0.57 90-perc 0.85 0.35 – 0.86 90-perc 1.3			Blok 2005
Sweden 1999 Canada 2002			5 3	0.04 – 0.10 0.11 – 0.52			Ricking 2003b
USA 1977 - 1999 UK 1999 - 2000	12 3	mean 10.35 7.1 – 33.9 3.7 – 13.2	12 3	mean 1.28 (3 0.02 – 2.0 0.6 – 2.7			Simonich 2002

Location	n	Influent	n	Effluent	n	Surface water	Reference
The Netherlands 1999	2	2.4 – 3.9	2	1.2			
USA, South-western			>5	0.027 – 0.092			Osemwengie 2001
USA, Nevada 2001			9 · 2	median 0.03 0.02 – 0.05	7 · 2	median 0.00008 nd – 0.0006	Osemwengie 2004
USA, Lake Michigan 1999 - 2000					13	95% confid. interval: 0.001 ± 0.0008	Peck 2004
USA, Iowa 2001					30 23 23	low-flow: max 1.2 normal-flow: 0.11 high-flow: max 0.18	Kolpin 2004
USA, San Francisco Bay, 1999 – 2000					2 · 13	0.001 – 0.008	Oros 2003

1 Unreliable results according to author (Eschke 1996, pers. comm.)

2 High contribution of sewage works effluents to surface water quality due to low surface water flows and high amounts of raw sewage produced by Berlin's 3.5 million population

3 Median without the extremely low figures from lagoon

4 'Free concentrations'

5 Quantification not reliable

Samples of primary, activated and tertiary sludge were taken from 6 STPs in The Netherlands during two sampling periods in 1997. In addition, the input and output of a compost facility were sampled three times. This facility treats digested activated sludge from several STPs. The median concentration in digested sludge was 16 mg/kg dwt (Blok 1998). One year later in a similar study, concentrations were slightly lower (Rijs and Schäfer 1998).

For the analysis of sewer slime, 17 samples were taken from sewer systems collecting waste water of domestic or industrial origin in Germany. The mean concentration in the industrial area was low, 2 mg/kg dwt, whereas in the domestic area the mean concentration was 23 mg/kg dwt. Furthermore two activated sludge samples were taken from domestic sewage treatment plants. The mean was 8 mg/kg dwt (Sauer et al. 1997).

In Hessen, Germany, the concentrations in activated sludge and in digested sludge of 6 domestic STPs were compared in 1996. There was no systematic relation between the concentrations in digested sludge and in activated sludge. From 1996 to 2000, the surplus sludge of 9 STPs was sampled once per year. Seven out of the 9 treatment plants have a sludge digestion step, but this is not reflected in the levels in the sludge. The median concentrations showed a steady downward trend: from 15 mg/kg in 1996 to 4.2 mg/kg in 2000 (HLUG 2001).

The concentrations on sludge were measured in the STP in Dortmund, Germany in 2002. The plant received 184,000 m^3 water per day from 350,000 inhabitants and industries. The system includes primary settling, activated sludge treatment, secondary settling and anaerobic digestion. The Solids Retention Time was 8-10 days in the aerator and 20 days in the digester. The mean concentration in digested sludge was 1.5 mg/kg (Bester 2004).

Samples were taken from 21 STPs in Germany in summer 2000 and in January 2001. The concentrations were measured in activated and digested sludge. The median concentration in activated sludge was 2.5 mg/kg dwt (range 0.8 – 4.7 mg/kg dwt). The median concentrations were similar in summer and winter but in winter the maximum was higher. In 9 STPs the concentrations in digested sludge were measured as well. These concentrations were 1.5 to twice as high as those in activated sludge. For an evaluation of the anaerobic degradability, however, the loss of organic matter during the digestion process should be taken into account (Müller et al. 2002).

In Switzerland, surplus sludge from different catchment areas was sampled in 1998. Seven samples were taken from systems mainly fed by domestic sewage, three were obtained from systems receiving a mixture of domestic sewage, storm water runoff and low amounts of industrial water, and two samples were from systems with a higher input of industrial waste water. The median concentration was 1.3 mg/kg dwt The concentrations in the area with higher industrial contribution to waste water seemed to be lower. The median in the sludge of mainly domestic origin was 1.5 mg/kg dwt (Herren and Berset 2000). The maximum concentrations here were below the median concentrations in The Netherlands in 1997 and 1998 and in Hessen (1996-2000), Germany.

In three municipal STWs in Berlin, Germany, 14 days-collective samples were taken of the sewage sludge. Two samples were taken in February 2000. The average concentration was 3.6 mg/kg dw, ranging from 2.5 to 5.1 mg/kg dw (Heberer 2002).

In a recent campaign in the UK the concentration on sludge was measured in the digested sludge of 14 wastewater treatment plants. The treatment plants varied largely in water flow as well as the origin of the influent, and also the levels of AHTN were extremely variable between different plants, ranging from 0.12 to 16 mg/kg dw. The median was 4.0 mg/kg dw. (Stevens et al. 2003).

In Switzerland samples were taken of the activated sludge in 16 STPs in 2001. The average concentration was 7.3 (range 3.4 to 11.2) mg/kg. A second sample in five of these plants was within 25% of the first value. These plants were generally small with 210 to 17140 inhabitants connected. The production of sludge varied between 31 and 96 g/d (Kupper et al. 2003a). In another study of this Swiss programme the weekly fluctuation in the concentrations on the activated sludge in a small STP (210 i.e.) were determined in a week in April 2001. The mean concentration was 1.7 mg/kg dwt, ranging from 1.2 to 2.1 mg/kg dwt, so a variation in time of ± 25% (Kupper et al. 2003b).

In Galicia, NW Spain in 2001 – 2002, concentrations were measured on sludge from an STP serving 100,000 inhabitants. The plant consists of three sections including pre-treament, primary treatment by sedimentation and secondary treatment in an activated sludge process. The concentration on sludge was determined by headspace SPME extraction and GC/MS analysis. The average concentration on the activated sludge as derived from the concentration in the outlet of the biological reactor and the total suspended solids was 5.8 (1.5 – 9.0) mg/kg dwt (Carballa et al. 2004). During the development of their analytical methods, the concentration in secondary sludge was 1.0 mg/kg dwt (converted from fresh weight, Llompart et al. 2003).

In a joint monitoring campaign in the Nordic countries a total of 27 STPs was sampled in Denmark, Sweden, Finland, Norway and Iceland in 2002. The type of sludge was not further specified. The median concentrations in Denmark, Sweden, Norway and Finland were 2.0,

1.5, 1.6 and 1.3 mg/kg dwt, respectively, whereas the levels were low in Iceland (0.12 mg/kg dwt) (Mogensen et al. 2004).

Table 3.17 Concentrations in sludge in STP

Sample	n	AHTN [mg/kg dwt]		Reference
NL 1997				Blok, 1998
Primary sludge	11	mean 8.3	range 3.3 - 14	
Activated sludge	12	mean 16.0	range 2.3 – 34	
Digested sludge [1]	13	median 16	range 0.9 – 22	
NL 1997-1998				Rijs and Schäfer 1998
Primary sludge	8	mean 8.2	range 3.7 - 11.7	
Activated sludge	7	mean 5.3	range 0 – 13.5	
Digested sludge	5	median 12	range 11 - 13	
Germany <1997				Sauer et al. 1997
STP activated sludge	2	mean 8.3	range 4.0 - 12.6	
Sewer slime urban/industrial area	17	mean 2.1	range 0.1 - 8.9	
Sewer slime rural area	2	median 23.1	range 9.5 - 36.7	
Germany, Hessen				HLUG 2001
Domestic waste water				
1996, activated sludge	6	median 14.3	range 3.5 – 20.8	
1996, digested sludge	6	median 15.6	range 14.3 – 20.1	
Wasted sludge (sometimes digested)				
1996	9	median 15.0	range 12.0 – 20.1	
1997	9	median 12.1	range 6.4 – 17.5	
1998	9	median 9.1	range 5.8 – 18.4	
1999	9	median 6.8	range 4.5 – 8.5	
2000	9	median 4.2	range 2.9 – 6.1	
Germany, Dortmund 2002	5	mean 1.5	range 1.3 – 1.7	Bester 2004
Digested sludge				
Germany, Nordrhein-Westfalen	19	median 2.3	range 1.2 - 7	Friedrich 2004
>2000				
Switzerland 1998				Herren and Berset 2000
Wasted sludge both domestic and with more industrial input	12	median 1.3	range 0.7 – 4.2	
Switzerland 2001				Kupper et al. 2003a
Wasted sludge both domestic and with more industrial input	16	mean 7.3	range 3.4 – 11.2	
Switzerland 2001	1	mean 1.7	range 1.2 – 2.1	Kupper et al. 2003b
Small STP (210 i.e.)				
Germany, Berlin, 2000	3 · 2	mean 3.6	range 2.5 – 5.1	Heberer 2002
3 municipal STPs				
Germany, 21 STPs, 2000 - 2001	21	summer: median 2.4	range 1.1 – 4.2	Müller et al. 2002

Sample	n	AHTN [mg/kg dwt]		Reference
activated sludge		winter: median 2.5	range 0.8 – 4.7	
dewatered/digested		winter: median 3.8	range 1.9 – 6.9	
Nordic countries, 2002				Mogensen 2004
Denmark	5	median 2.0	range 1.13 – 3.6	
Sweden	8	median 1.5	range 0.95 – 3.3	
Norway	5	median 2.0	range 0.07 – 3.5	
Finland	5	median 1.1	range 0.87 – 2.27	
Iceland	4	median 0.23	range 0.12 – 0.55	
UK (year ?), 14 WWTP Digested sludge	14	median 4.0	range 0.12 - 16	Stevens et al. 2003
Spain 2004 Activated sludge STP (100,000 i.e.)	3	mean 5.8	range 1.5 – 9.0	Carballa et al. 2004
Germany 2004	3 · 2	mean 2.9	range 1.4 – 3.8	Blok et al. 2005
Italy 2004	4 · 6	median 4.4	range 1.3 – 6.9 90th-perc 6.3	
Spain 2004	4 · 6	median 8.7	range 4.3 – 23.0 90th-perc 13.5	
Greece 2004	4 · 3	median 4.3	range 2.6 – 10 90th-perc 9.5	
Overal SEU-15	60		90th-perc 11.0	

1Concentrations in digested, thickened and composted sludge

d.l.: detection limit.

Median and 90th-percentile include samples with concentrations below d.l.

3.1.4.2.2 Concentrations in surface water, suspended matter and sediment

A summary of the concentrations in surface waters, suspended matter and sediment is given in **Table 3.16** and **Table 3.18**.

Thirty surface water samples were taken along a stretch of 160 km in the river Ruhr in Germany. These samples were taken before 1994. The total concentrations were generally at a level of 0.2 μg/l, but higher levels were found where tributaries enter the main stream, up to 11.2 μg/l. Under dry weather conditions, these tributaries are fed between 50 and 90% from effluents (Eschke et al. 1995) and as much as 25% of the water in the Ruhr itself may be effluent water.

The influence of STP effluents is even more pronounced in the water system of the Berlin area (Heberer et al. 1999, Fromme et al. 2001a), where certain sections of the water system contain a very high proportion of effluents (60 to 97%). Samples were taken of canals, rivers and lakes upstream and downstream of sewage works outlets. The median concentration, measured after solvent extraction, in the sections with a high contribution of effluents was 0.47 μg/l (Fromme et al. 2001a). This is below the median effluent concentrations by a factor of 4. Similar data were reported by Heberer et al. (1999) for 30 representative sampling locations in the same area, despite the different methodology (solid-phase micro extraction).

The median concentration in sections with low input of effluents is 0.02 μg/l (Fromme et al. 2001a).

In the River Elbe in Magdenburg, Germany, a monitoring programme was run from mid 1996 to mid 1997. Samples of river water and suspended matter were taken with one or 2 weeks intervals. The median surface water concentration was 0.05 μg/l (total) (Winkler et al. 1998). Other data reported for the Elbe by Bester et al. (1998) and Lagois (1996) show similar levels of total concentrations. Using SPME and GC/MS, the concentration of AHTN in 6 samples of the Elbe remained below the limit of quantitation (< 0.022 μg/l , Winkler et al. 2000).

The monitoring activities in the Elbe were continued and reported more extensively by ARGE (2000). Surface water samples were taken along the River Elbe starting from the Czech border going until the outflow to the sea 700 km downstream. During 1996-1997, 6 samples were taken with 2 months intervals on 7 sites along the River Elbe, including sites where two highly loaded tributaries join the river. Samples were also taken on 8 sites along the two tributaries in the region of Leipzig. Going downstream the concentrations of AHTN in the surface water significantly increased after the entry of the two tributaries and then decreased again. The lowest concentrations were found in summer (range 0.02 – 0.14 μg/l) and were highest in winter (0.06 – 0.33 μg/l). On all sites the concentrations in winter were above the summer concentrations by a factor of 4. The concentrations in the tributaries in the Leipzig region were on the high side of this range. During 1998 and 1999, each month samples were taken of suspended matter on 10 sites along the river in a sedimentation chamber. For the analyses they were combined to two-month samples. The results along the river profile parallel the concentrations found in surface water, showing an increase from 0.02 mg/kg dwt to 0.12 mg/kg dwt decreasing again more downstream. Also here the high concentrations in the suspended matter are related to the contribution of the tributaries. The concentrations fluctuate during the year with the lower concentrations in summer. The overall range was given as 0.004 to 0.13 mg/kg dwt, median 0.03 mg/kg for the Elbe. The highest median concentration in the tributaries was 0.09 mg/kg with a maximum of 0.23 mg/kg. It was noted that the concentrations decreased in 1999 as compared to 1998 by 25 to 40% and in the tidal area the decrease was even 50 to 70%. On 4 sites in one of the tributaries (Saale) sediment samples were taken. The concentrations ranged from 0.013 – 0.38 mg/kg dwt (ARGE 2000). These studies were continued over the following years. The concentrations on suspended matter seemed to be stable at a lower level than in 1998 (Wiegel und Stachel 2003). After the flooding in September 2002 along the Elbe, 37 sediments samples were taken covering the entire river stretch. The concentrations ranged from < 0.001 to 0.085 mg/kg dwt with some peaks observed on the Czech stretch of the river (Stachel et al.2005).

In Hessen, Germany, surface water samples were taken on 20 sites in rivers in 1999 and 2000, once per year (HLUG 2001). The total concentrations ranged from < 0.02 to 0.22 μg/l for AHTN. The median was 0.06 μg/l for 1999 and 0.05 μg/l for 2000. From 1996 to 2000, concentrations in suspended matter were analysed once per year in 17 samples from rivers and in 12 samples from brooks with a high effluent input. The concentrations in 16 rivers ranged from 0.02 to 0.86 mg/kg dw whereas the median decreased from 0.29 to 0.11 mg/kg dw. The number of sampled brooks with a high fraction of waste water sampled decreased from 11 in 1996 to 2 in 2000. The concentrations ranged from 0.4 to 12.7 mg/kg dw. For three sites a series of data is available for four years. Here the concentrations decreased by a factor of 3 to 7. In three brooks the sediment was also sampled. These concentrations were always considerably lower than the concentrations measured in suspended matter from the same sites. In one brook samples in three consecutive years showed a decrease by a factor of 7.3. The

trends observed in this monitoring programme in Hessen are illustrated in **Error! Reference source not found.**.

In Baden-Württemberg, Germany a monitoring programme was carried out along the Rivers Rhine, Neckar and Donau. Samples of suspended matter and sediment along the River Rhein (270 km) and Neckar (200 km) were analysed for AHTN in 1998 – 1999. The samples were extracted with cyclohexane and ultrasone treatment and analyses by GC/MS in SIM mode. In 26 out of 39 sediment samples (or 67%) AHTN was detected up to a level of 0.03 mg/kg dwt. The maximum sediment concentration was 0.18 mg/kg dwt. This particular sampling site showed to be an outlier in the Neckar where also the highest total organic carbon content was observed. The next lower concentration was lower by a factor of 2. AHTN was detected in almost all of 107 samples of suspended matter. The maximum concentration was 0.20 mg/kg dwt. The concentrations of organic carbon in the suspended matter ranged from 3 to 16% whereas for the sediment they ranged from 1 to 4.7%. In general the incidentally high concentrations correlate with low flow conditions in the rivers. Concentrations in 3 water samples were 0.05 – 0.14 μg/l. (LfU-BW 2001). It was remarkable that the concentrations were always lower in summer than in winter as also observed in the Elbe.

In 1999 in Nord-Rhein/Westfalen, Germany, samples were taken of river water and surface sediments on river banks on 19 sites on a longitudinal section along the River Lippe, tributary to the River Rhine. The median concentration in water was 0.04 μg/l. In general the concentrations were low (0.02 μg/l) in the upper reaches of the river and they increase more downstream (0.07 μg/l) when the river reached the more densely populated area whereas in the river mouth the concentrations decreased again (0.04 μg/l). Parallel to this profile, the concentrations in the sediment were low in the upper reach of the river and increased after the river crossed the more densely populated region (range < 0.002 to 0.13 mg/kg dwt or < 0.5 to 26 mg/kg TOC). The maximum concentration was found at the mouth of the river, 1.4 mg/kg dwt (1.8% TOC) or 77.7 mg/kg TOC, whereas on that site in a year 2000-sample the concentration on suspended solids was 0.823 mg/kg dwt (7.5% TOC), converted to 11 mg/kg TOC. This was not due to a local source but explained by the local favourable conditions for sedimentation due to low flow and the availability of terrestrial suspended matter for adsorption of contaminants. Based on analyses of the AHTN load (in $g.day^{-1}$) all over the river it was concluded that the decrease of the load caused by degradation and/or partitioning processes takes place on a short time scale of about 6 – 19 hours (Dsikowitzky et al. 2002a).

The investigations in the Lippe were continued twice per year untill March 2001 in a more generic screening programme in the Lippe River. The data for the concentrations in water per site were rather consistent in time (Dsikowitzky 2002). The overall median concentration was 0.03 μg/l, the 90^{th}-percentile was 0.05 μg/l. In all sediment samples in the second to fourth campaign the AHTN levels were relatively low (< 0.0005 to circa 0.040 mg/kg dwt, Kronimus et al. 2004). However, when the data of Dsikowitzky et al. (2002a) are combined with those of Kronimus et al. (2004), the concentrations on the sediment from the same sampling sites seem to be highly variable in time (e.g., from 107 to 1 μg/kg dwt within 6 months, or 1400 down to 23 and up to 90 μg/kg dwt in 1.5 years). The levels seem to be determined more by the time of sampling then by the site. Therefore the absolute value of these analyses is to be considered as doubtfull.

In the Netherlands, 67 samples of water and 28 suspended matter samples were taken from the rivers Rhine and Meuse at their point of entry into the country between 1994 and 1996. The analyses of the water samples included only truly dissolved material. Median concentrations were approximately 0.06 μg/l (Breukel and Balk 1996).

Samples of suspended solids were taken from representative main watercourses in The Netherlands in 1998. The median concentration was 0.04 µg/l (recalculated from concentrations in suspended matter from Rijs and Schäfer, 1998).

In a research project concentrations in 14 surface water samples in The Netherlands and Belgium were estimated from 'biomimetic extractions'. Median concentrations (dissolved) were 0.04 µg/l for AHTN (Verbruggen et al., 1999).

In 2001 concentrations were determined in the river Rhine in The Netherlands. Eight samples were taken on three different locations. The analytical procedure was specific for synthetic musks. The median concentration was 0.003 µg/l, the 90th-percentile and maximum were 0.0053 and 0.0055 µg/l, respectively (Geerdink en Schrap 2004).

In Switzerland 20 samples were taken from the River Aare and its tributaries and lakes in 1998. The median total concentration was 0.03 µg/l for AHTN (BUWAL 1998). Median concentrations in other Swiss rivers and a lake were at the same level (Schmid 1997 pers. comm. cited in OSPAR 2000). These levels are below the concentration found in one sample taken in the River Glatt, downstream of a STP (Müller et al. 1996).

In Switzerland in the Kanton of Basel surface water samples were taken between January and April 1997. The concentrations as determined by SPME ranged from 0.01 to 0.2 µg/l. The median concentration was 0.05 µg/l (Noser et al. 2000).

Surface water was sampled at several lakes and rivers in the Swiss midland region, a small mountain lake (Murgsee, altitude 1820 m) and in the Mediterranean Sea. The samples were taken between 2000 and 2001. Following solid phase extraction they were analysed by GC-MS or SIM. The concentrations in the lakes ranged from 1.1 to 6.7 ng/l. A higher level was found in one lake in the watershed area of the River Glatt, 7 to 18 ng/l. In the small mountain lake, AHTN was not detected (< 1 ng/l). Levels were higher in the rivers and streams, ranging from 2.3 ng/l upstream of an STP to (maximum) 186 ng/l in a small river downstream of an STP. A vertical concentration profile was produced for the Zürichsee between 1 and 130 m depth in March, May and July 2001. In March, during circulation of the lake, AHTN was more or less uniformly distributed (ca. 4 to 6 ng/l). In May, and even more so in July, significantly lower concentrations were observed in the epilimnion of the lake (1 to 2 ng/l) showing that elimination takes place near the water surface. AHTN was not deteted in the samples taken in the Mediterranean Sea at -5m and -150 m (Southern Spain; one sampling point was water primarily from the Atlantic Ocean) (Buerge et al. 2003).

The concentrations in a German lake and in the German and French river were below 0.006 µg/l, whereas in the Spanish river the concentration was 0.06 µg/l (showing a high effluent load). AHTN was not detected in drinking water (Mitjans and Ventura 2004).

Streamwater was collected in Pennsylvania, USA and two other locations. AHTN was detected in 58% of the samples (n=19). The mean concentration ± s.d. was 3.0 ± 7.7 ng/l. The concentrations ranged from below d.l. to 33 ng/l (Standley et al. 2000).

Surface water was sampled in Lake Mead, Nevada, USA, in 2001 twice during 7 months. The concentrations ranged from below detection level to 0.0006 µg/l (Osemwengie and Gerstenberger 2004). The lower concentrations were observed in summer and the higher in winter, as was also observed in the Elbe by ARGE (2000).

Surface water samples were taken in the San Francisco Estuary, California, USA, in the dry seasons (July) of 1999 and 2000. Whole water samples were taken at 1 meter below the water

surface. The sample extract fractions of the two years were combined to get respresentative samples of five different regions in the Bay area. The concentrations in the water were highest in the sewage impacted South Bay area, 0.008 μg/l. In the other regions the levels were 0.001 to 0.002 μg/l, whereas AHTN was not detected in the open ocean background site approximately 2 miles offshore (Oros et al. 2003).

The surface water of Lake Michigan, USA, was sampled during June 1999 and May 2000. AHTN was detected in 85% of the samples above the blank levels and it was found primarily, for 97%, in the dissolved phase. The concentration in the dissolved phase was 0.001 ± 0.0008 μg/l (Peck and Hornbuckle 2004).

Water samples were taken from 23 stream locations siturated upstream and downstream of 10 to 14 cities in IOWA, USA. A total of 76 samples were taken during high, normal and low streamflow conditions during 2001. The maximum concentration of AHTN during low-flow conditions was 1.2 μg/l. The concentrations were lower during normal and high-flow cinditions (Kolpin et al. 2004).

In California, USA, agricultural fields are irrigated with disinfected tertiary recycled wastewater or streamwater predominantly consisting of wastewater effluent. The recycled wastewater had undergone activated sludge treament with extended aeration, sedeimentation, nitrification/denitrification, sand filtration and chlorination. Six fields were sampled during the dry and wet wet weather over the course of two crop seasons. AHTN was tentatively indentified in the water phase from two fields (0.8 – 5.7 μg/l, Pederson et al. 2003).

Marine water samples were taken at seven stations in the German Bight of the North Sea from a depth of 5 m in the summer of 1990 and 1995. The highest concetration was 0.002 μg/l, the median concentration (total) was 0.0002 μg/l (Bester et al., 1998).

Table 3.18 Concentrations in sediment and suspended solids

Location	n	AHTN (mg/kg dwt)		Reference
Dutch borders 1994-1996	14	median 0.24	range 0.10-0.54	Breukel and Balk, 1996
Suspended matter Rhine				
Dutch borders 1994-1996	14	median 0.84	range 0.06-1.2	
Suspended matter Meuse				
90th percentile Rhine and Meuse 1994-1996	28	0.96		
NL surface waters 1998	24	median 0.12	90th perc. 1.0	Rijs and Schäfer 1998
Suspended matter		max. 1.7		
Germany, Elbe 1996-1997	31	median 0.47	range 0.19-0.77	Winkler, *et al.,* 1998
Susp. Particulate materials		90th perc. 0.61		
Germany, Elbe, suspended matter	82	median 0.03	range 0.004 – 0.13	ARGE 2000
1998 - 1999				
and tributaries	12	median 0.09	range 0.04 – 0.23	
	12	median 0.06	range 0.10 – 0.16	
	12	median 0.06	range 0.004 – 0.13	

Location	n	AHTN (mg/kg dwt)		Reference
Germany, Berlin area	19	*low effluent input :* median 0.02, 90-perc. 0.03		Fromme et al. 2001a
1996 - 1997	20	*moderate effl. input:* median 0.24, 90-perc. 0.52		
Sediment (10 cm depth)	20	*high effluent input:* median 0.93, 90-perc. 2.21		
Germany, Berlin Teltow Canal	4		range < d.l. to 0.34	Schwarzbauer et al. 2003
1998 – 1999				
and core 1985 - 1990				
Germany, Berlin Teltow Canal	9	median 0.22	range < 0.1 – 0.89	Blok et al. 2005
Dec 2003				
Sediment (0-5 cm)				
Germany, Hessen				HLUG 2001
Suspended matter, 1996	11	median 0.29	range 0.09 – 0.84	
1997	12	median 0.19	range 0.06 – 0.86	
1998	12	median 0.30	range 0.05 – 0.86	
1999	16	median 0.14	range 0.03 – 0.40	
2000	15	median 0.11	range 0.02 – 0.26	
Germany, Hessen, susp. matter in contaminated brooks, 1996	11	median 3.2	range 0.54 – 12.7	
1997	5	median 2.7	range 1.9 – 6.6	
1998	5	median 2.9	range 1.6 – 7.2	
1999	3	range 0.4 – 2.9		
2000	2	range 0.6 – 0.97		
Sediment in contaminated brooks, 1996 – 1999	5	range 0.7 - 4.8 *		
Germany, Elbe, susp. matter 1997	9	mean 0.047	range 0.007 – 0.104	Wiegel, cited in Fooken 2004
(Hamburg-Dresden)				
Germany Niedersachsen, sediment 5 rivers, 1996	8	median < 0.0005	range < 0.0005 – 0.004	(Lach and Steffen 1997 cited in Rimkus 1999)
Germany Nord-Rhein/Westfalen, sediment Lippe, 1999	19		range < 0.002 – 1.4	Dsikowitzky et al. 2002a
2000 - 2001	27	0.002	range <0.0005 – 0.090	Kronimus 2004
9 common points, 4 times 1999-2001	35	0.002	90th-perc. 0.10	
Suspended solids	1	0.82		Dsikowitzky et al. 2002a
Germany Baden-Württemberg, sediment	39	median 0.007	range <0.005 – 0.180	LfU-BW 2001
Rhein, Neckar,Donau, 1998 - 1999				
Suspended matter, 1998-1999	107		range <0.005 – 0.200	
Austria, Donau sediment	7		range < 0.001 – 0.007	Scharf et al. 2004
Tributary sediment	1	0.025		
Donau suspended solids	2		range 0.008 – 0.012	

Location	n	AHTN (mg/kg dwt]	Reference
Sediment			Biselli et al. 2005
German Bight 1997	3	<2, <2, n.d.	
North Sea 2000	3	< 2	
Baltic Sea 2001	4	n.d.	

* downward trend

Concentrations in suspended matter and river sediment are presented in **Table 3.18**. In 1994-1996, concentrations were determined in 28 samples of suspended matter in the Rhine and Meuse at their point of entry in The Netherlands. Median concentrations were 0.24 and 0.84 mg/kg in the Rhine and Meuse, respectively (Breukel and Balk, 1996). Levels in suspended matter in 24 samples taken in the main surface waters in The Netherlands (Rijs and Schäfer, 1998) were similar to levels in the Rhine.

Samples of suspended material were taken from the Elbe near Magdeburg in Germany during one year at weekly or 2-week intervals in 1996-1997. The median concentration was 0.47 mg/kg dwt (Winkler et al. 1998). These levels were similar to the levels in the Meuse, but with considerably lower variability.

Following these observations on suspended matter of Winkler et al. (1998), AHTN was also included in the following investigation. In the state Saxony-Anhalt, Germany, transects were made throught the floodplain of the River Elbe, from the dike to the riverbank and on 6 positions showing elevated concentrations of heavy metals, the depth distribution (to 1.5 m) of other contaminants was investigated. The concentrations of musk fragrances (in 'dry soil') were only in the range of the blank, 0.01 to 0.02 mg/kg dwt (Witter et al. 2003).

In Germany, 59 sediment grab samples were taken from the central bed of the rivers in the Berlin area. The levels were linked to the concentrations found in the surface water. Concentrations ranged from below the detection level (0.02) to 2.6 mg/kg dwt The observed high variability in the areas with high contributions of STPs was attributed to inhomogeneous sample materials (Fromme et al. 2001a). A total of 28 surface sediment samples from the Havel and Spree River representing the sedimenting record from 1979/1980 to 1994 were screened for the presence of a large number of organic pollutants. The median TOC content was 10.4%, the 90^{th}-percentile was 14.7% (ranged 0.9 – 55.6%). AHTN was detected in all samples but not quantified. The sum of AHTN and HHCB in a sediment core in the Teltow Canal, representative of approximately 20 years, was 0.2 mg/kg (Ricking et al. 2003a).

The Teltow Canal in Berlin was recognised as a particular area were high levels were found in the water as well as in the sediment. Four sediment samples were obtained from three locations, including one sample core representing current time (1998-1999) and an older accumulation time between 1985 and 1990. The concentrations were determined in the extractable fraction as well as in the non-extractable fraction after a separate application of different chemical degradation procedures. The concentrations ranged from below detection level to 0.34 mg/kg dwt in the extractable fraction and from below detection level to 0.95 mg/kg in the different non-extractable fractions. The total organic content of the samples varied between 7.1 and 12.1% (Schwarzbauer et al. 2003).

In 2003 samples were taken again on seven different places in the Teltow Canal (this was included in the high effluent input area of Fromme et al. 2001a). A core was taken deep frozen so a stratified sample of the sediment was obtained. The top 5 cm-layer was analysed

and samples were also taken from the 5 - 7 cm-layer and the 7 – 12 cm-layer on one site. The sediment samples were taken in the middle of the canal, 0.1 km upstream of the STP Wassmansdorf and 0.5, 1.0 and 1.5 km downstream. Other samples were taken 1 km downstream of the STP Ruhleben, 1 km downstream of the STP Stahnsdorf and at the entrance to the Havel River. The samples were freeze dried. The freeze dried samples were extracted by Accelerated Solvent Extraction (ASE) with a first Silicagel purification on line, followed by Dichloromethane extraction at 2000 psi. Extracts were treated for clean-up with an SPE column (details of extraction and GC/MS analysis in Bonnet et al. 2003 and Otten and Meijer, 2004). There was a clear relation with the distance to the STP: the OC content as well as the concentrations of AHTN and other substances increased right after the point of discharge (from < 0.1 to 0.23 mg/kg dwt) and decreased after 1 and 1.5 km (to < 0.1 mg/kg dwt) downstream. The content of organic carbon ranged from <1 to 14% (median 5%). Overall the concentrations in the sediment samples ranged from < 0.1 to 0.46 mg/kg dwt (Blok et al. 2005).

Furthermore, data were cited by Rimkus from other sources: In 1997, 9 suspended matter samples were taken from the River Elbe (Hamburg – Dresden), probably by a sedimentation chamber. The mean concentration was 0.047 mg/kg AHTN. In Niedersachsen sediment 8 samples were taken from 5 rivers in 1996: from Elbe, Weser, Ems each 2 samples, from Leine and Oker, each 1 sample. All concentrations were below the detection limit [Lach & Steffen 1997, cited in Rimkus 1999].

In the Austrian section of the River Donau between the borders with Germany and Slowakia the sediment was sampled at seven sampling points in August 2001. An additional sediment sample was taken in the confluent with a major tributary, the River Schwechat. The concentrations in the sediment samples in the Donau ranged from < 0.001 – 0.007 mg/kg dwt, whereas with 0.025 mg/kg dwt the level in the tributary was higher. In two samples of the suspended matter taken in the upper section of the Donau the concentrations were 0.008 and 0.012 mg/kg dwt (Scharf et al. 2004).

3.1.4.2.3 Trends in time

In Hessen, Germany, samples of sludge were taken once per year from 9 STPs over a 5 year period (1996 - 2000). During this period, samples of suspended matter were also taken from 17 sites along various rivers and in some particularly contaminated brooks. Effluents of the 9 STPs and surface water samples (20 sites) were taken once in 1999 and in 2000. This large scale project yielded information on the trend of the environmental concentrations during this period. The results show a general decrease in time. The concentrations in sludge and suspended matter decreased by a factor of 3 to 4, see Figure 3.6 (note the logarithmic scale). Concentrations in effluent and surface water were available only during the latter two years, but decreased to the same extent. This decrease coincides with the decrease in use volume since 1995.

Concentrations on sludge are directly reflecting the decreasing input of AHTN to the sewer systems over the years. The concentrations in the suspended matter of 17 sites in the Hessian rivers also generally decreased during the five years. Figure 3.7 and Figure 3.8 show the individual observations for these compartments. It is remarkable that not only concentrations in suspended matter and surface water follow the downward trend but this trend was also directly observed in the sediment of contaminated brooks. For one of those brooks, data were available for three consecutive years, showing that the concentrations in sediment closely followed the drop in the suspended matter concentrations (see isolated data points in Figure

3.6). Generally, sediment samples include materials settled during a series of years and thus include the history of the spot. Thus for non-degrading substances a reduction of the use volume would go unnoticed for some years. In this case the observed concentration drop suggests that degradation processes take place in the sediment without delay.

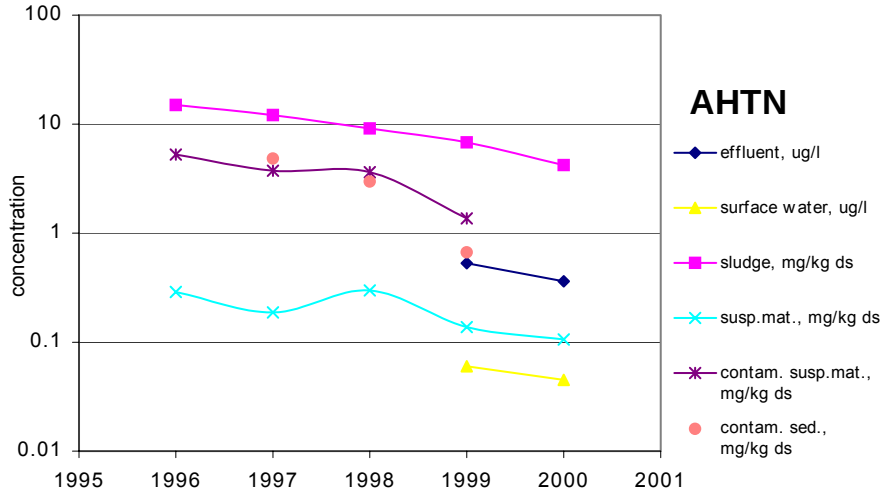


Figure 3.6 Trend in the median concentrations of AHTN in various environmental compartments, Hessen, Germany (based on data from HLUG 2001) (Remark: Contaminated suspended matter: mean for 3 sites; contaminated sediment: only one site. Logarithmic scale).

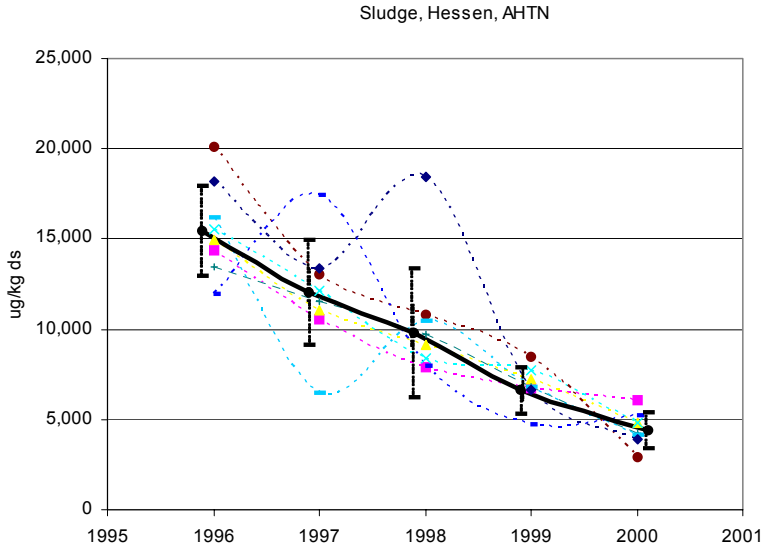


Figure 3.7 Trend in the concentrations of AHTN in wasted sludge from 9 domestic STPs, Hessen, Germany (HLUG 2001). The solid line connects the median per year. Standard deviations are included. Please note that each data point is one observation per STP per year. They are connected with a dotted line to show the individual STP, not to suggest a trend. The difference between the years 1996-2000 was statistically significant (t-test, $\alpha = 0.01$, $P < 1E\text{-}06$).

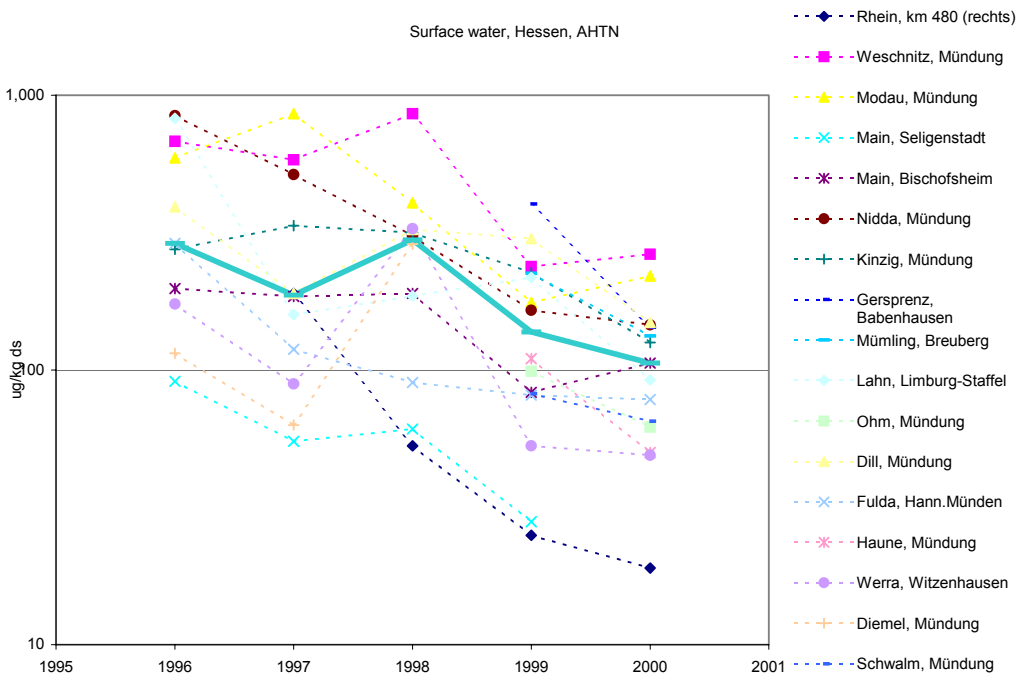


Figure 3.8 Concentrations of AHTN in suspended material in water systems in Hessen from 1996 – 2000 (based on data from HLUG 2001). Please note that the points represent one sample per year. Per river the points are connected by dotted lines; these are not suggesting the trend between the sampling periods. (Logarithmic scale)

A similar trend is observed in the concentrations in the STPs along the Teltow canal and the receiving compartments. Concentrations measured in the 1996-1997 were relatively high and stimulated further research. The results are summarised in **Table 3.19**. Between 1996/97 and year 2004 the concentrations in effluent and in sediment have decreased by a factor of 4 or more. Data for sludge are from 2000 and the concentrations in 2004 did not deviate from 2000.

This decrease is also observed in other regions, but not documented as completely. Therefore the year of sampling is an important piece of information of a sample.

The downward trend is also reflected in biotic samples, see section 3.1.7.2.

Table 3.19 Decrease of the AHTN concentrations in samples from STPs along Teltow Canal and sediment, Berlin (source: Heberer 2002, Fromme et al. 2000, Fromme et al. 2001a, Blok et al. 2005)

		1996/1997	2000	2004	reduction factor
Effluent (µg/l)	median	2.2		0.23	10
	90th-perc.	3.4			
	max	4.4		0.29	
Sludge (mg/kg dwt)	median		3.6	2.9	1
	90th-perc.				
	max		5.1	3.8	
Sediment (mg/kg dwt)	median	0.9		0.22	4
	90th-perc.	2.2			
	max	2.6		0.46	

3.1.4.3 Comparison between predicted and measured levels

When comparing measured concentrations with predicted concentrations, it should be realised that:

- measured concentrations in water are sometimes reported as total, sometimes as dissolved concentrations. However, with a suspended solids concentration of 10 mg/l in surface water, the fraction sorbed is less then 10%, and in general this source of variation is considered negligible;
- measured concentrations vary on a spatial scale, where higher concentrations are clearly related to the discharge of STP effluents into surface water;
- measured concentrations in Hessen, Germany, show a decrease in time, as a reflection of the decrease in use volume since 1995. This decrease is also observed but less well documented, in other regions of Europe. Therefore the year of observation is an important piece of information of the sample and in comparison with a prediction it determines the scenario to which it should be compared.
- environmental data are highly variable. Simply taking the highest value does not reflect a representative exposure. Therefore the data are summarised in median and 90^{th}-percentile values if possible in boxplots where the source of the data and periods of sampling can be taken into account. For a conservative comparison, the 90^{th}-percentile of the data is compared to the predicted concentrations.

For a proper comparison, the PEC based on use volumes for the year 2000 should be compared to concentrations measured in recent monitoring campaigns. When values from older studies are considered, it should be kept in mind that concentrations may have decreased by a factor of 2 to 4 since 1996. The aim of this section is, in fact, to identify the PECs that should be taken along in the risk characterisation section.

3.1.4.3.1 Influent

The comparison of predicted concentrations and those measured in influents is limited to the more recent data, starting from the year 2000. A large number of observations for AHTN in STP influents is reported from Germany and there are some from other European countries, for example The Netherlands, Switzerland, Austria, Spain and the UK. Influent concentrations as predicted by EUSES are taken from **Table 3.14**. The predicted influent concentrations in the scenarios for northern and southern European countries were based on the calculations where the release factor was reduced to obtain the observed effluent concentrations. **Table 3.20** shows that (1) the predictions by the TGD regional (10%) scenario are too high by almost two orders of magnitude and (2) that the predicted influent concentrations for Southern EU-15 (SEU-15) and Northern EU-15 (NEU-15) are just above the observed concentrations.

Table 3.20 Comparison of measured and predicted concentrations for STP influent (µg/l)

Scenario	Predicted influent STP, µg/l	Observations, location	n	Measured concentration influent	Reference
TGD regional (10%)	98.1				
southern EU-15	6.38 (in scaled predictions)	UK 2001	13	mean 4.2, 2.2 – 8.1	Kanda 2003
		Spain 2001-2002	3	0.9 – 1.7	Carballa 2004
		Spain 2003	1	0.61	Mitjans 2004
northern EU-15	2.94 (in scaled predictions)	Switzerland 2002	14	mean 1.4 – 1.5	Brändli 2002
		The Netherlands 2001	4	median 1.3	Artola 2002
		Germany 2002	5	0.43 – 0.71	Bester 2004
		Germany 2003	2	0.7 – 1.1	Fahlenkamp 2004
		Germany 2000	42	0.11 – 1.37	Müller 2002
		Austria 2000	7	0.09 – 1.8	Kreutzinger 2004

3.1.4.3.2 Effluent

Concentrations measured in the effluent of STPs were reported for larger campaigns in Germany, The Netherlands, Switzerland, Austria, Spain, Italy and Greece and from the USA, see section 3.1.4.2.1.

The estimations from the TGD regional (10%) scenario (see **Table 3.13**) predicted PECeffluent = 20.2 µg/l. For the Northern EU-15 Scenario the recent data for Germany (Hessen HLUG 2001, Müller et al. 2002, Fahlenkamp et al. 2004, Bester 2004, Dsikowitzky 2002, and Blok et al. 2005) were used as the start of the calculations for the Northern EU-15 (90^{th}-percentile, 0.54 µg/l), whereas the data for Italy, Spain and Greece were the basis for the calculations of the Southern EU-15 Scenario (overall 90^{th}-percentile, 1.3 µg/l, Blok et al. 2005).

The results of larger recent campaigns are summarised in a boxplot in Figure 3.9. For each country the boxplot shows the median, the interquartile range, outliers and extremes (values between 1.5 and 3 boxlengths or more then 3 boxlengths from the upper edge, respectively). The boxplot in the figure shows that the value of 1.3 µg/l used in the SEU-15 Scenario is higher than the levels in the other countries. The boxplot also suggests that only Switzerland and Germany suit the Northern EU-15 Scenario for AHTN.

When the level of 1.3 µg/l used for the Southern EU-15 Scenario is compared to the other data listed in section 3.1.4.2.1, some studies report observations above this level, e.g., reports from Germany (Sachsen-Anhalt, Berlin) and from Switzerland. However, these studies were carried out before the year 2000 and later studies have shown that concentrations in those regions had decreased significantly. Recently reported data from Austria and Sweden are also in line with the Northern EU-15 Scenario, see Table 3.16. The maximum observed in Spain (considered as an outlier in Figure 3.9) is above the 90^{th}-percentile of Southern EU-15 by a factor less than 2.

For the risk characterisation the Southern EU-15 Scenario is used:

PECeffl = 1.3 µg/l (total).

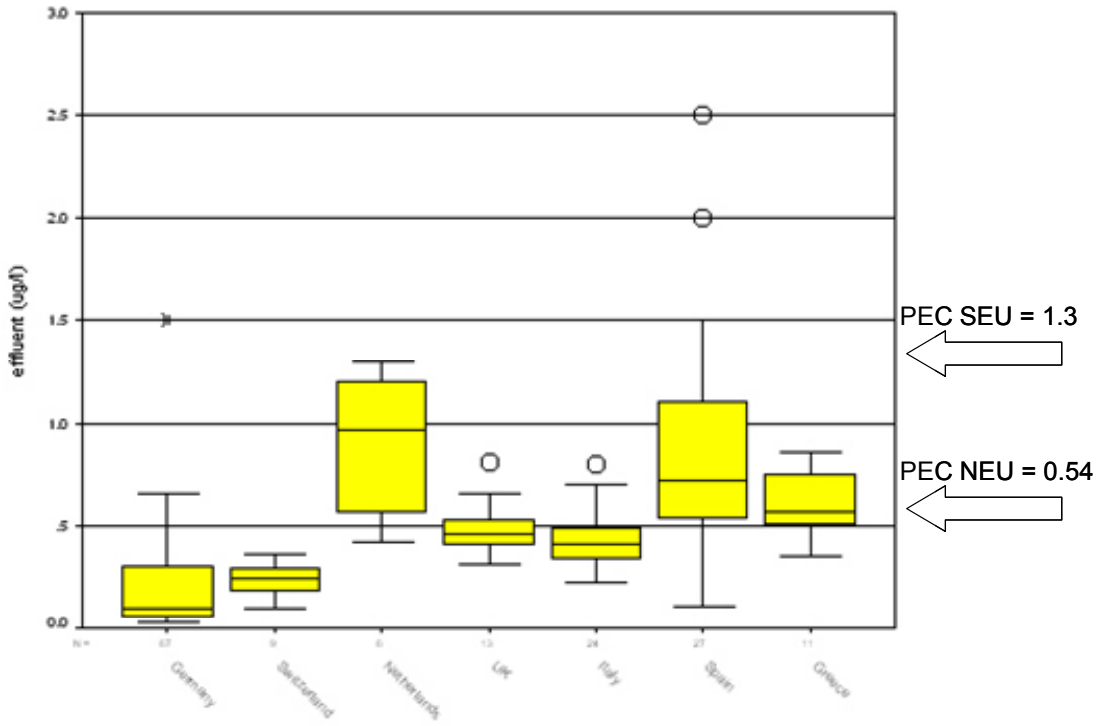


Figure 3.9 AHTN concentrations in effluents in recent campaigns (Source: HLUG 2001, Müller 2002, Blok et al. 2005, Brändli 2002, Mitjans 2004, Simonich 2002, Artola 2002)

3.1.4.3.3 STP sludge

Concentrations measured in sludge were reported from The Netherlands, Germany, Switzerland, UK, the Nordic countries Denmark, Sweden, Norway, Finland, Iceland and a from southern countries Greece, Spain and Italy, see **Table 3.17**.

The concentration in sludge predicted by the TGD regional (10%) scenario = 174 mg/kg dwt. For the Northern EU-15 Scenario the calculations were made using the 90th-percentile of recent data for Germany, 5.9 mg/kg dwt (HLUG 2001, Heberer 2002, Müller et al. 2002, Friedrich et al. 2004, Bester 2004, Blok et al. 2005) and for the Southern EU-15 Scenario, the overall 90th-percentile of the results in Spain, Italy and Greece was used, 11.0 mg/kg dwt (Blok et al. 2005).

The boxplot in Figure 3.10 summarises the results of recent campaigns. The boxplot shows that the value of 11.0 mg/kg dwt is above the levels in all other countries. It also shows that the Nordic countries and Germany belong to the Northern EU-15 Scenario whereas Switzerland and the southern countries are within the Southern EU-15 scenario. When the other data in **Table 3.17** are considered, it is clear that the results from older studies are above 11 mg/kg dwt. However, results of recent studies in the same region as the old studies show that the current concentrations in sludge are below 11 mg/kg dwt, so they are in line with the Southern EU-15 Scenario.

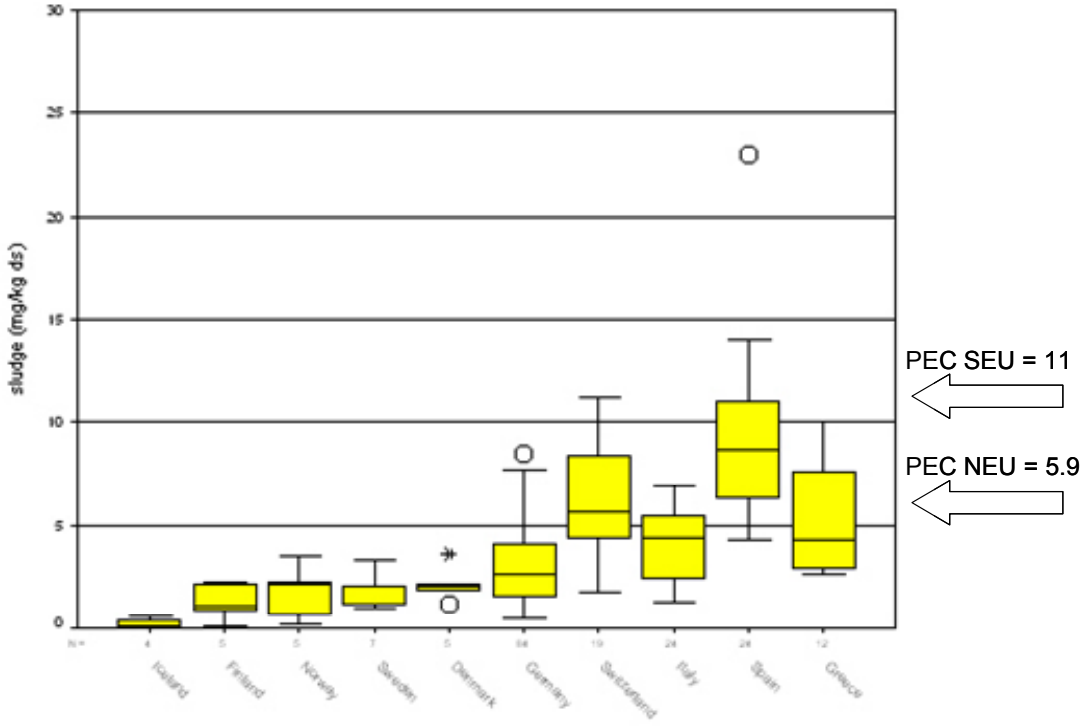


Figure 3.10 AHTN concentrations in sludge in recent campaigns (Source: HLUG 2001, Müller et al. 2002, Blok et al. 2005, Heberer 2002, Kupper 2003a, Friedrich 2004, Mogensen et al. 2004)

It should be remarked that when sludge is applied to soil, fluctuations in sludge concentrations from the same source are smoothed and therefore the median concentration in the wasted sludge is relevant for the calculation of PECsoil. However, in the present calculations the 90th-percentile was used in accordance with the EU-TGD (EC 2003).

3.1.4.3.4 Surface water

Concentrations were measured in a large number of rivers and brooks, lakes and canals in Germany (Ruhr, Elbe including its tributaries, Main and tributaries in Hessen, Lippe), in The Netherlands (Rhine, Meuse and other main waterways), Switzerland (Aare and tributaries, Basel area, major lakes in midland region and mountains) and outside Europe in Southwest USA, Lake Michigan, Iowa and the San Francisco Bay area. The samples are not randomly distributed over the EU-15+2. The results of the monitoring campaigns are summarised in a graph in Figure 3.11. The graph shows the median (top of column), 90^{th}-percentile (dash) or maximum (triangle) of the data reported in Table 3.16. The higher values observed in surface water are clearly related to the discharge points of treated sewage and they are to be compared to $PEClocal_{water}$. In remote areas the AHTN concentration will have been decreased by losses process and dilution, so concentrations in larger rivers are lower, in between the $PEClocal_{water}$ and PECregional.

The figure also includes the levels of the predicted local concentrations:

$PEC_{TGD\ regional\ (10\%)}$ = 2.02 µg/l, PEC_{SEU} = 0.13 µg/l and PEC_{NEU} = 0.05 µg/l.

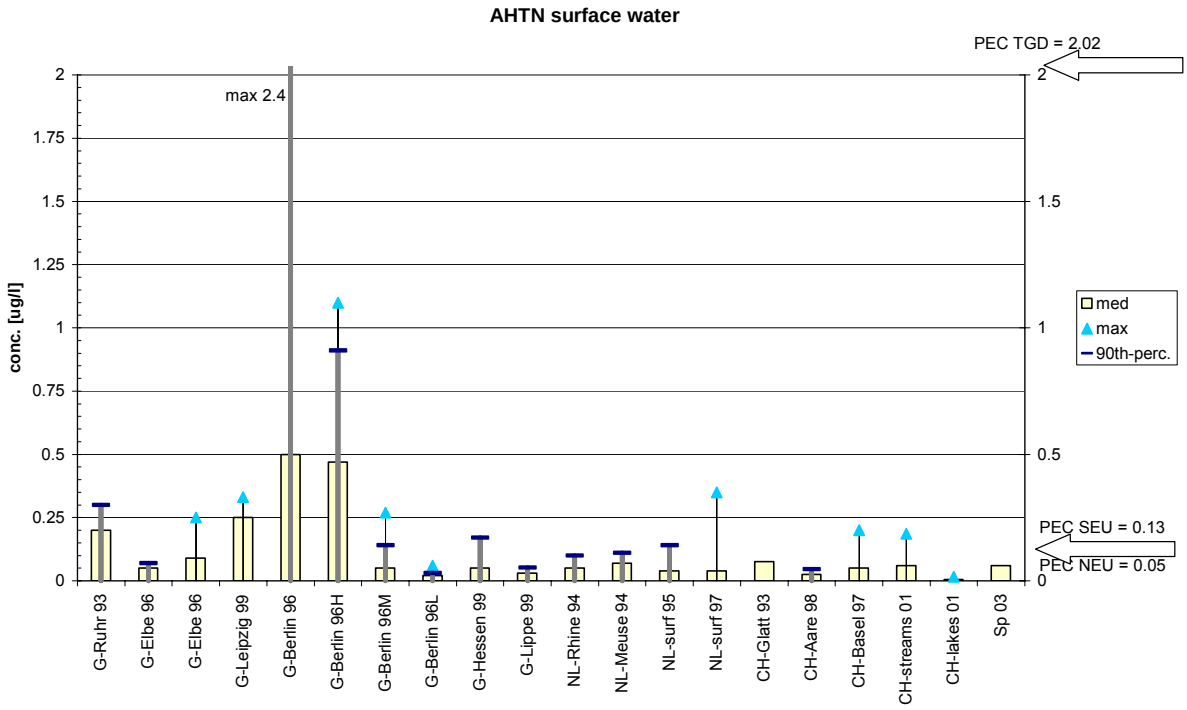


Figure 3.11 Summary of the measured AHTN concentrations in surface water; median, 90th-percentile and/or maximum (µg/l)

The major part of the samples was taken in Germany and they cover a period of more than a decade. During the past ten years the use of polycyclic musks has decreased and this is reflected in lower levels in the surface water, although it should be remarked that the number of recent monitoring campaigns in surface water is relatively low. The observation is, however, supported by the lower recent *effluent* levels reported in the previous section (3.1.4.3.2). The picture confirms that most recent levels in Germany are at the level of the SEU-15 and NEU-15 scenarios. Exceptions are the older data from Germany, in particular from the area in Berlin with high effluent input. Since presently the effluent concentrations in the STPs in Berlin have dropped (see 3.1.4.2.3), it is expected that the overall surface water concentrations will have decreased proportionally.

The Northern EU-15 Scenario predictions are based on effluent concentrations recently measured in Germany by applying a dilution of 1:10. It is concluded that in general these predictions are at the same level as the most recent values in Germany. As the SEU-15 Scenario is also based on recent effluent concentrations, it is concluded that the SEU-15 Scenario is acceptable except maybe for places with a lower dilution potential than the default 1:10.

For the risk assessment the Southern EU-15 Scenario will be used:

$PEClocal_{water}$ = 0.13 µg/l.

The 90th-percentile of the surface water samples in the high effluent input area in Berlin (1996/1997) was 0.91 µg/l.

3.1.4.3.5 Sediment

Concentrations measured in sediment are reported from Germany, viz. the Elbe, the Berlin area, for contaminated brooks in Hessen, rivers in Niedersachsen, the Lippe, Baden-Würtemmberg. In addition there is one report for sediment taken from the Donau. The predictions of the concentration in sediment are based on the concentrations observed in current effluents and sludge in Germany for the northern EU-15 Scenario: $PEClocal_{sediment}$ = 0.035 mg/kg wwt equaling ~ 0.2 mg/kg dwt. The levels in the contaminated brooks in Hessen in 1996-1998 were clearly above this level as well as the levels from the areas in Berlin highly influenced by effluents in 1996/1997. Later observations in the programme in Hessen showed a clear decrease. A specific study set up to verify the high concentrations in the sediment of the Berlin Teltow Canal (December 2003) also showed that the concentrations had decreased and are now at the level of the SEU-15 $PEC_{sediment}$. The other studies report sediment concentrations that are below 0.2 mg/kg dwt, see **Table 3.21**. Thus the predictions based on the current effluent and sediment concentrations predict the sediment concentrations relatively well. Therefore the sediment concentrations predicted based on effluents and sludge concentrations measured in the Southern EU-15 can be used for the risk assessment:

$PEClocal_{sediment}$ = 0.086 mg/kg wwt ~ 0.395 mg/kg dwt.

The maximum of the concentrations measured in the Teltow Canal in Berlin (2003) is 0.46 mg/kg dwt.

Table 3.21 Comparison of measured and predicted concentrations for sediment

Scenario	$PEClocal_{sediment}$ mg/kg		Measured concentrations in sediment mg/kg dwt	Reference
	wwt	dwt		
TGD regional (10%)	1.31	3.4		
southern EU-15	0.086	0.4		
northern EU-15	0.035	0.2		
			Germany Hessen, contam. 1996 – 1998 n=5 per year range 4.8 - 0.7 (decreasing) Germany Hessen, 1999 – 2000 n=5 per year contam. 0.7	HLUG 2001
			Germany, Berlin area 1996-1997 n=59 low input: median 0.02, 90% 0.03 moder. input: median 0.24, 90% 0.52 high input: median 0.93, 90% 2.21	Fromme 2001a
			Germany, Berlin Teltow Canal 2003 n=9 range < 0.1 – 0.46	Blok et al. 2005
			Germany, Niedersachsen, 1996 n=8 range < 0.0005 – 0.004	Lach and Steffen 1997 cited in Rimkus 1999
			Germany Lippe 1999-2001 n=36 median 0.002, 90-perc. 0.10, max 1.4	Dsikowitzky. 2002, Kronimus 2004
			Germany, large rivers Baden-Württemberg 1998-1999 n=39 median 0.007, max 0.180	LfU-BW 2001
			Austria Donau range < 0.001 – 0.007, and 0.025 n=8	Scharf 2004

3.1.5 Terrestrial compartment

The exposure of the terrestrial compartment follows from atmospheric deposition and application of sludge on agricultural land. The following scenarios are used:

1 production of AHTN on one site;
2 fragrance compounding for the sites described in section 3.1.2.2.1 and for a generic medium/large and a small site;
3 end product formulation by a generic large and small formulator (see section 3.1.2.2.2);
4 private use in northern and southern EU-15 countries, see section 3.1.2.3.

During production and compounding, no waste is discharged directly to the soil compartment. The wasted sludge produced in the industrial wastewater treatment plant on site 4 is treated in an incinerator. On site 6 no waste water is produced.

3.1.5.1 Calculation of PEC_{local}

For the industrial sites discharging to a municipal STP, the concentration of AHTN in sludge is given in **Table 3.11** and **Table 3.12**. The concentration in sludge due to private use is given in **Table 3.14**. **Table 3.22** presents the concentrations in sludge for the scenarios as well as the estimated concentrations in soil.

Table 3.22 Local PECs for the terrestrial compartment.

Scenario	Csludge, mg/kg	$PECsoil_{local}$ mg/kg wwt, 180 d	$PECsoil_{regional}$ mg/kg wwt	$PECgroundwater_{local}$, µg/l
Site				
Production	0.17	0.00042	0.00012	0.00080
Compounding Site 1	0.31	0.000092	0.00016	0.175 E-03
Compounding Site 2	0.46	0.00113	0.00012	0.0022
Compounding Site 3	2.85	0.00696	0.00012	0.0132
Compounding Site 4	<56.8> WWTP	--	--	--
Compounding Site 5	0.06	0.000163	0.00016	0.39 E-03
Compounding Site 6	--			
Compounding Site 7 (Large-medium generic)	30.2	0.074	0.00016	0.14
Compounding Site 8 (Small generic)	4.51	0.011	0.00016	0.0209
Formulation Large company	7.37	0.018	0.00016	0.0342
Formulation Generic scenario	9.0	0.022	0.00016	0.0418
Consumer use				
TGD regional (10%)	174	0.425	0.003	0.808
southern EU-15	11.0	0.0269	0.00016	0.051
northern EU-15	5.9	0.0144	0.00012	0.0274

3.1.5.2 Measured levels

Few data are available on concentrations measured in sludge amended soils.

An average sludge application rate on a field in Georgetown, DE was 7000 wet gallons per acre (or 0.6 to 1.1 kg sludge per m^2). The AHTN concentration in sludge was in the order of 50 mg/kg dwt. With a ploughing depth of 15 cm the expected intitial concentration would be 0.13 mg/kg dwt. The concentration measured immediately after application was 0.12 mg/kg dw. Analysis of the pre-application sample demonstrated that the residue of AHTN from the previous application had dissipated below quantification limits (<0.05 mg/kg dwt). It was concluded that AHTN dissipated faster in the field than in an experimental set-up reported in section 3.1.3.1.3, which may be explained by the biological activity in the field (DiFrancesco et al 2004).

In the state Saxony-Anhalt, Germany, transects were made through the floodplain of the River Elbe, from the dike to the riverbank and on 6 positions showing elevated concentrations of heavy metals, the depth distribution (to 1.5 m) of other contaminants was investigated. The concentrations of musk fragrances were only in the range of the blank, 0.01 to 0.02 mg/kg (Witter et al. 2003).

In the state of Baden-Württemberg, Germany, 13 study locations were selected comprising a sewage sludge field with known history of sludge applications and a reference field. Total quantities of sludge applied to the fields ranged from 3.2 ton/ha to 31.5 ton/ha over a known number of years. The load on two experimental plots was 85 and 510 ton/ha. (N.B. the default load according to the EU-TGD is 5 ton/ha during 10 years). Concentrations were expressed as the sum of polycyclic musks (PCM). The concentrations in 6 sludge amended fields measured in 2002 were below 1 μg PCM/kg dwt except on one field (2.1 μg PCM/kg) where the concentration in the reference field was also elevated. Also the concentrations in the reference plots of the experimental areas were elevated: 0.54 μg PCM/kg ds. The concentrations in the experimental fields were 1.1 and 5.4 μg PCM/kg ds (LfU-BW 2003).

3.1.5.3 Comparison between predicted and measured levels

The predicted concentrations in agricultural soil after 10 years of sludge application are 0.014 and 0.027 mg/kg wwt (0.03 mg/kg dw) for the Northern and the Southern EU-15 Scenarios respectively. Measured concentrations in soil are scarce and hardly suitable for comparison. The observations from the field in the US where sludge is regularly applied twice per year show concentrations < 0.05 mg AHTN/kg dw after one half year. The study in Baden-Württemberg, Germany suggests that after applications similar to the scenario described in the TGD, concentrations were below 0.001 mg AHTN+HHCB/kg dw. The concentrations found in the floodplains of the river Elbe were below 0.01-0.02 mg/kg dw. It is concluded that all reported concentrations are below PEClocal. The detection levels limit the comparison with PECregional.

For the risk assessment the SEU-15 scenario will be used:

$PEClocal_{soil}$ = 0.027 mg/kg wwt.

3.1.6 Atmosphere

3.1.6.1 Calculation of PEC_{local}

3.1.6.1.1 Emission during production and fragrance compounding

During the production of AHTN, emission to the atmosphere may take place from the exhaust system and when stock containers are being filled. The emission from the exhaust filter was quantified on the basis of measured concentrations (max. 0.3 mg/Nm^3 (=Normal cubes)) and flow (1,510 Nm^3/h). The system runs 4480 h per year and therefore the estimated emission amounts to 2 kg per year. Stock containers are filled at a rate of 0.75 m^3/h during 8h/d, 7d per week and 40 weeks/yr. The vapour concentration measured inside the containers was 203 mg/m^3, resulting in an estimated emission of 0.34 kg per year. The total emission to air is 2.4 kg/y or 0.0004% of the total production (Van Hooren 2001).

During compounding of AHTN, emission to the atmosphere may take place from the ventilation systems of the plant. The air passes filtration systems before emission, but no information is available on the concentration in filtered air. Therefore estimates are based assuming no removal in the filter systems. The highest emission as percentage of use estimated in site 1-6 is 0.06%. This emission is taken as default for the generic scenarios.

Table 3.23 Emission to air

	Air flow/hour · hours/day · days/year	**Measured conc. in air (indoors)**	**Estimated emission per year**	**% of use**
site 1	74,000 · 10 · 240 = 177.6 · 10^6 m^3		<0.001% of use for HHCB → < 45 g AHTN	
site 2	50,000 · 16 · 250 = 200 · 10^6 m^3	AHTN < 0.03 mg/m^3	< 0.0002% of use for HHCB → < 12 g AHTN	
site 3	300,000 · 16 · 250 = 1.2 · 10^9 m^3	max. 10 mg/m^3 total org. material (licence)	max 12,000 kg/yr, 0.16% of use volume is AHTN = 19.2 kg	max 0.06%
site 4	total 50,750 m^3/h in various rooms	av. 0.007 – 0.012 mg/m^3	max. 2.25 kg	max. 0.01%
site 5	6000 · 14 · 250 = 21,000 m^3	0.012 mg/m^3 (other site)	0.25 kg	0.003%
site 6	natural		47 kg	0.05% estimated for total loss

3.1.6.1.2 Calculation of PEC_{local} for end product formulation

A specific large end product formulator used 5% of the use volume in 345 days per year. The emission factor (ESD in TGD) is 0.00002. Therefore the emission per year is 358 ton · 0.05 · 0.00002 = 358 g AHTN/year. Accordingly, the daily emission is calculated to be 1.04 g AHTN/day (based on formulation 345 days per year).

For a larger small-scale formulator the use volume is 1430 kg per year. Therefore the emission is 0.00002 · 1430 kg = 29 g AHTN/year.

3.1.6.1.3 Calculation of PEC_{local} for private use

In the intial calculations the tonnage used in compounding was completely discharged to the sewer, implying that there was only an indirect release to air from the STP and from evaporation after partitioning over the environmental compartments. With the refinements described in sections 3.1.4.1.3 and 3.1.4.1.4 the release factors were adapted in a way that equal fractions were released to waste water and air. The daily release to air and the regional and local concentrations in air due to consumer use of AHTN are summarised in **Table 3.24**

Table 3.24 Release to air and concentrations in air after consumer use

Scenario	Release factor to air *	Regional release to air, kg · d^{-1}	Regional PECair mg · m^{-3}	Local PECair mg · m^{-3}	Total deposition flux, mg · m^{-2} · d^{-1}
TGD regional (10%)	0	0	4.84 E-07	5.50 E-06	7.33 E-06
southern EU-15	0.065	6.38	1.17 E-07	3.66 E-06	5.65 E-06
northern EU-15	0.03	2.94	4.83 E-08	1.68 E-06	2.61 E-06

- based on Figure 3.5.

The predicted concentrations in air **PEClocal $_{air}$** related to private use in northern and southern European countries range from 1.7 to 3.7 ng/m^3.

3.1.6.2 Measured levels

Concentrations were measured in ten ambient air samples taken in the south of Norway in 1998. The concentrations were below 0.01 ng/m^3 (Kallenborn et al. 1999, Kallenborn and Gatermann 2004). For comparison, median and maximum concentrations in indoor air were 44 and 107 ng/m^3 (Fromme et al. 2004).

Concentrations were measured in ambient air sampled above the water of Lake Michigan during June 1999 and May 2000. Samples were also taken in urban Wilwaukee in June 2001.. The concentration in the gas phase of the urban area was 2.5 ± 1.0 ng/m^3 and 0.49 ± 0.3 ng/m^3 over the lake. The airborne particulate-phase concentration was 0.42 ± 0.20 ng/m^3 or 1.4 ± 0.70 ng/mg. The fraction of AHTN in the airborne particulate phase was 17% (Peck and Hornbuckle 2004).

In a study for Greenpeace by TNO, rainwater samples (actual deposition) were taken on 47 locations in The Netherlands, on two locations in Germany and one in Belgium in February 2003. Rainwater was collected in funnels with a diameter of approximately 30 cm during a

few weeks. AHTN was found in almost all samples with concentrations ranging from <2 to 19 ng/l, median 4.1 ng/l (Peters 2003). In a confirmatory study the concentrations were measured on six locations in the centre of The Netherlands in February 2004. This time the concentrations ranged from 8.4 to 40 ng/l, median 13 ng/l. The higher levels in 2004 are explained by the weather conditions: sunny weather in 2003, whereas the wet and cloudy weather in 2004 would have caused a higher wash-out of the atmosphere resulting in higher concentrations in the precipitation (Peters 2004a).

The concentration in rainwater was also measured in the Nordic countries in 2002. The concentration was above the limit of quantitation (20 ng/l) only once in 23 samples (Iceland, 35 ng/l) (Mogensen et al. 2004). The validity of this value for a sample taken in the central highlands, a rural background/northern pristine area, may be disputable since this was the highest value by far in the data set whereas overall the concentrations reported for Iceland showed extremely low levels.

3.1.6.3 Comparison between predicted and measured levels

The concentrations observed in ambient air in Norway are below PECregional air. The concentrations over Lake Michigan were below PEClocal level in the TGD scenario and just at PECregional in the TGD scenario (but conditions are not related).

From the concentrations measured in rainwater a wet deposition flux may be derived, assuming 700 mm rain/year. 700 mm per year equals 1.92 l of rain per m^2 per day. With the medians of 4.1 and 13 ng/l of rain, the deposition is 0.007 - 0.025 µg/m^2/d. These results are above the total deposition flux estimated for The Netherlands by a factor of 2 to 10. In view of the variability in weather conditions, rainfall, sunshine, the results seems to match relatively well.

3.1.7 Secondary poisoning

3.1.7.1 Calculation of PECs

The measured BCF for fish is 597 l/kg. The BCF for earthworms is estimated from K_{ow} (see section 3.1.3.4.3), resulting in a value of 3015 l/kg wwt.

$PECfish = PECwater * BCF_{fish} * BMF$

where:

BCF_{fish} = 597 l/kg ,

$PECwater = 0.5 (PECwater_{local} + PEC\ water_{regional})$.

BMF, the biomagnification factor, depends on BCF_{fish}. With BCF < 2000, BMF = 1.

Likewise, PECworm is calculated on the basis of the concentration in porewater:

$PECworm = Cporewater \cdot BCF_{worm} + F$ (F is an additional correction for gut contents),

BCF_{worm} = 3015 l/kg wwt,

$Cporewater = 0.5\ (Cporewater_{local} + Cporewater_{regional})$ (agricultural soil),

The results are presented in **Table 3.25.**

Table 3.25 PECs in fish and worm

Scenario	PECwater $_{local}$ (µg/l)	PECwater $_{regional}$ (µg/l)	$PEC_{oral,fish}$ mg/kg wwt	PECground-$water_{local}$, µg/l	PECground-$water_{regional}$ µg/l	$PEC_{oral,worm}$ mg/kg wwt
Production	0.021	0.00173	0.00675	0.00080	0.00022	0.00141
Compounding Site 1	0.008	0.00785	0.00473	0.175 E-03	0.00030	0.00245
Compounding Site 2	0.059	0.00173	0.0182	0.0022	0.00022	0.00327
Compounding Site 3	0.002	0.00173	0.00115	0.0132	0.00022	0.0186
Compounding Site 4	0.009	0.00173	0.00326			
Compounding Site 5	0.010	0.00785	0.00525	0.39 E-03	0.00030	0.000846
Compounding Site 6	0.008					
Compounding Site 7 (Large-medium generic)	0.42	0.00785	0.128	0.14	0.00030	0.194
Compounding Site 8 (Small generic)	0.069	0.00785	0.024	0.0209	0.00030	0.0293
Formulation Large company	0.109	0.00785	0.0335	0.0342	0.00030	0.0477
Formulation Generic scenario	0.123	0.00785	0.039	0.0418	0.00030	0.0581
TGD regional (10%)	2.02	0.0844	0.628	0.808	0.0061	1.12
southern EU-15	0.132	0.00785	0.0418	0.051	0.00030	0.0709
northern EU-15	0.053	0.00173	0.0165	0.0274	0.00022	0.0381

3.1.7.2 Monitoring data

For earthworms or other terrestrial invertebrates, no measured concentrations are available.

Monitoring data are available for fish and shellfish, sampled in Germany, The Netherlands, Italy, Norway and Czech Republic. A distinction is made between eel and other fish species because the lipid content of eel is considerably higher than for the other species. Therefore, even though the concentrations of AHTN in lipids sometimes seems to be lower for the eel than for the other species, the total body burden of the eel (fresh weight) is always higher.

Fish samples were taken in the River Ruhr, Germany. Species were chub, crucian carp, perch, bream, roach, pike and eel. Concentrations were determined in the edible parts (muscle), in adipose tissue and the liver and the results were expressed as the concentration in fat. median concentrations were 134 µg/kg wwt for eel and 29 µg/kg wwt for the other fish (Eschke *et al*. 1995).

Rimkus (1997) presented data for fish sampled from fish ponds in Denmark (rainbow trout), eel, pike and perch from the River Elbe, brown trout from the River Stör, 3 km downstream of a STP and herring from the East Sea and seas around Denmark and Ireland. Using the fat content from the publications, these concentrations were recalculated to fresh weight concentrations. The concentrations in fish from the Elbe are clearly below those found in the river Ruhr by Eschke et al. (1995) by a factor of 10. Rimkus as well as Eschke reported elevated concentrations from fish found in effluent ponds that are related to the elevated concentrations found in effluents. In addition Rimkus (1999) reported on concentrations in shellfish: in the blue mussel (*Mytilus edulis*) found on the North Sea coast, the concentration was < 30 – 60 µg/kg lipids, whereas in shrimps (*Crangon crangon*) the concentration ranged from < 40 – 60 µg/kg lipids.

Eel caught in the main waters of The Netherlands were analysed by Rijs and Schäfer (1998). Concentrations were below those in the Ruhr by a factor of 4.

Eel and other fish were sampled in Berlin (Fromme et al., 1999, 2001a). A classification was made according to the fraction of effluent input into the water system. Of 165 eel samples, the median was 0.04 mg/kg wwt, 90th-percentile 0.89 mg/kg wwt. In 43% the AHTN concentrations were below the detection limit of 0.02 mg/kg wwt. For the other species sampled (176 samples), 54% was below the detection limit and the 90th-percentile was 0.05 mg/kg. Median concentrations in eel in the high effluent input areas (up to 97%) were above those in the Ruhr by a factor of 5, whereas for the other fish species the one with the highest concentrations, the common bream, the median concentrations were higher than the median in the Ruhr by a factor of 10. In his review Heberer (2002) gives the overall median and 95th-percentile of 324 fish samples as 0.04 and 0.90 mg/kg wwt respectively, or 0.55 and 10.8 mg/lg lipids.

In Italy, fish were sampled in eight Italian rivers and lakes. The median concentration in 28 fish samples was 0.004 mg/kg wwt (Draisci et al. 1998).

In the Czech Republic a monitoring programme on fish was set up. In a first publication on the River Elbe, the highest concentrations were found downstream of a detergent production plant: 3.2 mg/kg lipids (Hajslova et al. 1998). Within 4 years, from 1997 to 2000, over 800 fish were collected using electrofishing on four sites on the Elbe River, four sites on the Moldau River and three sites on the Tichá Orlice River. Fish species sampled were Chub, Bream, Barbel, Perch and Trout. The monitoring continued for three or four consecutive years, from 1997 to 2000. Concentrations were reported in mg/kg lipids but other characteristics including the lipid content was reported as well. Median concentrations ranged from 0.2 to 11.4 mg/kg lipids. The highest concentrations were found in Barbel, a bottom-dwelling species. A significant correlation existed between the concentrations in sediment on a site and the fish collected there. It was also observed that different age/size classes (younger and older fish) showed no differences in tissue levels. This demonstrated that the uptake is due to bioconcentration only and not to biomagnification through the food chain (Hajslova and Setkova 2004). A decrease in time in the concentrations in fish was *not* observed, suggesting that the downward trend observed in northern Europe has not occurred in the Czech Republic.

Eel were sampled in the River Elbe near the Czech border and in the Stör, a tributary downstream of Hamburg, Germany, in 2001. The median concentration in eel muscles caught near the Czech border was 0.013 mg/kg wwt, whereas the maximum was 0.024 mg/kg wwt. More downstream, the median concentration in eel from the Stör was lower, 0.003 mg/kg wwt, maximum 0.020 mg/kg wwt. In 5 tributaries along the Elbe bream were caught in 2000

and 2001. The median concentrations varied from 0.002 to 0.006 mg/kg wwt, whereas the maxima ranged from 0.004 to 0.044 mg/kg wwt. Flounders were caught on two sites in the Elbe estuarium. AHTN was detected in 11 out of 14 flounders caught near the mouth of the Stör and the median concentration was around the detection limit of 0.001 mg/kg wwt, the maximum was 0.004 mg/kg wwt. Further out into the sea, AHTN was detected in only 4 out of 16 fish. The median was below the detection level, the maximum 0.004 mg/kg wwt (Wiegel and Stachel 2002).

Samples of marine organisms taken on three sites along the shore of the Wadden Sea and the Baltic Sea in northern Germany showed AHTN at detectable levels in bladder wrack (*Fucus vesiculosus*, brown algae) only in 1996, not in 1998 and 2000 and only on one site at 0.0003 mg/kg wwt. Between 1994 and 2000, the concentration in eelpout samples ranged from 0.0002 to 0.001 mg/kg wwt. In blue mussles (*Mytilus edulis*) the concentration varied between 0.0004 and 0.0025 mg/kg between 1986 and 2000, in general showing a downward trend. At another location the levels turned below detection levels in 1998 - 2000. The concentrations in herring gull (*Larus argentatus*) eggs in the same area ranged from 0.0006 – 0.002 mg/kg wwt, apparently at the same level of their food organisms (Müller et al. 2004, Rüdel and Schröter-Kermani 2004).

In a recent publication of the same work Rüdel et al. (2006) present extensive results on biotic samples in the German Environmental Specimen Bank from the period of 1986 to 2000, The levels systematically decreased from a maximum around 1994-96 to clearly lower levels in this century. The most conspicuous was the decrease of AHTN in bream in the River Saale which decreased by 87% from 4.8 to 0.6 mg/kg lipids between 1995 and 2003.

A recent report on concentrations in trout from Danish farms showed a downward trend with concentrations of 1.1 µg/kg wwt in 1999 to less than 0.2 µg/kg wwt in 2003/2004. In 1999, AHTN was detected in 98% of all samples, whereas in 2003/2004 it was detected only in 34% of all samples (Duedahl-Olesen et al. 2005).

Fish were caught in remote alpine lakes in Switzerland that are exclusively fed by direct or indirect atmospheric input. In seven alpine lakes between 2000 and 2650 meter above sea level, fish were caught in August and September 2003: brown trout (*Salmo truuta fario*), alpine char (*Salvelinus alpinus*), brook trout (*Salvelinus fontinalis*), lake trout (*Salvelinus namaycush*). The average fat content of the fish ranged from 1.3 to 3.8% in the different lakes. The sum of the concentration of AHTN and HHCB ranged between 0.064 to 0.12 mg/kg lipids for 6 lakes (Schmid et al. 2004). In the fish in one lake a relatively high level (0.28 mg/kg lipids) was observed which was not at all in proportion to the concentrations of the other substances in the study. This seems to suggest an additional route of entry next to the atmospheric deposition. The study included no analytical control samples so contamination can not be ruled out.

Schmid et al (2007) produced a new publication on the same study results. In this paper the individual polycyclic musks were quantified in fish caught in the different lakes. In comparing both papers we remark that the lake originally showing the relatively high level was now recognised as an area with slight tourism, but at the same time apparently the samples had been exchanged and thus the concentration was reduced to the level found in the other lakes. The elevated concentration is now attributed to a lake nearby a pass road. It is remarkable that in particular HHCB is elevated (0.23 as compared to the median 0.050 mg/kg lipids), whereas for AHTN the increase is less clear (from median 0.027 to 0.05 mg/kg). Usually the concentrations of the different musks are correlated but in this case neither the

other polycyclic musks nor the nitromusks show an increase in this sample suggesting that some external factor is the cause of the elevated concentration.

In Nevada in 2001, each month seven to eight carp (*Cyprinus carpio*), 2 kg each, were caught in Lake Maed. The concentration of AHTN as well as the total lipid content was determined. The concentration of AHTN ranged from 0.0014 in October-November (lipid content 11%) to 0.0036 mg/kg wwt in February-March (lipid content 17 – 20%) (Osemwengie and Gerstenberger 2004).

Concentrations found in fish are summarised in **Table 3.26.** Concentrations are presented in mg/kg fresh weight and/or mg/kg lipids.

Table 3.26 Monitoring data for AHTN in fish (mg/kg)

Sample	N	AHTN		Reference
		mg/kg lipids	mg/kg fresh weight	
Germany, RIver Ruhr Non-eel	7	median 3.5 (2.2 - 7.1)	median 0.029 (0.018 – 0.034)	Eschke *et al*., 1995
Germany, River Ruhr, Eel	2	mean 0.6 (0.5 – 0.7)	mean 0.13 (0.119 – 0.146)	
Non-eel fish effluent pond	8	median 15.3 (3.0 – 37.2)	median 0.29 (0.07 – 0.647)	
Eel from effluent pond	5	mean 36 (10.2 – 57.9)	mean 10 (2.39 – 17.49)	
Denmark fish pond Rainbow trout	4	mean 0.36	mean 0.011	Rimkus, 1997
Germany River Elbe, Eel	5	mean 0.056	mean 0.016	
River Elbe Non-eel	4	mean 0.58	mean 0.004	
Fish river Stör near STP outfall	3	mean 14.0	mean 0.28	
East Sea Herring	1	0.53	0.046	
Denmark Herring	1	0.07	0.005	
Ireland Herring	1	<0.01	<0.0008	
The Netherlands Eel	6		median 0.03	Rijs and Schäfer 1998
Germany Berlin area, Eel , 1996-1997	54	low effluent input median < d.l. 90-perc. < d.l.	low effluent input median < d.l. 90-perc. < d.l.	Fromme et al. 2001b
	53	moderate effluent input median 0.186 90-perc. 0.545	moderate effluent input median 0.032 90-perc. 0.112	
	58	high effluent input median 2.833 90-perc. 5.170	high effluent input median 0.668 90-perc. 1.380	

Sample	N	AHTN		Reference
		mg/kg lipids	mg/kg fresh weight	
Germany, Berlin area, Other fish 1996-1997				
Perch	19	low effluent input median < d.l. max. < d.l.	low effluent input median < d.l. max. < d.l.	
	9	high effluent input median 7.1 max. 43.7	high effluent input median 0.047 max. 0.332	
Common bream	37	low effluent input median < d.l. max. 2.7	low effluent input median < d.l. max. 0.042	
	10	high effluent input median 18.4 max. 35.3	high effluent input median 0.324 max. 0.851	
Roach	48	low effluent input median < d.l. max. 2.0	low effluent input median < d.l. max. 0.050	
	6	high effluent input median 4.5 max. 18.4	high effluent input median 0.064 max. 0.339	
Pike	12	low effluent input 'all –1' < d.l max. 2.2	low effluent input 'all –1' < d.l max. 0.021	
	2	high effluent input 8.0 – 10.0	high effluent input 0.044 – 0.060	
Pike perch	25	low effluent input median < d.l. max. < d.l.	low effluent input median < d.l. max. < d.l.	
	8	high effluent input median 10.0 max. 88.3	high effluent input median 0.037 max. 0.362	
All fish in Berllin area	351		90th percentile 0.57	
Germany, Elbe various tributaries 2000 – 2001			eel median 0.003 – 0.013 max 0.020 – 0.024 bream med 0.002 – 0.006 max 0.004 – 0.044	Wiegel and Stachel 2002
	30		flounder median ≤ 0.001 max 0.004	
Italy, various waters	28	median 0.132 90-perc. 1.075 <0.2 – 4.0	median 0.004 90-perc. 0.026 nd – 0.105	Draisci et al. 1998
Switzerland 2003 7 alpine lakes		median 0.08 range 0.064 – 0.12 * for sum of AHTN and HHCB		Schmid et al. 2004 * sample with max 0.29 out of range with other substances

Sample	N	AHTN		Reference
		mg/kg lipids	mg/kg fresh weight	
		median 0.027 range 0.020 – 0.038 extreme 0.054		Schmid et al. 2007 (revision of Schmid et al. 2004)
Czech Republic				Hajslova and Setkova 2004
Chub	302	median ranges 0.6 – 2.4		
Bream	164	median ranges 0.9 – 3.5		
Barbel	50	median ranges 0.5 – 11.4		
Perch	156	median ranges 0.4 – 3.7		
Trout	117	median ranges 0.3 – 3.1		
Norway				Kallenborn et al. 2001
Thornback ray filet	1	0.089	0.0008	
Haddock filet	2	mean 0.254	mean 0.0014	
Atlantic cod filet	3	mean 0.008, max. 0.010	mean 0.002, max 0.003	
Saithe filet	1	0.093	0.002	
Thornback ray liver	1	0.003	0.001	
Haddock liver	3	mean 0.024, max. 0.034	mean 0.0162, max. 0.023	
Atlantic cod liver	13	mean 0.096, max. 0.38	mean 0.035, max. 0.13	
Saithe liver	1	0.001	0.0004	
USA Nevada *Cyprinus carpio*	12 · 7		0.0014 – 0.0036	Osemwengie 2004

In Norway a total of 10 pooled Atlantic cod liver samples (*Gadus morhua)* was collected from harbours, fjords and the open coast, winter 1997/1998. The samples were collected in recipients for industrial and municipal wastewater. Seven fish samples were taken from an inner harbour (two thornback ray (*Raja clavata)* and 5 haddock (*Melanogrammus aeglefinus*)), and 8 fish samples (6 Atlantic cod and 2 Saithe (*Pollachius virens*) were from another harbour in 1999. Concentrations were estimated in liver and filet and expressed on the basis of lipid content (Kallenborn et al. 2001). With the lipid content given in the paper the figures were converted to freshweight concentrations. Due to a lack of details the fresh weight data are probably overestimating the real values.

In a large monitoring programme in the Nordic countries blue mussels were sampled in 20 sites in Denmark, Sweden, Norway, Finland and Iceland in the vicinity of urban as well as pristine areas. AHTN was detected in 5 samples but below the Limit of Quantitation (> 0.18 and < 0.53 mg/kg lipids) (Mogensen et al. 2004). In the same programme red fox or polar fox livers were sampled and analysed. AHTN was not detected in any of the 15 samples (< 0.77 mg/kg lipids, Mogensen et al. 2004).

The occurrence of AHTN was reported in the liver of a Danish otter. The concentration of AHTN was 95 mg/kg lipids (Leonards and De Boer 2004).

The concentration of AHTN was determined in Canadian and Arctic samples of Ringed Seal blubber, Arctic char, blue mussels and lake trout. The values for AHTN were not reliable due

to matrix co-elution problems. The concentration of AHTN in the char, blue mussels and trout seemed to be elevated as compared to the background, whereas the concentration in ringed seal was at the background level (Hühnerfuss et al. 2002), giving no indication for biomagnification.

BAFs and BMFs in Beluga whale (*Delphinuapterus leucas*) tissues were substantially lower than expected based on the physical-chemical properties indicating metabolic transformation (Kelly et al. 2004).

Concentrations were determined in samples of mammals and sharks collected from Japanese coastal waters. AHTN was detected only in one adult at the detection limit (9.6 µg/kg wwt) in 11 samples of Finless Porpoises and their fetuses that were stranded along the coastal area or accidentally caught by fishing nets during 1999 and 2002. In the one adult porpoise AHTN was detectable in the blubber only (lipid content 83%), and not in any of the other tissues. Hammerhead sharks were collected from a Japanese coastal water in 2004. The AHTN level in their livers (27-58% lipid content) was also below the detection limit (< 9.1 µg/kg lipids, Nakata 2005).

AHTN concentrations were determined in tissues from marine mammals, water birds and fish collected from US waters. Concentrations were between < 1 and 2.7 µg/kg wwt in the liver of seals, sea lions, Atlantic sharpnose shark, river otter, mink, common merganser, lesser scaup, greater scaup and mallard. The same levels were found in Atlantic salmon and smallmouth bass. No data were available for the blubber of bottlenose dolphin and striped dolphins in Florida, whereas the level in the liver of a pygmy sperm whale was below the detection limit (< 1 µg/kg wwt). This was also the case for the livers of polar bear (*Ursus maritmus*) from the Alaskan Arctic (Kannan et al. 2005).

Female Chinese sturgeons (*Acipenser sinensis*) were caught between 2003 and 2005, ranging in weight from 140 to 263 kg (total length 2.85 – 3.39 m), age 17 to 25 years old (Wan et al. 2007). They migrate between the sea and their spawning grounds in the Yangtze river at intervals of more than 2 years. No details on the sampling place and times were given. The highest levels of AHTN were detected in all 4 adipose tissue samples and in some of the 11 roe samples. Means were 2.4 and 2.2 µg/kg wwt, respectively. Lower levels were found in 2 out of 7 liver samples, but not in muscle, heart, gonads, stomach, intestines, gills, pancreas, kidney and gallbladder. The concentrations in adipose tissue are below those found in fish in Europe but they were higher than those in blubber of dolphins and whales on the coast of Florida. The concentration of AHTN in liver was below those in liver tissues of hammerhead sharks along the Japanese coast. When expressed on a lipid weight basis, the concentrations of AHTN were similar among the different tissues. For fish, increasing trends with age are found for truly persistent substances like DDT, HCB and some PCBs in both female and male brown trout. However, in this study no statistical relationship was found. The concentration ratios for roe to adipose tissue in two fish were 0.39 to 0.78 (Wan et al. 2007). The age-related accumulation is not evident from the figures, the variation is very high and r^2 is only 0.13. It is known that fish are able to metabolise AHTN with a short half-life period, so the variable concentrations will reflect the recent exposure more then their life history.

Bioaccumulation of AHTN in a marine food chain was investigated by analyzing marine organisms at various trophic levels including lugworm, clam, crustacean, fish, marine mammal and bird samples collected from tidal flat and shallow water areas of the Ariake sea, Japan between 2000 and 2005 (Nakata et al. 2007). Tissues of whole body, soft tissue, hepatopancreas, liver or blubber were analysed. The highest concentrations were detected in clams (converted from freshweight results, $\leq$180 µg/kg lipids) whereas the levels in mallard

and black-headed gull were low (< 4 µg/kg lipids), mainly below the detection limits and comparable with concentrations in fish and crab. It is concluded that no trend of increasing concentrations at higher trophic levels could be detected. In analogy to the findings for HHCB, it is concluded that bioaccumulation of AHTN in the food chain does not occur and that there is a lack of long-term transportation potential in the environment (Nakata et al. 2007).

3.1.7.3 Comparison between predicted and measured concentrations

Concentrations measured in fish are reported from both very heavily polluted areas and from more remote regions, in Germany, The Netherlands, Italy, Switzerland, Czech Republic, Norway, the North Sea and USA. AHTN was detected in most samples except in fish caught in remote areas, lakes and on sea. The highest concentrations by far were observed in the areas classified as 'high effluent input' areas in Berlin, Germany, in 1996-1997. The levels found in the Czech Republic (1997-2000) are reported based on the fraction of lipids. The data for the species that are shared with the Berlin study indicate that the maximum levels in Czech fish are below those in fish from the high effluent input area in Berlin by a factor of 10. It has been shown that the levels in effluents discharged into the high input areas in Berlin have decreased considerably, as is also reflected in the current sediment concentrations in the Teltow Canal. Thus it may be expected that the levels in fish are also reduced considerably.

No recent data are available for comparison with the Northern European Scenario (0.0165 mg/kg wwt). When comparing $PECoral_{fish}$ for the Southern European Scenario (0.04 mg/kg wwt) to the data other than from the high effluent input area in Berlin, this PEC is exceeded also in areas with lower levels of contamination. Apparently the input for the predictions (the current effluent and sludge concentrations in southern Europe) is at a lower level than it was in Germany at the time the fish were sampled. The data in **Table 3.16** and **Table 3.17** confirm that this is the case indeed.

Thus for the risk assessment the Southern EU-15 Scenario cannot be used. The risk assessment will be based on the TGD regional (10%) scenario since it covers all monitoring data except for some historic extremes in the Berlin area:

$PECoral_{fish}$ = 0.628 mg/kg wwt.

The 90th-percentile for all fish in the Berlin area (1996/1997) was 0.57 mg/kg wwt.

3.1.8 Marine compartment

For an assessment of the exposure of the marine environment a local exposure assessment was performed for the generic compounding sites (site 7 and 8), for the generic formulators and for the private use scenarios for northern and southern European countries.

3.1.8.1 Compounding and formulation

For a default assessment industrial trade effluents of sites along the coast are not treated in a municipal biological STP. The dilution factor of the effluent in the marine environment is 100 (instead of 10 in the freshwater environment). In the standard freshwater environment the water flow is 18,000 m^3. After discharge of the STP (2000 m^3), the water flow becomes 20,000 m^3 per day. The TGD (EC 2003) assumes that the dilution factor in the marine

environment is 100 instead of the factor of 10, so the water flow for dilution in the marine environment is 200,000 m^3 per day. By default the dilution factor for mixing of river water into the coastal sea is 10, so $PECregional_{seawater} \simeq 0.1 \cdot PECregional_{water}$. $PECregional_{seawater}$ is estimated by EUSES.

Since the losses in the trade effluents are not treated in a municipal STP, the amounts released from the generic sites are directly diluted in 200,000 m^3 of seawater. Most of the characteristics of the coastal environment (regional) are similar to the freshwater compartment apart from the suspended matter concentration. The concentration of suspended matter in the local coastal environment is 15 mg/l and in the regional marine zone it is set to 5 mg/l. The results of the default calculations are given in **Table 3.27**.

Table 3.27 PEClocal for the marine environment, compounding and formulation (default scenario without treatment in STP) for the year 2000

Site	Emission kg/d [in 200,000 m^3]	$Clocal_{seawater}$ µg/l (total) → Clocal (diss)	$PECregional_{sea\ water}$ (diss), µg/l	$PEClocal_{sea\ water}$ µg/l	Marine $Clocal_{sediment}$ (equil. part.) mg/kg wwt	PECregional mg/kg wwt	$PEClocal_{sediment}$ mg/kg wwt
Compounding Site 7 (Large-medium generic)	0.0384	0.192 → 0.184	0.000718	0.184	0.119	0.000731	0.120
Compounding Site 8 (Small generic)	0.0057	0.0287 → 0.0274	0.000718	0.0282	0.018	0.000731	0.018
Formulation Large company	0.0094	0.045 → 0.043	0.000718	0.0437	0.0284	0.000731	0.0284
Formulation generic	0.0114	0.057 → 0.0546	0.000718	0.0553	0.036	0.000731	0.036

A survey was carried out to verify whether compounders or formulators using AHTN and HHCB discharge their wastewater into the marine environment only after treatment in a sewage treatment plant (Letter of IFF and PFW, July 2006). The conclusions of the survey are that in the EU-15+2:

- No compounder discharges its wastewater directly into the marine environment;
- Of the more then 1000 formulators, only 16 are located in a radius of 500 metres from the sea/ocean shore. These are among the very small formulators: the **total** amount used by these formulators is below the volume used in the generic scenario by more than an order of magnitude.
- The treatment of wastewater before discharge is common practice in this industry chain.

When the presence of an STP is taken into account in the calculations, PECmarine roughly equals $0.1 \cdot PECfreshwater$ (see **Table 3.11**). As the fraction discharged with the effluent is 0.206 (according to EUSES, see **Table 3.8**), the values after treatment are roughly 0.206 of the values predicted in **Table 3.27** for the default scenario, see **Table 3.28**.

Table 3.28 PEClocal for the marine environment, compounding and formulation (scenario with wastewater treatment) for the year 2000

Site	Emission kg/d	$Clocal_{seawater}$ µg/l (total) → Clocal (diss	PECregional $_{sea\ water}$ (diss), µg/l	$PEClocal_{sea\ water}$ µg/l	Marine $PEClocal_{sediment}$ (equil. part.) mg/kg wwt
Compounding Site 7 (Large-medium generic)	0.0384 *0.206 = 0.008	0.0396 → 0.0379	0.000718	0.0386	0.025
Compounding Site 8 (Small generic)	0.0057 *0.206 = 0.0012	0.0059 → 0.0056	0.000718	0.0063	0.004
Formulation Large company	0.0094 *0.206 = 0.0019	0.0097 → 0.0093	0.000718	0.0100	0.006
Formulation generic	0.0114 *0.206 = 0.0023	0.0117 → 0.0103	0.000718	0.0110	0.007

3.1.8.2 Local emissions from private use

According to the TGD (EC 2003), it can be assumed that for releases to municipal waste water of substances used for private or public use (IC5 and IC6), the degree of treatment in a biological STP corresponds to the inland scenario. Therefore the effluent concentration from the STP is used as a starting point for the assessment. $PEClocal_{seawater}$ (dissolved) is simply derived from Ceffluent with a dilution factor of 100 and a correction for the sorbed fraction, see **Table 3.29.**

Table 3.29 PEClocal for the marine environment, private use

Scenario	Ceffluent STP, µg/l	$Clocal_{sea\ water}$ µg/l (total) → Clocal (diss)	Marine PECregional (diss) µg/l	Marine PEClocal µg/l	Marine $Clocal_{sediment}$ (equil. part.) mg/kg wwt	Marine PECreg. sediment mg/kg wwt	Marine PEClocal $_{sediment}$ mg/kg wwt
TGD regional (10%)	20.2	0.202 → 0.193	0.00759	0.201	0.130	0.0077	0.138
southern EU-15	1.30	0.0130 → 0.0124	0.000718	0.0131	0.008	0.000731	0.0087
northern EU-15	0.54	0.0054 → 0.0052	0.000156	0.0054	0.004	0.000158	0.004

3.1.8.3 Secondary poisoning

According to the TGD (EC 2003) the concentration in the food of predators and top-predators is calculated from:

$$PECoral_{predator} = 0.5 \cdot (PEClocal_{seawater} + PECregional_{seawater}) \cdot BCF_{fish} \cdot BMF_1$$

$$PECoral_{top\text{-}predator} = (0.1 \cdot PEClocal_{seawater} + 0.9 \cdot PECregional_{seawater}) \cdot BCF_{fish} \cdot BMF_1 \cdot BMF_2$$

BMF is the biomagnification factor in fish (BMF_1) and in the predator (BMF_2). The value of the BMF is determined by the BCF_{fish} as in this way the potential for metabolism in biota (i.e. fish) is taken into account. The BCF for AHTN is below 2000, leaving both biomagnification factors at 1.

Accordingly, $PECoral_{predator}$ and $PECoral_{top\text{-}predator}$ are calculated for all scenarios taken in consideration for the marine risk assessment, see **Table 3.30**.

Table 3.30 Predicted concentrations in fish, exposure of marine predators

Scenario, mg /d per capita	**PECregional seawater, µg/l**	**PEClocal seawater, µg/l**	**PECoral predator mg/kg**	**PECoral top-predator mg/kg**
Production, compounding and formulation				
Compounding Site 7 (Large-medium generic)	0.000718	0.184	0.0554	0.0114
Compounding Site 8 (Small generic)	0.000718	0.0274	0.0086	0.0021
Formulation Large company	0.000718	0.0437	0.0133	0.0030
Formulation generic	0.000718	0.0553	0.017	0.0037
Private use				
TGD regional (10%)	0.00759	0.201	0.062	0.0161
southern EU-15	0.000718	0.0131	0.0041	0.0012
northern EU-15	0.000156	0.0054	0.0017	0.00041

3.2 EFFECTS ASSESSMENT: HAZARD IDENTIFICATION AND DOSE (CONCENTRATION) - RESPONSE (EFFECT) ASSESSMENT

3.2.1 Aquatic compartment (incl. sediment)

It should be noted that AHTN is reported to be photodegradable (See section 3.1.3.1.1). Under atmospheric conditions the half-life of AHTN was determined to be 7.3 hours (Syracuse estimation programme AOP) and in a laboratory test with lakewater a half-life of 4 hours was reported (Buerge et al. 2003).

Generally, the wave length of UV ranges from 180 to 400 nm. The wave length of visible light is > 400 nm. The UV/Vis spectrum of AHTN (PFW Aroma Chemicals B.V. (2001), shows that there is absorption at wavelengths below 325 nm with peaks at 215, 257 and 297 nm. The molar absorptivity of AHTN decreased from 967 cm^{-1} $.M^{-1}$ at 297.5 nm to 20 cm^{-1} $.M^{-1}$ at 320 nm and extinguishes at higher wave lengths. The maximum specific rate of light absorption was given as 315 nm (Buerge et al. 2003). AHTN does not absorb at wavelengths above 325 nm (in the visible light spectrum).

In the laboratory test on photodegradation in water the light source was a mercury fluorescent lamp emitting UV-radiation between 300 and 460 nm, max 365 nm on the water surface or through a quartz test tube (Buerge et al. 2003). Hence, the photodegradation-induced half-life of 4 hours in the study by Buerge et al. is a consequence of absorption of UV light.

In alga growth inhibition tests generally fluorescent lighting tubes are used emitting wavelengths ranging between 400 and 700 nm at an intensity of approximately 8000 lux. Hence, the wave length of the illumination in the alga test is clearly above 325 nm, thus it will not cause photodegradation of the AHTN molecule. Moreover, there is a glass wall between the alga suspension and the light source, which would disrupt the penetration of UV radiation. The illumination in the other aquatic toxicity tests is at most daylight filtered by glass windows (aquaria) and glass vessel walls (so no UV light) or artificial light in the visible light spectrum. Hence, although photodegradation of AHTN may be considered a relevant degradation process in the atmosphere and in the upper layer of surfacewaters through penetration of UV light, it is concluded that photodegradation is of no significant influence on the outcome of the aquatic toxicity studies performed under normal laboratory conditions since the amount of UV light penetrating the test solutions will be negligible.

In view of the high log K_{ow}, the toxicity was not tested with short-term tests according to the base set, but in tests ensuring longer term exposure. Standard tests were carried out with algae, Daphnia and fish, and in addition a marine crustacean was tested. Because of the low water solubility of AHTN, it was necessary in the aquatic toxicity tests described below to prepare stock solutions using DMF as a solvent and Tween 80 as a dispersant or using triethylene glycol or ethanol as a solvent. These stock solutions were then diluted to reach the desired concentrations in the tests. The residual level of the solvent in the test vessel was always below the maximum level allowed by the test guidelines. Solvent controls containing equivalent levels of the solvents were used in all cases along with undosed water controls. AHTN has a strong tendency to sorb, thereby reducing the concentrations in solutions over time. Final concentrations in water were determined by HPLC or GC or, for radiolabelled AHTN, by LSC and the test concentrations are expressed as measured concentrations. Except

for the highest concentration in the fish growth test, the tested concentrations did not exceed the water solubility limit. The results are summarised in **Table 3.31**[13].

Table 3.31 Aquatic toxicity of AHTN (GLP and completely documented) Results expressed as mean measured concentrations

Test and reference	Results [1] [mg/l]	Remarks [2]
Pseudokirchneriella	*Test A*	carrier: 0.005% DMF and 0.005% Tween 80
subcapitata [3]	NOECr = 0.438	n=5
72-h static	NOECb = 0.204	*Test A*
Van Dijk 1997	LOECr = 0.797	HPLC identification
	LOECb[4] = 0.438	start conc. 81-90% of nominal
	ErC50 > 0.797	end conc. 31-85% of nominal
	EbC50 = 0.468 <0.434 - 0.508>	*Test B*
	Test B	start conc. 77-90% of nominal
	NOECr = 0.374	end conc. 53-142% of nominal
	LOECr[4] = 0.835	geometric mean NOEC = 0.276
	ErC50 > 0.835	DQ 1
	EbC50 ≈ 0.835	
Daphnia magna	NOECrep = 0.196, LOEC[4] = 0.401	carrier: 0.008% DMF and 0.002% Tween 80
21-d semi-static	ErC50 = 0.244 <0.239 - 0.249>	n=5
Wüthrich 1996a	IC50 = 0.341 <0.243 - 0.433>	HPLC identification
		conc.fresh 84-103% of nominal
		conc.used 70-85% of nominal
		DQ 1
Bluegill sunfish	NOECgrowth = 0.089, LOEC[4] = 0.184	carrier: 0.005% DMF and 0.005% Tween 80
Lepomis	LC50 = 0.314 <0.226 - 0.448>	n=5
macrochirus		HPLC identification
21-d flow-through		conc. 57-111% of nominal
Wüthrich 1996b		DQ 1
Fathead minnow	LOEChatch > 0.140	solvent triethylene glycol
Pimephales promelas	NOECsurv. = 0.067,	n= 5
flow-through	LOECsurv. = 0.140	GC identification
32 days post hatch,	LC50 = 0.100 <0.097 - 0.100>	conc. 55-108% of nominal
36 days overall	NOECgrowth = 0.035,	DQ 1

[13] For the qualification of the quality of the tests the following classification scheme was used: DQ 1: Valid without restriction. The test is carried out to internationally recognised protocols (or equivalent protocols) and all or most of the important experimental details are available; DQ 2: Use with care. The test is carried out to internationally recognised protocols (or equivalent protocols) but some important experimental details are missing, or the method used, or endpoint studied, in the test means that interpretation of the results is not straight forward; DQ 3: Not valid. There is a clear deficiency in the test that means that the results cannot be considered as valid; DQ 4: Not assignable. Insufficient detail is available on the method used to allow a decision to be made on the validity of the study.

Test and reference	Results [1] [mg/l]	Remarks [2]
Croudace 1997	LOECgrowth[5] = 0.067 NOECdevelop.= 0.035, LOECdevelop.= 0.067	
Zebrafish *Brachydanio rerio* 34 d intermittent flow-through Hooftman 1999	LOEChatch > 0.075 LOECsurv. > 0.075 NOECgrowth = 0.035 (length) LOECgrowth = 0.050 NOECdevelop. = 0.035 LOECdevelop. = 0.050	solvent triethylene glycol or generator column n= 5 HPLC conc. 104 – 110% of nominal DQ 1
Marine copepod *Acartia tonsa* 6d-static, daily feeding Bjørnestad 2007	NOECdevelop. = 0.022 LOECdevelop[4] .= 0.044 EC10develop.= 0.0282 <0.017 - 0.037> EC50develop. = 0.072 <0.061 – 0.087>	Radiolabelled ^{14}C-AHTN solved in ethanol (< 0.01%) n=7 LSC identification conc. > 80% of nominal DQ 1

1 measured concentrations, <95% confidence limits>

2 The number of concentrations tested (n) excludes control and solvent control

3 Former name *Selenastrum capricornutum*

4 Dunnet's test (p=0.05)

5 Wilcoxon rank sum test (p=0.05)

In addition to the above tests that were carried out under GLP and concentrations controlled by analyses, in the meantime more test results are reported. The experiments are summarised here (**Table 3.31**). These studies have in common that they are presented in a highly aggregated form and sufficient details are not always available. Examples of the information that is sometimes unavailable are control survival or performance, dose-response relationships, variability of replicates, or actually measured concentrations.

Table 3.32 Other aquatic toxicity tests (details not complete)

Test and reference	Results [1] [mg/l]	Remarks [2]
Midge larvae, 4th intstar *Chironomus riparius* 96h, semi-static Artola 2002	No effects up to 0.5 mg/l (max. water solubility)	solvent iso-propanol n = 6 GC/MS identifcation DQ 4: publication does not specify more details. Insufficient detail to assess validity of test
Worm *Lumbriculus variegatus* 5-d, static Artola 2002, 2003	EC50immob. = 0.397 <0.396 – 0.399 >	solvent iso-propanol n = 6 GC/MS identification DQ 4: publication does not specify more details. Insufficient detail to assess validity of test
Marine copepod	LC50 = 0.61 <0.58 – 0.65>	solvent acetone (<0.01%)

Test and reference	Results [1] [mg/l]	Remarks [2]
Nitocra spinipes 96-h, static Breitholtz et al. 2003		No analysis, results expressed as nominal concentrations 96h-LC50: DQ 2
Marine copepod *Nitocra spinipes* 7–8.5 d, partly renewal Breitholtz et al. 2003	NOEC larval devel. > 0.06 NOEC pop. growth > 0.06	solvent acetone (<0.01%) n = 4 GC/MS identifications, results expressed as nominal concentrations DQ 3: Concentration levels could not be maintained (circa 20% of nominal), presence of organic matter caused also oral exposure; not a water-only test
Marine copepod *Acartia tonsa* 48-h, Wollenberger et al. 2003	LC50 = 0.71 <0.62 – 0.83> LC10 = 0.45 ,<0.30 – 0.55>	solvent acetone (<0.01%) no analysis, results expressed as nominal concentrations 48h-LC50: DQ 2
Marine copepod *Acartia tonsa* 5-d, partly renewal Wollenberger et al. 2003	EC50 = 0.026 <0.020 – 0.035> EC10 larval development ratio= 0.0072 <0.0042-0.012>	solvent acetone (<0.01%) n = 6 DQ 3: No analysis of concentration levels algae added as food source were left as residue. potentially causing oral exposure; not a water-only test. Test was repeated, see **Table 3.31**
Freshwater mussel *Lampsilis cardium* 48-h Gooding et al. 2004	glochidia: 24h-LC50 = 0.30 and 48h-LC50 =0.44 juveniles: small dose dependant impact on growth	no data on preparation, no details on effects GC/MS analysis and identification actual conc. 5 to 19% of nominal DQ 4: no details, LC50 24h < LC50 48h
Zebrafish *Brachydanio rerio* 48-h, static Carlson and Norrgren 2004	NOEC larval devel. $\geq$ 1.0 NOEC heart rate = 0.010 [3] LOEC heart rate = 0.033 [3]	solvent DMSO (0.1%) n = 7(devel.); 6 (survival) no analysis, nominal concentrations DQ 2: development: actual concentrations are missing
Zebrafish *Brachydanio rerio* 13-d e.l.s. Carlson and Norrgren 2004	NOECsurvival to starvation $\geq$ 0.1	DQ 2: actual concentrations are missing,
Zebrafish *Brachydanio rerio* 96-h Dietrich and Chou 2001	EC50hatching = 0.18	DQ 4: no details
Zebrafish	NOECdevelopment $\leq$ 0.3	DQ 4: no details

Test and reference	Results [1] [mg/l]	Remarks [2]
Brachydanio rerio 3-d Schreurs et al. 2001		
Clawed frog *Xenopus laevis* 96-h Dietrich and Chou 2001	LC50embryo-adult> 2.0 LC50embryo growth> 1.0 LC50embryo-development > 4.0	DQ 4: no details

1 <95% confidence limits>

2 The number of concentrations tested (n) excludes control and solvent control

3 heart rate: biological significance not clear

3.2.1.1 Toxicity test results for aquatic organisms

3.2.1.1.1 Fish

A 21-d prolonged toxicity test was carried out with bluegill sunfish (*Lepomis macrochirus*) according to OECD Test Guideline 204 under flow-through conditions (Wüthrich 1996b). The fish weights at the start of the experiment varied between 1.4 and 1.6 g. Nominal concentrations ranged from 0.125 to 2.0 mg/l (step size 2). Concentrations were measured at the start, halfway through and at the end of the test period. Concentrations up to and including 0.184 mg/l did not significantly affect survival of the fish. Mortality was 70% at the next higher concentration of 0.392 mg/l, and 100% in 1.00 mg/l after 11 days and in 2.22 mg/l after 4 days. The 21-d LC50 was 0.314 mg/l. Clinical signs such as loss of equilibrium, enhanced and/or irregular respiration and cessation of food intake were observed before the onset of death. Fish growth was significantly reduced at 0.184 mg/l. Therefore the NOEC (0% growth reduction) is 0.089 mg/l.

An early life stage test was carried out with fathead minnow (*Pimephales promelas*) according to OECD Test Guideline 210 under flow-through conditions (Croudace, et al. 1997). Eggs less than 24 h old were exposed to nominal concentrations ranging from 0.0125 to 0.2 mg/l (step size 2). Concentrations were measured 13 times at regular intervals during the 36-day test period. Hatchability of eggs was not significantly affected in any of the test concentrations. Larval survival after 32 days was not affected in concentrations of 0.067 mg/l and below. In 0.14 mg/l larval survival was 18%. Larval growth was not affected in concentrations of 0.035 mg/l or below. At 0.067 and 0.14 mg/l, mean standard lengths were reduced by 7 and 38%, whereas weights were reduced by 7 and 75%, respectively, as compared to the solvent control. In these two higher concentrations, physical abnormalities were recorded in the surviving larvae. For the majority (84%) of the larvae surviving in 0.067 mg/l, and in all survivors in 0.14 mg/l, the caudal (tail) fin was absent. The relative tail length of the other 16% of the larvae in 0.067 mg/l was not affected at all as compared to the control. These effects were completely absent at lower concentrations. The NOEC from this early life stage study is 0.035 mg/l.

Another early life stage test was performed with the zebrafish, *Brachydanio rerio*, to determine whether this unusual effect was induced also in other species. The test was carried out in accordance with OECD TG 210 in an intermittent-flow-through system during 34 days. Test concentrations ranged from 0.010 to 0.075 mg/l. To investigate the possible contribution of the solvent triethylene glycol to the effects, eggs/larvae were also exposed to an AHTN solution prepared using a generator column. The mean actual concentration was 0.065 mg/l. The eggs/larvae showed a normal development and in concentrations up to 0.035 mg/l the phenomenon of the missing tail fin was not observed. Higher concentrations induced the lack of the caudal fin and curling or curving of the body. These effects were concentration-dependent. The reduced length of the fish and the observed disturbed swimming behaviour at concentrations above 0.035 mg/l (NOEC) is explained by the absence of the tail fin (Hooftman and Borst 1999). These findings confirm the results of the study on fathead minnow.

Apart from the completely reported GLP studies, some other test results are available on zebrafish. Carlson and Norrgren (2004) carried out a short test on an early life stage with zebrafish. Selected newly laid zebrafish eggs were individually exposed in 96-well styrene plates in 250 µl of test medium with nominal concentrations from 1 to 1000 µg/l during 2 days. Concentrations were not measured. No effects were found on the development of the embryos after 2 days. At 33 µg/l a slight reduction in heart rate was observed. When the results (Manuscript Carlsson and Norrgren, 2002, Swedish University of Agricultural Sciences, Uppsala) are analysed more closely, as is done in Figure 3.12 it is clear that the effect is minimal and that there is no dose-effect relation: the heart beat is still at the same level at a concentration 33 times the so-called NOEC of 10 µg/l. Only in the concentration of 1000 µg/l the line has started to descent. This also makes the ecological relevance of these effects unclear. In an embryo-larval study 20 selected eggs (with 6 or 7 replicates per concentration) were exposed in concentrations from 1 to 100 µg/l. The medium was refreshed daily but no food was supplied. Exposure to AHTN did not result in reduced survival time at any concentration up to 100 µg/l. The observations are in line with the adverse effects observed in the full E.L.S. test, see Figure 3.12.

In an early life stage test with zebrafish ventro-dorsal curvature of the tail was observed after 96h of exposure to concentrations ≥ 0.2 mg/l. The 96h-EC_{50} for embryo hatching was 0.18 mg/l (Dietrich and Chou 2001).

In another early life stage test with zebrafish, four hours to three days after fertilisation of eggs, abnormal tail curving was observed at a concentration of 0.9 mg AHTN/l and less at 0.3 mg/l (Schreurs et al. 2001).

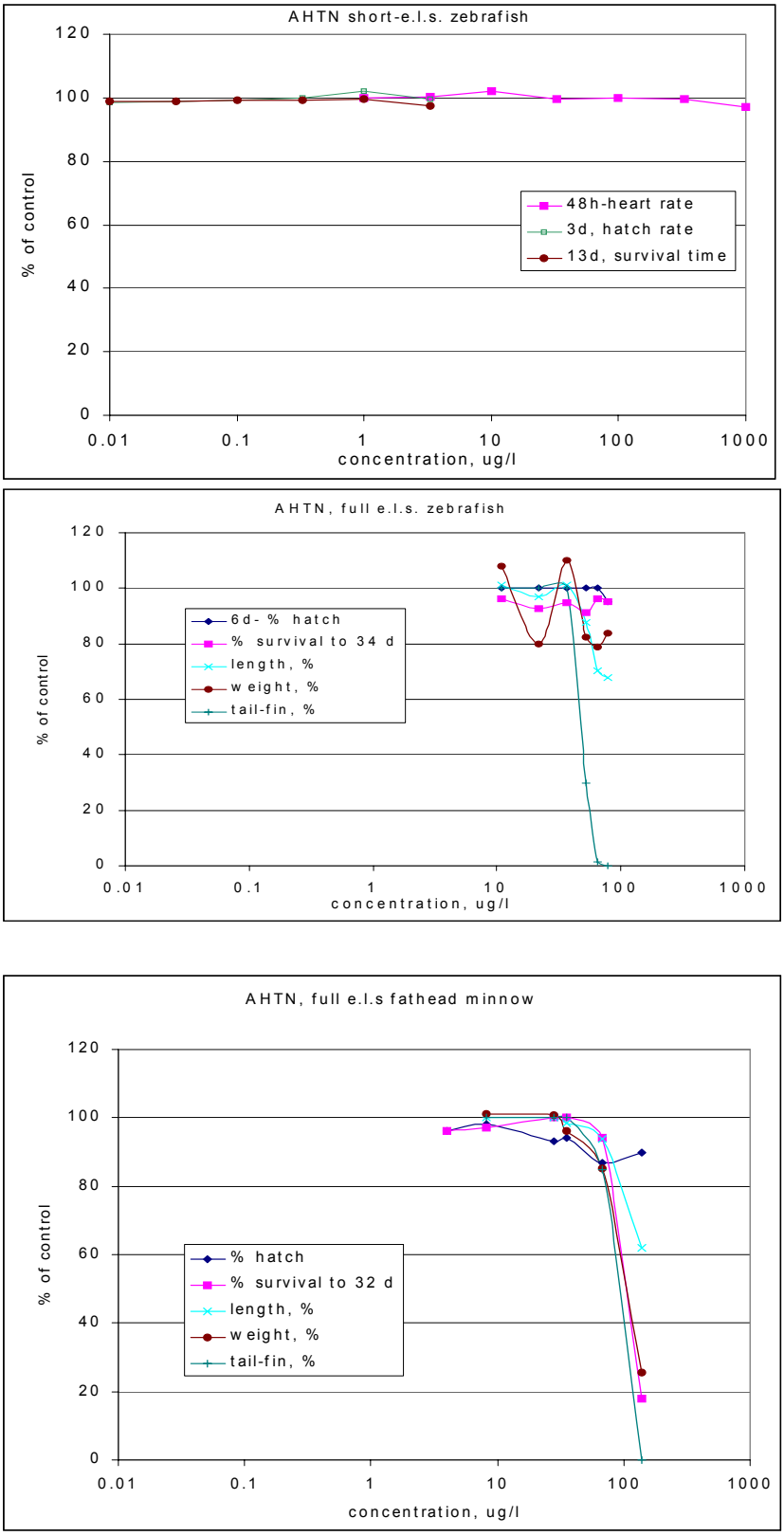


Figure 3.12 Comparison of the results of three different studies on the effects of AHTN on early life stages of fish.

3.2.1.1.2 Aquatic invertebrates

For *Daphnia magna*, a semi-static 21-d toxicity test was carried out according to OECD Test Guideline 202, part II, proposed updated version of June 1993 (Wüthrich 1996a). The test medium was refreshed three times per week. Nominal concentrations ranged from 0.062 to 1.0 mg/l (step size 2). Concentrations were measured at the start and end of the first and last exposure period. Immobility of the parent generation was 80% at 0.401 mg/l and the 21d-EC50 was 0.341 mg/l. The mean reproduction in test concentrations up to and including 0.196 mg/l ranged between 80 and 114% as compared to the solvent control (93% in 0.196 mg/l). In the next higher test concentration of 0.401 mg/l, reproduction of the surviving adults was inhibited almost completely. Thus, 0.196 mg/l can be considered the NOEC in this study.

The chronic toxicity to marine copepod *Acartia tonsa* was tested according to the OECD Draft Guideline for Testing of Chemicals (2004) describing a life cycle test, with specific adaptations to prevent volatilisation and accumulation of organic debris (Bjørnestad 2007). For this test AHTN was mixed with radio-labelled [phenyl - ^{14}C(U)]-AHTN (1%) with a radiochemical purity of 94.8%. Ethanol was used as a solvent. Test concentrations were 5.5, 11, 22, 44, 88, 176 and 352 µg/l, with four replicates per concentration and 6 replicates in the control as well as in the solvent control. For the test 110-ml glass flask were used, closed with screw caps with Teflon seals and filled almost completely with natural seawater, salinity 32.5‰ and test substance leaving a headspace of 5 ml. The test was carried out at 20 ± 1.0°C in a climate room with a daily light/dark period of 16:8 hours. One day before addition of the eggs, the flasks were saturated with the appropriate test concentration. At the initiation of the test, fresh solutions were added to the bottles. The test was initiated with approximately 80 eggs in each flask and approx. 7,000 cells/ml of the algae *Rhodomonas salina.* The algal concentration was readjusted daily to 7,000 cells/ml and the flasks were slowly rotated to keep the algae from settling. This was confirmed to be the optimal design to prevent the formation of organic debris and the loss of test substance by sorption and volatilisation. Samples of the test solution for LSC were taken daily. After 5.5 days the number of nauplii, copepodites and non-hatched eggs was counted and the lengths of the larvae was measured.

The actual test concentrations were >80% of nominal during the whole study, implying that the nominal concentrations can be used for the calculations. The larval mortality in the controls was below the quality criterion of 30%: 19.5 and 15.3 % in the control and solvent control, respectively. The larval development ratio (LDR) was 69% in the control and 75% in the solvent control. The NOEC(LDR) was 22 µg/l with an inhibition of 3% as compared to the solvent control, whereas the inhibition was 20% in 44 µg/l (LOEC). At 176 µg/l no copepodites were observed and the length of the remaining nauplii was significantly smaller than in the controls. Moreover no copepodites were observed. Up to and including 88 µg/l, there was no impact on growth. The EC10(LDR) was 28.2 µg/l (95% confidence interval 17.0 – 36.8 µg/l) and the EC50 was 71.7 µg/l (60.5 – 87.2 µg/l).

Apart from these completely reported GLP studies, results are available for a number of other invertebrate species, see **Table 3.32**.

The GLP-study on *A. tonsa* described above was initiated as a result of the publication of Wollenberger et al. (2003) on this species. The effects of AHTN on the larval development ratio of the marine copepod *Acartia tonsa* was investigated according to draft ISO/DIS 14669 (1997). The 48h-LC_{50} was 0.71 mg/l, whereas the 5d-EC_{50} for larval development was 0.026 mg/l and 5d-EC_{10} is given as 0.007 mg/l (Wollenberger et al. 2003). The test showed a number of methodological deficiencies, e.g., (1) the test concentrations were not measured and it is known from a similar set-up that concentrations were decreased to only 10-30% after

2 days; (2) the renewal of the medium by only 50% combined with daily feeding probably left residual algae and faeces in the medium to which the test substance could readily sorb. This probably lead to oral exposure in addition to the intended exposure throught the water phase. In view of the newer test results of the GLP study with this species, the results of the Wollenberger study are not used for the risk assessment.

A population growth experiment was carried out with the marine harpactoid copepod *Nitocra spinipes*. After short-term exposure (96 h) the LC50 was 0.61 mg/l (0.58-0.65). To investigate the chronic toxicity, eight replicates of 10-15 nauplii per concentration were tested in 10 ml test medium. The test was carried out at 22 °C in artificial seawater (low salinity: 6‰). Every other day, 70% of the medium was renewed and new food (salmon feed) was added. The test included the period between hatching of the F_0 to hatching of F_1-generation. Concentrations in the chronic test ranged from 0.002 to 0.06 mg AHTN/l. The larval development ratio was established after 7 to 8.5 days and the exposure for the life-cycle experiment was terminated at day 18, although for ovigerous females the exposure was continued until day 22. No effects were observed on larval development or mortality nor on the true population parameter intrinsic rate of increase (r_m) up to and including 0.06 mg/l. The NOEC is above 0.06 mg/l (Breitholtz et al. 2003). Measurements showed that the initial concentrations were highly variable (60-70% of nominal) and that concentrations in the water were not maintained at all after the 2 days interval before renewal (10 – 30% of nominal). The loss of AHTN is partly explained by volatilisation, as in the above described *Acartia* test, the concentrations were well maintained in a closed system. An explanation of the variability of the concentrations may be the sorption to organic residues remaining in the system after incomplete (70%) refreshment of the medium. *Nitocra spinipes* is a benthic organism so it would also be exposed to the test substance sorbed to organic residues. The test conditions did not ensure a 'water-only' exposure and therefore the results of this test can not be expressed on the basis of the concentration in water. This is also remarked by the authors in Breitholtz et al. (2003).

A more detailed evaluation of the long-term aquatic toxicity study with *Nitocra spinipes* is included in Appendix 1 of this RAR. In this evaluation it is reasoned that *Nitocra spinipes* could be more sensitive for HHCB than the other sediment-dwelling organisms tested and this possibly also applies to AHTN. However, this calculation is prone to some uncertainties. From the acute tests with water-only exposure it can not be concluded that *Nitocra spinipes* is more sensitive than other benthic species. For this reason, there is no need for a new sediment toxicity study with *Nitocra spinipes* (regardless of the fact that no test protocol is available for such study).

Although also in the *short term* tests with *Acartia tonsa* (Wollenberger et al. 2003) and *Nitocra spinipes* the test concentrations have not been measured, in view of the relatively short exposure period, the LC50 values based on nominal concentrations are considered valid (only) for the classification and labelling of the substance.

The toxicity of AHTN to midge larvae *Chironomus riparius* was determined in a 96h test with mortality as endpoint. Per concentration 10 midge larvae were exposed individually. The test medium was renewed every 24 hours. AHTN was solved in isopropanol and 6 concentrations were tested, ranging from 0 to 1.9 µMol (0.5 mg/l). The concentrations were chemically analysed in freshly prepared medium and before renewal. An LC50 could not be determined as no toxicity was observed even at concentrations close to maximum solubility and in the range of predicted baseline toxicity levels. The measured body residues were all below 0.02 mmol/kg wwt. They were independent of aqueous concentrations and far below reported lethal body burdens for narcosis type compounds indicating that AHTN is indeed biotransformed but does not act directly by narcosis (Artola 2002, Artola et al. 2003).

The same author also reported on the toxicity to the worm *Lumbriculus variegatus* in a 120h static test with immobilisation as endpoint. AHTN was dissolved in isopropanol and 6 concentrations were tested, ranging from 0 to 1.9 µMol (0.5 mg/l). The concentrations were chemically analysed in freshly prepared medium and at termination. Ten worms were exposed in triplicate. The LC50 was 0.397 mg/l. The concentrations measured in surviving organisms in the highest test concentration (27 mmol/kg wwt) are somewhat higher than the critical body residues for narcosis (2-10 mmol/kg wwt). This was explained by the relative insensitivity of *L. variegatus* (Artola 2002).

The effect of AHTN on early life stages of the freshwater mussel *Lampsilis cardium* was studied. Glochidia (larvae) were tested in 24 and 48-h static tests with concentrations up to 1.2 mg/l. The concentrations were measured by GC/MS and were shown to drop to only 5 to 19% of the nominal concentrations. The LC50 values ranged from 0.30 to 0.44 mg/l. In tests with juveniles there was no significant mortality, but a slight dose dependant reduction of growth rate was observed (Gooding et al. 2004). As the experimental details are not available the interpretation cannot be evaluated.

3.2.1.1.3 Algae

The toxicity to algae was studied in a static test according to OECD Test Guideline 201 with *Pseudokirchneriella subcapitata* (Van Dijk, 1997). Nominal concentrations ranged from 0.0625 to 1.0 mg/l (step size 2). Concentrations were measured at the start and end of the test; maintenance of the concentrations proved to be difficult and in some cases pH-values rose to above 10 indicating an insufficient buffering capacity of the medium. The loss of test material in the algal test was studied for a related polycyclic musk and it was shown that sorption to algal cells was the most relevant 'sink', whereas sorption to glass might play a role as well as volatilisation (see Appendix I, Table A1-2). The results were expressed as the mean concentration over the exposure time. The test was carried out twice (test A and B) due to the sharp concentration decrease in the first study with the goal to confirm the observed toxicity levels in a second study. Growth rate (µ) was the less sensitive parameter. In test (B) the growth rate reduction at 0.835 mg/l was 16% (LOECr) with a NOECr of 0.374 mg/l. In test (A), the NOECr for effects on growth rate was slightly higher, 0.438 mg/l, whereas the inhibition at 0.797 mg/l (LOECr) was 33%. The geometric mean NOECr for both tests was 0.40 mg/l. The 72-h ErC50 was > 0.797 and > 0.835 in test (A) and (B) respectively. Growth measured as Area Under the Curve was not significantly inhibited in concentrations up to and including (A) 0.204 and (B) 0.374 mg/l. In the next higher concentration, the LOECb, (A) 0.438 and (B) 0.835 mg/l, the biomass production was inhibited by (A) 36% and (B) 54%,.

3.2.1.1.4 Microorganisms

In the standard tests on biodegradation that have been conducted (section 3.1.3.1.2), the toxicity controls proved that AHTN was not toxic to the inoculum at concentrations far above the water solubility (NOEC > 30 mg/l).

3.2.1.1.5 Amphibians

The acute toxicity of AHTN was tested on the South African clawed frog larvae (*Xenopus laevis*) in a procedure analogous to ASTM guideline E 1439-91. The 96h-LC50 for embryo-

adult was > 2.0 mg/l, the 96h-EC50 was > 1.0 mg/l for embryo growth and > 4.0 mg/l for embryo malformation (Dietrich and Chou 2001).

3.2.1.2 Calculation of Predicted No Effect Concentration ($PNEC_{water}$)

For the determination of the PNEC various results of prolonged toxicity tests are available for algae, the invertebrates Daphnia and Acartia, and fish that were fully reported and carried out according to GLP requirements. Tests are also available for other species of the class of crustaceans, insects, molluscs, annelids and amphibians, however, the validity of these data cannot be established as critical pieces of information are lacking (information on actual test concentration, dose-response, variability of replicates, control survival, etc.). Based on the results of the tests summarised in **Table 3.31**and **Table 3.32** (see **Table 3.33**) the lowest value is the EC_{10} of 0.028 mg/l for the larval development of the marine crustacean *Acartia tonsa.* Therefore with an assessment factor of 10, **$PNEC_{water}$ is 2.8 µg/l**.

Table 3.33 Summary of aquatic toxicity data from prolonged tests

	Test organisms	Results [mg/l]
Algae	*Pseudokirchneriella subcapitata*	72h-NOECr = 0.40 (mean of test A&B)
Crustaceans Cladocera	*Daphnia magna*	21d-NOEC(rep) = 0.196
Copepods	*Acartia tonsa*	5d-EC_{10} = 0.028
Fish	Bluegill sunfish *Lepomis macrochirus*	21d-NOEC = 0.089
Fish	Fathead minnow *Pimephales promelas*	36d-NOECgrowth, develop. = 0.035,
Fish	Zebrafish *Brachydanio rerio*	34d-NOECgrowth, develop. = 0.035

For microorganisms no specific toxicity tests have been carried out. In the biodegradation tests, no inhibition was observed, implying that the NOEC is above 30 mg/l. With an assessment factor of 10, the **$PNEC_{STP}$** would be > 3 mg/l. This PNEC is above the water solubility of AHTN of 1.25 mg/l.

3.2.1.3 Toxicity test results for sediment organisms

Toxicity tests were carried out with three species of sediment organisms, according to or in line with the OECD TG 218 (Draft December 2002): Sediment-water chironomid toxicity test using spiked sediment. The sediment was formulated from 5% Sphagnum moss peat, 75% quartz sand (>50% in range 50-200 µm), 20% kaolinite clay and 0.05% calcium carbonate to adjust the pH between 6.5 and 7.1. The organic carbon content was 2%. At the same time 0.2-0.25% Urtica powder was added as feed. The formulated sediment was conditioned for 7 days prior to application of the test material. The test material was solved in acetone to prepare the stock solutions for each concentration. The proper volumes were mixed first with dry quartz

sand allowing the solvent to evaporate. Next the sand was mixed with the formulated sediment to achieve the intended nominal concentration levels. Each glass vessel contained a layer of 1.5 to 3 cm of sediment and the water (Elendt medium M4) volume was 3.5 to 4.5 times the sediment volume. Both a control and a solvent control were included. The test animals were introduced after an equilibration period of 1 week. The tests were carried out at 20 ºC under a 16/8 hours L/D cycle with a light intensity of 400 to 600 lux. The overlying water was slightly aerated during the test. No additional food was given during the test. Test concentrations were measured. Samples of porewater and overlying water were extracted by SPE using Speedisks. Sediment samples were freeze-dried and analysed by GC/MS after solvent extraction. The results are reported by Belfroid and Balk (2005). These tests were carried out under GLP and they are completely documented. The results are summarised in **Table 3.34**.

Table 3.34 Sediment toxicity of AHTN (GLP and completely documented)

Test and reference	Results [1] [mg/kg dwt] <95% c.l.>	Remarks [2]
Insectae	NOEC = 125 (development rate)	solvent: acetone, 2.6% OC
Chironomus riparius	LOEC = 250 (development rate)	n=5
28 d	NOEC = 500 (emergence ratio)	identification by GC/MS
Egeler & Gilberg 2004a	LOEC = 1000 (emergence ratio)	start conc. 87% of nominal
	EC50 = 785 <714 - 863> (emergence ratio)	end conc. 67% of start
Crustaceae, Amphipoda	NOEC = 28.9 (survival)	solvent; acetone, 2.15% OC
Hyalella azteca	LC50 = 77.3 <68.6 – 87.1>	n=5
28 d	NOEC = 28.9 (growth)	identification by GC/MS
Egeler 2004	LOEC = 63.6 (growth)	start conc. 66% of nominal
	EC50 = 57.6 <45.7 – 72.4> (biomass)	end conc. 92% of start
Oligochaeta	NOEC ≥ 140 (survival)	solvent; acetone 2.06% OC
Lumbriculus variegatus	NOEC = 26.5 (reproduction)	n=5
28 d	LOEC = 60.9 (reproduction)	identification GC/MS
Egeler & Gilberg 2004b	EC50 = 87.2 <65.1 – 128.9> (reproduction)	start conc. 62% of nominal
	NOEC = 11.5 (biomass)	end conc. 91% of start
	LOEC = 26.5 (biomass)	
	EC50 = 120.6 <81.1 – 254.2> (biomass)	

1 nominal concentrations; <95% confidence limits>

2 The number of concentrations tested (n) excludes control and solvent control

For the midge larvae *Chironomus riparius* the test was carried out with five concentrations ranging from 62.5 to 1000 mg/kg dwt with step size 2 (Egeler and Gilberg 2004a). Twenty animals, first instar larvae, were used in each of the four replicates per test concentration, in the control and in the solvent control. The test concentrations were measured on day 0, 14, 20 and 28 in the control, 125 and 1000 mg/kg. After the equilibrium period at the start of the test the concentration was on average 87% of the intended nominal test concentration. At termination of the test, the average concentration was 67% of the concentration at the start.

The development rate was not affected up to 125 mg/kg for both males and females (NOECdev., -4 and -7%). The emergence ratio was a less sensitive endpoint. A clear dose response relation was found for the emergence of the midges. The data for males and females were pooled for the statistical analysis. The NOECemerg. was 500 mg/kg, the EC_{15} was 549 mg/kg dw with 95% confidence limits <472 – 639> and the EC_{50} was 785 <714 – 862> mg/kg dw. The actual concentration on day 0 at the NOECdev. level was 101 mg/kg. During the test the level decreased by 17 to 27%. The NOEC in this study is taken to be **101 mg/kg dwt (measured concentration)**.

The Amphipoda *Hyalella azteca* was tested in five concentrations ranging from 6 to 140 mg/kg sediment with step size 2.2 (Egeler 2004). The test animals were 7 to 14 days old, and between 355 and 500 μm. Four replicates each with 10 animals were used per test concentration and in the solvent control, whereas six replicates were used in the control. The test concentrations were measured on day 0, 9, 19 and 28 in the control, 29 and 140 mg/kg. At the start of the test the test concentration was on average 66% of the intended nominal test concentration. At termination of the test, the average concentration was 92% of the concentration at the start. Survival was not affected up to 29 mg/kg, whereas the mortality in 64 mg/kg was 28% and 98% in 140 mg/kg. Thus the LC_{50} was 77.3 <68 – 87> mg/kg. Growth was inhibited at 64 mg/kg (LOEC). The length of the amphipods was 15% below the pooled control at 64 mg/kg, whereas the (total) biomass per replicate and the individual biomass were 54% and 38% below the control, respectively. At 29 mg/kg the inhibition was 3.5% for length and 15% for biomass. The EC_{15} for total biomass was 30.2 <15.9 – 39.6> mg/kg, the EC_{50} was 57.6 <45.7 – 72.4> mg/kg. The actual concentration on day 0 at the NOEC level was measured: 18.2 mg/kg. During the test the concentration remained at this level. Thus **18.2 mg/kg dwt** is considered as **the NOEC** in this study.

The aquatic oligochaete worm *Lumbriculus variegatus* was tested in concentrations ranging from 5 to 140 mg/kg with step size 2.3 (Egeler and Gilberg 2004b). The test animals were 'synchronised' before the start of the test to avoid high variation in the test results. Four replicates each with 10 regenerated animals were used per test concentration and in the solvent control, whereas six replicates were used in the control. The test concentrations were measured on day 0, 9, 20 and 28 in the control, 26.5 and 140 mg/kg. At the start of the test the test concentration was on average 62% of the intended nominal test concentration. At termination of the test, the average concentration was 91% of the concentration at the start. Survival was not affected up to the highest test concentration. The total number of worms (including adult and regenerated worms) at the end of the test was evaluated as a parameter of reproduction. Reproduction was significantly inhibited by 43% in 61 mg/kg, whereas the inhibition was 13% at 26.5 mg/kg (NOECrepr.). The $EC_{50repr.}$ was 87.2 <65.1 – 128.9> mg/kg, the $EC_{15repr.}$ was 26.4 <11 – 39.1> mg/kg. Growth as measured by biomass was significantly inhibited by 20% at 26.5 mg/kg, whereas at 11.5 mg/kg the inhibition was 13% (NOECbiomass). The EC_{50}(biomass) was 120.6 <81.1 – 254.2> mg/kg, the EC_{15}(biomass) was 19.4 <6.5 – 31.6> mg/kg. The lowest NOEC was 11.5 mg/kg. The actual concentration on day 0 was not measured at the level of the NOEC. Therefore the actual concentration is based on the mean results stated above: 62% of 11.5 mg/kg or 7.13 mg/kg sediment. During the test the level decreased to 91%. The concentration in the porewater decreased from 140 to 50 μg/l. The NOEC in this study is taken to be **7.1 mg/kg dwt (measured concentration)**.

Data are also available for sediment organisms (*Chironomus riparius* and *Lumbriculus variegatus*) tested in water during 4 to 5 days. They were summarised under 3.2.1.1.

3.2.1.4 Calculation of Predicted No Effect Concentration ($PNEC_{sediment}$)

PNECsediment is determined from the results of the three tests described in section 3.2.1.3, with the midge larvae, amphipoda and worms. These tests were carried out, according to the protocol, in a substrate containing 2% organic carbon. In the TGD, PECsediment is derived for a sediment containing 5% organic carbon and thus NOEC needs to be standardised to 5% organic carbon. This is shown in**Table 3.35.** The lowest NOEC is 17.2 mg/kg for the growth of *Lumbriculus variegatus*. Therefore an assessment factor of 10 is applied to the lowest of the NOECs, giving **$PNEC_{sediment}$** of **1.72 mg/kg dwt**.

Table 3.35 Summary of sediment toxicity data

	Test organisms	Results [mg/kg dwt], measured, 2% OC	Result standardised, 5% OC [mg/kg dwt],
Insecta	*Chironomus riparius*	28d-NOECdevelopment = 101 (OC 2.4%)	28d-NOECdevelopment = 210
Crustaceans Amphipoda	*Hyalella azteca*	28d-NOECgrowth = 18.2 (OC 2.15%)	28d-NOECgrowth = 42.3
Worms Oligochaeta	*Lumbriculus variegatus*	28d-NOECgrowth = 7.1 (OC 2.06%)	28d-NOECgrowth = 17.2

For comparison, $PNEC_{sediment}$ was also be derived according to the equilibrium partitioning theory:

$$PNEC_{sed} = \frac{K_{susp.-water}}{RHO_{susp}} \cdot PNEC_{water}$$

where:

$PNEC_{sediment}$: PNEC for sediment-dwelling organisms (kg/kg_{wwt})

$PNEC_{water}$: PNEC for aquatic organisms (kg/m^3)

$K_{susp.-water}$: suspended matter-water partition coefficient ($746\ m^3/m^3$)

RHO_{susp}: bulk density of suspended matter ($1150\ kg_{wwt}/m^3$)

With $PNEC_{water}$ = 2.8 µg/l, $PNEC_{sediment,\ EqP}$ = 1.83 mg/kg wet weight. This is converted to dry weight by multiplication with a factor of 4.6 (susp. solids) and thus **$PNEC_{sediment,\ EqP}$ = 8.42 mg/kg dwt**.

The PNECsediment based on sediment toxicity tests and the one derived by equilibrium partitioning from PNECwater differ by a factor of 5.

3.2.2 Terrestrial compartment

3.2.2.1 Toxicity test results for terrestrial organisms

3.2.2.1.1 Plants

No data available. In section 3.1.3.4.4 it was noted that in a mixture of soil and sludge with a total concentration of 30 mg/kg dwt the growth of plants was inhibited (Müller et al. 2002). However, the cause and the extent of the inhibition were not clarified.

3.2.2.1.2 Earthworm

Toxicity tests were carried out with earthworms and springtails (see **Table 3.36**). The test materials at appropriate concentrations were dissolved in equal amounts of acetone, mixed with the quartz sand and allowed to slowly evaporate and mixed with the standard soil containing 10% Sphagnum peat, 20% kaolinite clay, approximately 70% fine quartz-sand (grain size 0.1-0.5 mm) and 0.5% calcium carbonate to adjust to pH 6.0±0.5. After preparation of the test concentrations and an equilibrium period of one week, the test organisms were added to the soil. The test medium was not refreshed during the test period.

The earthworm test was carried out according to ISO 11268 (Gossman, 1997). Adult worms (*Eisenia fetida*) were exposed to nominal concentrations in soil of 8, 19, 45, 105 and 250 mg/kg. Weights of the adult worms ranged between 340 and 540 mg, but did not differ more than 100 mg within this range in each test container. The worms were fed weekly with finely ground cattle manure. Adult worms were removed after 4 weeks of exposure, counted and weighed. The remaining offspring remained in the test containers for another four weeks. No mortality or growth inhibition of the adults was observed after 4 weeks in concentrations up to and including 250 mg/kg. In the range finding test 100% mortality occurred after 14 days exposure to 1000 mg/kg. Reproduction was not significantly affected up to concentrations of 105 mg/kg (14% inhibition – not statistically significant, NOEC). At the level of the LOEC (250 mg/kg), the reproduction was 39% of the control.

3.2.2.1.3 Microorganisms

No data are available on the toxicity to specific microorganisms in soil.

3.2.2.1.4 Other terrestrial organisms

The springtail test was carried out according to the draft ISO/CD 11267 (Klepka 1997). The test was started with juvenile springtails of the species *Folsomia candida* 10 to 12 days of age and survival and reproduction after 28 days were determined. Nominal test concentrations were 1, 3, 8, 19, 45 and 105 mg/kg soil. The animals were fed with granulated dry yeast. No significant mortality (-7%) or effects on reproduction (0%) were observed in concentrations up to and including 45 mg/kg. Mortality was significant (18%) in the highest concentration of 105 mg/kg. The reproduction, expressed as the number of juveniles per container, was also significantly reduced (51%) in 105 mg/kg. The NOEC from this study was, therefore, 45 mg/kg.

Table 3.36 Toxicity data for soil organisms

Test and reference	Results for AHTN (nominal concentrations)	Remarks
Earthworm *Eisenia foetida* ISO 11268 (OECD 207) Gossmann1997	8wk-NOEC = 105 mg/kg, LOEC[2] = 250 mg/kg, reproduction and food consumption 4wk-NOEC ≥ 250 mg/kg, mortality and growth	inital weight adults 0.34-0.54 g test range 8-250 mg/kg solvent: acetone artificial soil pH 6.1, 10% sphagnum DIN[1] temp. 17-23°C
Springtail *Folsomia candida* ISO /CD 11267 Klepka 1997	4wk-NOEC = 45 mg/kg, LOEC[3] = 105 mg/kg, mortality and reproduction	10-12 d old juveniles test range 1-105 mg/kg solvent: acetone temperature 17-25°C artificial soil, 10% sphagnum DIN[1]

[1] Sphagnum DIN standard: organic material minimum 90%, organic carbon 52%

[2] Dunnet's test (p=0.05)

[3] Student's t-test (p=0.05)

3.2.2.2 Calculation of Predicted No Effect Concentration ($PNEC_{soil}$)

For AHTN two long term tests are available, allowing an assessment factor of 50 to be applied to the lowest NOEC. However, first this lowest NOEC is normalised to the standard soil of the TGD containing 3.4% of organic material: 45 / 0.1 ·0.034 = 15.3 mg/kg. Therefore **$PNEC_{soil}$ = 0.31 mg/kg dwt or 0.28 mg/kg wwt**.

If $PNEC_{soil}$ were derived from $PNEC_{aqua}$ by equilibrium partitioning, $PNEC_{soil, equil}$ = 1.84 mg/kg wwt or 2.1 mg/kg dwt.

3.2.3 Atmosphere

No data are available on exposure of organisms via the air. Therefore, no $PNEC_{air}$ can be derived.

3.2.4 Secondary poisoning

No specific toxicological data are available on e.g. (fish-eating) birds. The PNEC for secondary poisoning will therefore be based on mammalian toxicity data for AHTN

3.2.4.1 Effect data

See relevant paragraphs in Human Health Chapter (4.1.2). Two relevant NOAEL values were identified: a NOAEL of 5 mg/kg bw/d is derived from the 90-day oral study with rats and a NOAEL of 15 mg/kg/d in a 21-day reproduction and development toxicity study.

3.2.4.2 Calculation of Predicted No Effect Concentration ($PNEC_{oral}$)

A NOAEL of 5 mg/kg bw/d is derived from the 90-day oral study with rats (see Chapter 4.1.2). As toxicity is based on the P-generation (rats > 6 weeks) a conversion factor of 20 has to be used resulting in a NOEC of 100 mg/kg food (e.g., in fish). For the derivation of $PNEC_{oral}$, the test duration of 90 days implies an assessment factor of 90, giving $PNEC_{oral}$ = 1.1 mg/kg food.

In a 21-day reproduction and development toxicity study, the NOAEL was $\geq$ 20 mg/kg/d (no LOAEL established). With the same conversion as above, the NOEC in food is $\geq$ 400 mg/kg. With an assessment factor of 300 (as for a 28 day test) $PNEC_{oral}$ is >1.3 mg/kg food.

In conclusion, **$PNEC_{oral}$ = 1.1 mg/kg food.**

3.2.5 Marine effects assessment

For the marine effects assessment, the available toxicity data of freshwater and marine test organisms may be pooled. According to the TGD (2003) there are no reasons to believe that a systematic bias to freshwater or marine species would exist. In view of the larger diversity of taxa in the marine environment it is expected that there is a wider distribution of the species sensitivity. This is reflected by a larger assessment factor in the derivation of the PNEC for the marine environment.

Results are available from long-term tests with species from three trophic levels: algae as the primary producers, *Daphnia* and *Acartia* as primary consumers and fish as secondary consumers (see **Table 3.33**). Therefore the Assessment Factor is 100 (instead of 10 used in the freshwater compartment), applied to the lowest EC_{10} of 28 µg/l for the marine copepod *Acartia tonsa*. Therefore the **$PNEC_{marine\ water}$ = 0.28 µg/l**.

The PNEC for the marine sediment is derived from three long-term sediment tests with species representing different living and feeding conditions, implying that an assessment factor of 50 is applied to the lowest NOEC of 3.75 mg/kg wwt (OC-normalised), see section 3.2.1.3. Thus **$PNEC_{marine\ sediment}$ = 0.075 mg/kg wwt or 0.345 mg/kg dwt.**

3.2.6 Other effects

This section summarises other effects reported in literature, such as endocrine interactions and other subcellular interactions. Studies on endocrine interactions are also described in section 4.1.2.9.3 of the Human Health Risk Assessment. Here the studies with possible environmental relevance are described.

3.2.6.1 Endocrine interactions

In vitro studies

The activity of AHTN (purity; >98%) on the estrogen induced vitellogenin (Vtg) production in carp (*Cyprinus carpio*) was investigated by using cultured primary hepatocytes from genetically uniform strains of carp. Vtg production was measured by indirect competitive ELISA, using a polyclonal antiserum against goldfish Vtg that cross-reacts with carp Vtg (described in Smeets et al., 1999). The Vtg production by carp hepatocytes, a sensitive marker of estrogenic activity, was not affected by AHTN (cited in Seinen et al., 1999).

The interaction of AHTN (purity unknown) with the hepatic estrogen receptor(s) of rainbow trout, carp and the amphibian *Xenopus leavis* was investigated in a competitive binding assay. In the *X. leavis* assay a concentration of 258 mg AHTN/l competitively inhibited the binding of 17β-estradiol to the receptor to 40%, whereas no inhibition was found at 258 µg/l. Very weak binding of AHTN was found in the rainbow trout receptor-binding assay. No competitively binding took place in the carp receptor-binding assay, corroborating the results cited by Seinen et al., 1999 (Dietrich and Chou, 2001).

The ecsysteroid agonist and antagonist activity of AHTN was assessed in an assay with the *Drososphila melanogaster* BII-cell line. The concentration of 20-hydroxyecdysone used in the antagonist assay was $5 * 10^{-8}$ M (i.e. 0.024 mg/l). AHTN did not show specific agonistic or antagonistic activity in this bioassay up to the highest concentration (10^{-4} M i.e. 26 mg/l). However, AHTN was cytotoxic at concentrations $\geq 2.5 * 10^{-5}$ M (6.5 mg/l) (Breitholtz et al., 2002).

Human embryonal kidney 293 (HEK293) cells were transient transfected with plasmids containing the human and zebrafish estrogen receptor isoforms (hERα and hERβ, and zfERα, β and γ) and an estrogen responsive reporter gene construct (ERE-luciferase). AHTN (purity; >98%) was tested up to 10 µM. AHTN alone showed a marginal transcriptional activation of hERα at the highest test concentration. The other receptors were not affected at all. The anti-estrogenic activity was tested using a submaximal dose of 0.01 nM estradiol (E2) for hERα, 0.1 nM E2 for hERβ and zfERγ and 1 nM E2 for zfERα and zfERβ, together with 0.1, 1 or 10 µM AHTN. A dose-dependent suppression by AHTN of E2 induction was shown toward hERβ and zfERγ. A weak antagonistic effect could be observed on hERα and zfERβ only at the highest test concentration of 10 µM (i.e. 2.6 mg/l), whereas no effect was seen at zfERα (Schreurs et al., 2004).

In vivo studies

This same group also investigated the possible anti-estrogenic effects in zebrafish *in vivo* (Schreurs et al., 2004). Transgenic zebrafish, containing a similar reporter gene construct as used in the above mentioned *in vitro* experiments, were exposed to AHTN with and without E2. AHTN at a concentration of 10 µM was toxic to the fish. AHTN did not show any estrogenic effect with the tested concentrations of 0.01, 0.1 and 1 µM. The concentrations of 0.1 and 1 µM (258 µg/l) resulted in a dose-dependent antagonistic effect on E2 (at 0.01 µM E2) in the juvenile zebrafish. The repression was down to 20% of the E2 induction at the highest test dose. The authors state that the actual concentrations at which they observe anti-

estrogenic effects are around or below the no-observed effect levels in the e.l.s tests and growth tests with fish and they conclude that no developmental disorders were or will be observed at the concentrations used in their transgenic zebrafish assay (Schreurs et al., 2004).

3.2.6.2 Other effects

The interaction of AHTN with multixenobiotic resistance (mxr) transporters was studied in gill tissue of the marine mussel *Mytilus californianus* (Luckenbach et al. 2004a). Mxr transporters are ATP dependant efflux pumps that remove a broad spectrum of chemically unrelated xenobitoics from the cell. A competitive substrate transport test using rhodamine B (RB), a fluorescent substrate of mxr transporters, was used to assay modulation of transport activity by AHTN. If the efflux of RB is inhibited, it accumulates in the cell to a higher degree which is indicated by increased fluorescence. Discs were cut from gill tissue and mucus was removed. The tissue was incubated in seawater with 1 µmol RB and AHTN for 90 min at 15 °C and next rhodamine B was extracted and measured. AHTN caused increased accumulation of RB in a dose-dependent way compared to control levels. The IC50 was listed as 2.05 µMol ~ 0.5 mg/l. The IC10 was 0.095 mg/l. The reversibility of the inhibitory effect seemed to be delayed by 24 hours (Luckenbach and Epel 2004b). This may be related to the high lipophilicity of the polycyclic musks. Effective inhibitory concentrations were similar to quinidine and approximately 100 times higher than for verapamil. The relevance of these *in vitro* observations for the risk assessment is not clear. The mucous membrane plays a protective role. Removal of this membrane in this test system raises questions as to the relevance of these observations.

The transporter activity in mussel gill is as sensitive as the effects observed in the standard toxicity tests with aquatic organisms. Thus at the exposure level where the protective transporter efflux is decreased rendering the cell more accessible to other potential toxicants, other effects of the synthetic musks show up also in algae or fish tests studying development and growth.

3.3 RISK CHARACTERISATION [14]

The risk characterisation ratios for compounding and formulating are based on use volumes for the year 2000. It should be remarked that the total use volume for compounding and formulation has declined by 30% between 2000 and 2004, implying that the PEC/PNEC ratios for individual sites or scenarios presented here may be overestimating the risk. This does not affect the ratios for private use as these are based on concentrations actually measured between 2000 and 2004.

The PECs were discussed in section 3.1.4.3 for the aquatic environment, in section 3.1.5.3 for the terrestrial compartment and section 3.1.7.3 for secondary poisoning. In most cases the results of the Southern European scenario were selected for the risk assessment for private use. For completeness the results of measurements in Berlin in 1996/1997 are also included but it should be realised that the situation has improved since then. For comparison, also other scenarios are shown, like the TGD regional (10%) and the scenario for the northern European countries. The data used for the risk assessment are underlined in the tables.

3.3.1 Aquatic compartment (incl. sediment)

Surface water and STP

The PEC/PNEC ratios for the aquatic compartment are presented in **Table 3.37**. The PNECs used are > 3000 mg/l for the STP and 2.8 µg/l for aquatic organisms. The PECs were discussed in section 3.1.4.3. For private use, the results of the SEU-15 scenario are used. All ratios are below 1, hence a **conclusion (ii)** is drawn for all scenarios.

Table 3.37 PEC/PNEC ratios for STP and surface water (µg/l)

	PEC_{STP}	**$PEC/PNEC_{STP}$**	**$PEC_{surface\ water}$**	**RCR Surface water**
		PNEC > 3000		**PNEC = 2.8**
Production, formulation and compounding				
Production	0.02	6.67E-06	0.021	0.008
Compounding Site 1	0.04	1.33E-05	0.0008	0.0003
Compounding Site 2	0.06	2.00E-05	0.059	0.021
Compounding Site 3	0.39	1.30E-04	0.002	0.001
Compounding Site 4	7.8 (WWTP)	2.60E-03	0.009	0.003
Compounding Site 5	0.01	3.33E-06	0.010	0.004
Compounding Site 6	--			
Compounding Site 7 (Large-medium generic)	4.13	1.14E-03	0.42	0.15
Compounding Site 8	0.62	2.07E-04	0.069	0.02

[14] Conclusion (i) There is a need for further information and/or testing.
Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.
Conclusion (iii) There is a need for limiting the risks; risk reduction measures which are already being applied shall be taken into account.

	PEC_{STP}	**$PEC/PNEC_{STP}$**	**$PEC_{surface\ water}$**	**RCR Surface water**
		PNEC > 3000		**PNEC = 2.8**
(Small generic)				
Formulation Large company	1.01	3.37E-04	0.109	0.04
Formulation Generic scenario	1.2	4.00E-04	0.131	0.05
Private use				
southern EU-15	**1.30**	**4.33E-04**	**0.132**	0.05
Private use (other scenarios)				
TGD regional (10%)	20.2	6.73E-03	(2.02)	(0.72)
northern EU-15	0.54	1.80E-04	0.053	0.02
PEC regional SEU-15			0.008	0.003
PEC regional NEU-15			0.002	0.0007
90th percentile Berlin high effluent input area 1996/1997			(0.91)	(0.33)

Sediment

The results of the comparison of PEC with PNEC are shown in Table 3.38. $PNEC_{sediment}$ = 0.375 mg/kg wet weight or 1.72 mg/kg dry weight, derived directly from toxicological data, where the intake of AHTN by ingestion of food is taken into account. Thus the risk characterisation is expressed as PEC/PNEC without an additional factor. The PECs were discussed in section 3.1.4.3.

Table 3.38 shows that for all compounding and formulation scenarios as well as for the production scenario, PEC/PNEC is below 1. Also for the private use scenario which is based on the Southern EU-15 Scenario, the ratio is below 1 (underlined in table).

The assessment based on recent measured concentrations is carried out for the sediment in the Teltow Canal in Berlin, which was a cause for concern in earlier risk assessments. For completeness the measurements in Berlin in 1996/1997 where the risk quotient was above 1, are included. The current data for the Teltow Canal show that PEC/PNEC is now below 1.

Table 3.38 PEC/PNEC ratios for sediment (mg/kg wwt)

	$PEClocal_{sediment}$ mg/kg wwt	**RCR Sediment**
		PNEC = 0.375 mg/kg wwt **PNEC = 1.72 mg/kg dwt**
Production, formulation and compounding		
Production	0.014	0.04
Compounding Site 1	0.005	0.01
Compounding Site 2	0.038	0.10
Compounding Site 3	0.0014	0.004

	PEClocal$_{sediment}$ mg/kg wwt	**RCR Sediment**
		PNEC = 0.375 mg/kg wwt **PNEC = 1.72 mg/kg dwt**
Compounding Site 4	0.006	0.02
Compounding Site 5	0.006	0.02
Compounding Site 6	0.005	0.01
Compounding (Large-medium generic)	0.273	0.73
Compounding (Small generic)	0.045	0.12
Formulation Large company	0.070	0.19
Formulation Generic scenario	0.085	0.22
Private use		
Southern EU-15	**0.086**	**0.23**
Private use (other scenarios)		
TGD regional (10%)	(1.31)	(3.5)
northern EU-15	0.0347	0.09
PECregional SEU-15	0.009	0.02
PECregional NEU-15	0.002	0.005
Measured 90th-perc. Berlin high effluent input area 1996/1997	(2.21 mg/kg dw)	(1.3)
Measured max. Berlin, Teltow Canal 2003	0.46 mg/kg dw	0.27

Conclusions to the risk assessment for the aquatic compartment:

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

Conclusion (ii) applies to production, compounding, formulation and private use.

3.3.2 Terrestrial compartment

The PEC/PNEC ratios for the soil compartment are presented in **Table 3.39**. The PNECsoil of 0.31 mg/kg dwt or 0.28 mg/kg wwt is used for this comparison. The PEC/PNEC ratios for production, compounding and formulation are all below 1 (**conclusion ii**).

For the risk assessment for private use the Southern European Scenario is used. The PEC/PNEC ratio for this scenario is below 1. Therefore **conclusion ii** is justified.

Table 3.39 PEC/PNEC ratios for the terrestrial environment

	PECsoil mg/kg wwt	**RCR Soil**
		PNEC = 0.274 mg/kg wwt **PNEC = 0.31 mg/kg dwt**
Site		
Production	0.00042	0.002
Compounding Site 1	0.000092	0.0003
Compounding Site 2	0.00113	0.004
Compounding Site 3	0.00696	0.03
Compounding Site 4		
Compounding Site 5	0.000163	0.001
Compounding Site 6		
Compounding (Large-medium)	0.074	0.27
Compounding (Small generic)	0.011	0.04
Formulation Large company	0.018	0.07
Formulation Generic scenario	0.022	0.08
Private use		
southern EU-15	**0.027**	**0.101**
Private use (other scenarios)		
TGD regional (10%)	0.425	(1.55)
northern EU-15	0.0144	0.05

Conclusions to the risk assessment for the terrestrial compartment:

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

Conclusion (ii) applies to production, compounding, formulation and private use.

3.3.3 Atmosphere

As no $PNEC_{air}$ could be derived, a risk characterisation for the atmosphere is not possible.

3.3.4 Secondary poisoning

The PNECoral for the assessment of secondary poisoning is 1.1 mg/kg. This PNEC is compared with PEC_{oral} for fish as well as for worms. The concentrations selected for the risk assessment were discussed and selected in 3.1.7.3. For fish-eating predators, the PECs for

private use based on the TGD regional (10%) scenario was used, whereas the PEC for worm-eaters is based on the SEU-15 scenario. In addition, PNEC is compared to levels measured in fish in the area of Berlin, see **Table 3.40**.

All PEC/PNEC ratios are below 1 (**conclusion ii**).

Table 3.40 PEC/PNEC ratios for fish-eating and worm-eating predators.

	$PEC_{oral,fish}$ mg/kg wwt	**PEC/PNEC fish**	**$PEC_{oral,worm}$ mg/kg wwt**	**PEC/PNEC worm**
	PNEC = 1.1 mg/kg wwt		**PNEC = 1.1 mg/kg wwt**	
Site				
Production	0.0068	0.006	0.00141	0.001
Compounding Site 1	0.0047	0.004	0.00245	0.002
Compounding Site 2	0.0182	0.02	0.00327	0.003
Compounding Site 3	0.0012	0.001	0.0186	0.02
Compounding Site 4	0.0033	0.003		
Compounding Site 5	0.0053	0.005	0.000846	0.001
Compounding Site 6				
Compounding Site 7 (Large-medium generic)	0.128	0.12	0.194	0.18
Compounding Site 8 (Small generic)	0.0224	0.02	0.0293	0.027
Formulation Large company	0.034	0.03	0.0477	0.043
Formulation Generic scenario	0.039	0.04	0.0581	0.053
Private use				
TGD regional (10%)	**0.628**	**0.57**		
southern EU-15			**0.0709**	**0.06**
Private use (other scenarios)				
TGD regional (10%)			(1.12)	(1.0)
southern EU-15	0.0418	0.04		
northern EU-15	0.0165	0.02	0.0381	0.03
measured 90th-percentile all fish Berlin 1996/1997	0.57	0.52		

Conclusions to the risk assessment for secondary poisoning:

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

Conclusion (ii) applies to production, compounding, formulation and private use.

3.3.5 Marine risk assessment

3.3.5.1 Aquatic marine compartment

With the approach using additional assessment factors of 10 to derive a marine PNEC and a simple approach of a conservative additional dilution factor of 10 in the marine environment, the risk for the marine environment is screened, see **Table 3.41**. For the private use scenario the marine PEC/PNEC ratios are similar to the freshwater and marine sediment ratios, well below 1.

As indicated in the TGD, a generic scenario for an industrial site must use a default assessment, unless site specific information is available, for PEClocal. This default assumes that industrial effluents are not treated in a municipal biological STP but are discharged directly to the marine aquatic environment. As described in section 3.1.8.1 the results of a survey confirmed that compounders and formulators using AHTN and HHCB discharge their wastewater into the marine environment only after treatment in a sewage treatment plant (Letter of IFF and PFW, July 2006). Therefore the default marine scenario used in the calculations is not realistic. When the presence of an STP is taken into account in the calculations, the PECs for marine water and sediment are considerably lower and thus all PEC/PNEC ratios are well below 1.

Table 3.41 PEC/PNEC ratios for the aquatic marine environment

	Default PEClocal seawater, µg/l	**Default PEC/PNEC seawater**	**With STP PEC/PNEC seawater**	**Default Marine PEClocal sediment, mg/kg wwt**	**Default PEC/PNEC sediment**	**With STP PEC/PNEC sediment**
		PNEC = 0.28 µg/l			**PNEC = 0.075 mg/kg wwt**	
Site						
Compounding Site 7 (Large-medium)	0.184	2.56	0.14	0.120	1.6	0.33
Compounding Site 8 (Small generic)	0.0282	0.39	0.02	0.018	0.24	0.05
Formulation Large company	0.0437	0.61	0.04	0.0284	0.38	0.08
Formulation generic	0.0553	0.77	0.04	0.036	0.48	0.09
Private use						
southern EU-15	**0.0131**		**0.05**	**0.0087**		**0.12**
Private use (other scenarios						
northern EU-15	0.0054		0.02	0.004		0.05
TGD regional (10%)	0.201		0.71	0.138		(1.84)

Conclusions to the risk assessment for the aquatic marine compartment:

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

Conclusion (ii) applies to compounding, formulation and private use.

Note that for compounding this conclusion is now based upon evidence that currently no medium-large compounders located within a radius of 500 m from a sea or ocean shore discharge their waste water directly (i.e. without treatment) into the marine environment. If such would be the case a risk would be identified and the need for limiting the risks would be indicated (conclusion iii). See **Table 3.41**

3.3.5.2 Secondary poisoning

The risk for food chain effects is expressed as the PEC/PNEC ratio for a predator in the marine food chain and for a marine top-predator, see Table 3.42. The risk ratios are below 1 for the private use scenario. Also for the default compounding and formulation scenarios the PEC/PNEC ratios are below 1. Therefore no calculations were performed with inclusion of the STP. The concentrations measured in marine fish in Norway are also below the PNEC.

Table 3.42 PEC/PNEC ratios for the aquatic marine environment

	PECoral predator mg/kg wwt	PEC/PNEC predator	PECoral top-predator mg/kg wwt	PEC/PNEC top-predator
PNEC	**1.1**		**1.1**	
Site				
Compounding Site 7 (Large-medium generic)	0.0554	0.05	0.0114	0.010
Compounding Site 8 (Small generic)	0.0086	0.008	0.0021	0.002
Formulation Large company	0.0133	0.012	0.003	0.003
Formulation generic	0.017	0.015	0.0037	0.003
Private use				
southern EU-15	**0.0041**	**0.004**	**0.0012**	**0.001**
Private use (other scenarios)				
northern EU-15	0.0017	0.002	0.00041	0.0004
TGD regional (10%)	0.062	0.06	0.0161	0.015

Conclusion to the risk assessment for secondary poisoning in the marine food chain:

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

Conclusion (ii) applies to compounding, formulation and private use.

3.4 PBT ASSESSMENT

In order to protect the marine environment against unpredictable or irreversible long-term effects, substances must be submitted to the PBT-assessment. Available data must be tested to the PBT-criteria from the TGD (2003). For substances that do not fulfil all three PBT criteria, but are known to be persistent and bioaccumulating, vPvB (very persistent and very bioaccumulating) criteria are set.

3.4.1 Persistence

The TGD (2003) criteria for persistence for PBT substances as well as vPvB substances are summarised in **Table 3.43**.

Table 3.43 PBT criteria for persistence

PBT		**vPvB**	
criterion (days)	Environment	criterion (days)	environment
t ½ > 60	marine waters	t ½ > 60	water (marine and freshwater)
t ½ > 40	fresh waters	t ½ > 180	sediment (marine and freshwater)
t ½ > 180	marine sediments		
t ½ > 120	fresh water sediments		

For AHTN no data are available from tests that simulate the marine environment in water or sediment. Evidence for rapid degradation is available from tests with river water with 5 µg/l, resulting in 42% degradation of the parent material in 28 days and an overall t½ of 9 days. Rapid primary biodegradation was observed and polar metabolites were readily formed (Schaefer and Koper 2005). Moreover, it was also shown that the substance is rapidly metabolised in fish (t½ 1–3 days) and in midge larvae.

Although there is a lack of data for AHTN on the fate in sediment, the available data show that there is a high similarity with the behaviour of HHCB. The rate and pattern of metabolisation in the die-away tests as well as in fish for AHTN were similar to the ones in HHCB.

Rapid photodegradation in water is observed by Willenborg and Butte (1998), Buerge et al. (2003) and Sanchez-Prado et al. (2004). The half-life in lake water in summertime was 3.5 to 7 days. It may be expected that this photodegradation also takes place in the upper water layer of the marine environment. The estimated atmospheric half-life time is 7.3 hours.

Thus, based on the available information it can be concluded that AHTN does not meet the persistence criterion.

3.4.2 Bioaccumulation

The bioaccumulation criterion for PBT substances is BCF > 2000. For vPvB substances, the criterion is BCF > 5000.

The bioconcentration in Bluegill sunfish (*Lepomis macrochirus*) was studied according to OECD Guideline 305E. The measured BCF is 597 (Van Dijk, 1996). This study was carried out under GLP. In another bioconcentration study with zebrafish (*Brachydanio rerio*)

according to OECD Guideline 305E the measured BCF is 600 (Butte and Ewald 1999). BAF-values determined from actual measurements in fish and surface water are available. The individual BAF in 165 eel samples in the Berlin area ranged from 250 to 1791 (Fromme et al., 2001b). BAF-values for rudd (40), tench (280), crucian carp (670), eel (400) and zebra mussel (570) are also reported (Gatermann et al., 2002).

Therefore, the BCF for fish is below 2000 although there is an indication that it may accumulate in a lower invertebrate species that is not capable of metabolising the substance.

Evidence for the absence of food chain accumulation or biomagnification is presented in section 3.1.7.2. In general concentrations in predatory organisms in Arctic and marine species were reported to be substantially lower than expected based on the physical-chemical properties of AHTN. In the beluga whale concentrations were relatively low indicating metabolic transformation (Kelly et al. 2004). The concentrations were at or below the background level in the ringed seal (Hühnerfuss et al. 2002), pygmy sperm whale and Alaskan polar bear (Kannan et al. 2005), sperm whale and South polar skua eggs (Nakata et al. 2007), red fox and/or polar fox livers (Mogensen et al 2004). The extensive studies of Kannan et al. (2005) and Nakata et al. (2007) show that bioaccumulation in the food chain does not occur.
It is concluded that AHTN does not meet the criterion for bioaccumulation.

3.4.3 Toxicity

The criterion for environmental toxicity for PBT substances is NOEC (long term) < 0.01 mg/l.

Several tests on aquatic species were performed under GLP:

Algae: the toxicity to algae was studied twice in a static test according to OECD Test Guideline 201 with *Pseudokirchneriella subcapitata* (Van Dijk, 1997). The measured geometric mean 72 hours NOEC of these 2 tests was 0.276 mg/l.

Invertebrates: for *Daphnia magna*, a semi-static 21-d toxicity test was carried out according to OECD Test Guideline 202, part II, proposed updated version of June 1993 (Wüthrich 1996a). The measured 21 days NOEC was 0.196 mg/l.

For the marine copepod *Acartia tonsa*, a 5-d toxicity test was carried out with larval development ratio as the endpoint according to a specific adaptation of the draft OECD Test Guideline (2004). The 5 days EC_{10} was 0.028 mg/l.

Fish: A 21-d prolonged toxicity test was carried out with bluegill sunfish (*Lepomis macrochirus*) according to OECD Test Guideline 204 under flow-through conditions (Wüthrich 1996b). The measured 21 days NOEC was 0.089.

An early life stage test was carried out with fathead minnow (Pimephales promelas) according to OECD Test Guideline 210 under flow-through conditions (Croudace, et al. 1997). The 36 days NOEC from this early life stage study is 0.035 mg/l.

Another early life stage test was performed with the zebrafish, *Brachydanio rer*io. The test was carried out in accordance with OECD TG 210 in an intermittent-flow-through system during 34 days. The 24 days NOEC from this early life stage study is also 0.035 mg/l (Hooftman and Borst 1999).

Conclusion: the lowest (long-term) test result is 0.028 mg/l. Thus, based on the results of 6 GLP studies, AHTN does not meet the criterion for environmental toxicity.

The criterion for human toxicity for PBT substances is classification with one or more of the following R-phrases: 25, 28, 40, 45, 46, 48, 60, 61, 62, 63, 64, 68. All toxicological tests performed on mammals only justify the classification harmful when swallowed (R22).

The criterion for endocrine disrupting effects for PBT substances is evidence of ED potential, e.g. listed in the Community Strategy for Endocrine Disrupters. There is no evidence of ED potential; AHTN is not listed in the Community Strategy for Endocrine Disrupters (COM(2001)262final) as a substance with suspected or proven ED potential.

It is concluded that AHTN does not meet the criteria for PBT substances.

4 HUMAN HEALTH

4.1 HUMAN HEALTH (TOXICITY)

4.1.1 Exposure assessment

4.1.1.1 General discussion

See also section 2.2. for the use pattern of AHTN.

AHTN (6-Acetyl-1,1,2,4,4,7-hexamethyltetraline) is a member of a group of substances used in fragrances and known collectively as the polycyclic musks.

AHTN is used to make a fragrance long lasting and have a positive technical effect on its balance. The balance is important because AHTN possesses fragrance properties that bring the initial and residual smell into harmony. Because of these properties, it is used in many fragrances and is found in a wide variety of consumer products like soaps, shampoos, detergents, cosmetics and perfumes.

AHTN is produced in one plant in The Netherlands.

4.1.1.2 Occupational exposure

From the use pattern described in section 2.2 the following stages of the life cycle will be described in this chapter:

Scenario 1. Production and crystallisation of AHTN (section 4.1.1.2.1)

Scenario 2. The compounding of fragrance oils (Occupational exposure from formulation; section 4.1.1.2.2)

Scenario 3. Formulation of consumer products that contain fragrance oils (section 4.1.1.2.3)

Scenario 4. The use of cleaning agents by professional cleaners (section 4.1.1.2.4)

For each of the scenarios measured exposure data (where available) and exposure models have been used to estimate the exposure levels. Where the EASE model is used, estimates were calculated using EASE 2.0 for Windows.

4.1.1.2.1 Scenario 1. Production and crystallisation of AHTN

All European production of AHTN occurs in one plant in the NL (brand name Tonalid®).

The following specific description of workers exposure is based on site visits and audits by an independent consultant for industry to this plant.

The following activities can be described:

- Process operation
- Crystallisation and packing (1 person in each shift)
- Analytical measurements (1 person in each shift)
- Odour quality control (2 persons, daily basis)
- Wastewater treatment (1 person, daily basis)

1. Process operation

Activities performed

The tanks are filled by pumping the raw materials with remote control of the pumps. The entire process operates without direct handling and at a pressure below atmospheric to prevent any escape of vapour from the equipment. Vessels are cleaned a few times per year by pumping a solvent into them and this solvent is collected in barrels for incineration.

Exposure at process operation

Direct handling by operators is rare and therefore exposure of the skin to liquid is negligible. From the storage tank outside on the roof of the factory there is no inhalation of vapour.

2. Crystallisation of AHTN

In the crystallisation room liquid AHTN is crystallised, so it can be stored and transported as a powder. The process is shown in Figure 4.1.

The crystallisation process starts with liquid AHTN at a temperature of about 60 °C, which is stored in vessels located on the roof of the building. Liquid AHTN flows from a vessel between two cold crystal rollers and on a cold conveyor belt. This conveyor belt is cooled by water of about 6 °C. The cooling system is called the belt cooler. The AHTN, which crystallises, is broken into big pieces by another crystal roller. A crusher at the end of the conveyor belt crushes the big pieces of AHTN, and the powder that comes out is kept in the collecting bunker. From here 50 kilograms at a time is poured into drums. After the filling station, the drums are transported to a second station where packing takes place. Now the drums are ready for storage and transport.

Exposure at crystallisation

In the crystallisation room, one person is working and is responsible for the maintenance of the installation, filling of the drums and the packing of the drums. It is obliged to wear a helmet and safety goggles at the plant. Gloves and dust masks are not obliged, but are available for the workers. During an eight-hour working day two persons work 4 hours each in the crystallisation room and where they are exposed to AHTN dust by inhalation and dermal contact. Oral exposure may be excluded as under occupational conditions no food is consumed. The inhalation exposure is dependent of the concentration of AHTN dust in the workplace atmosphere.

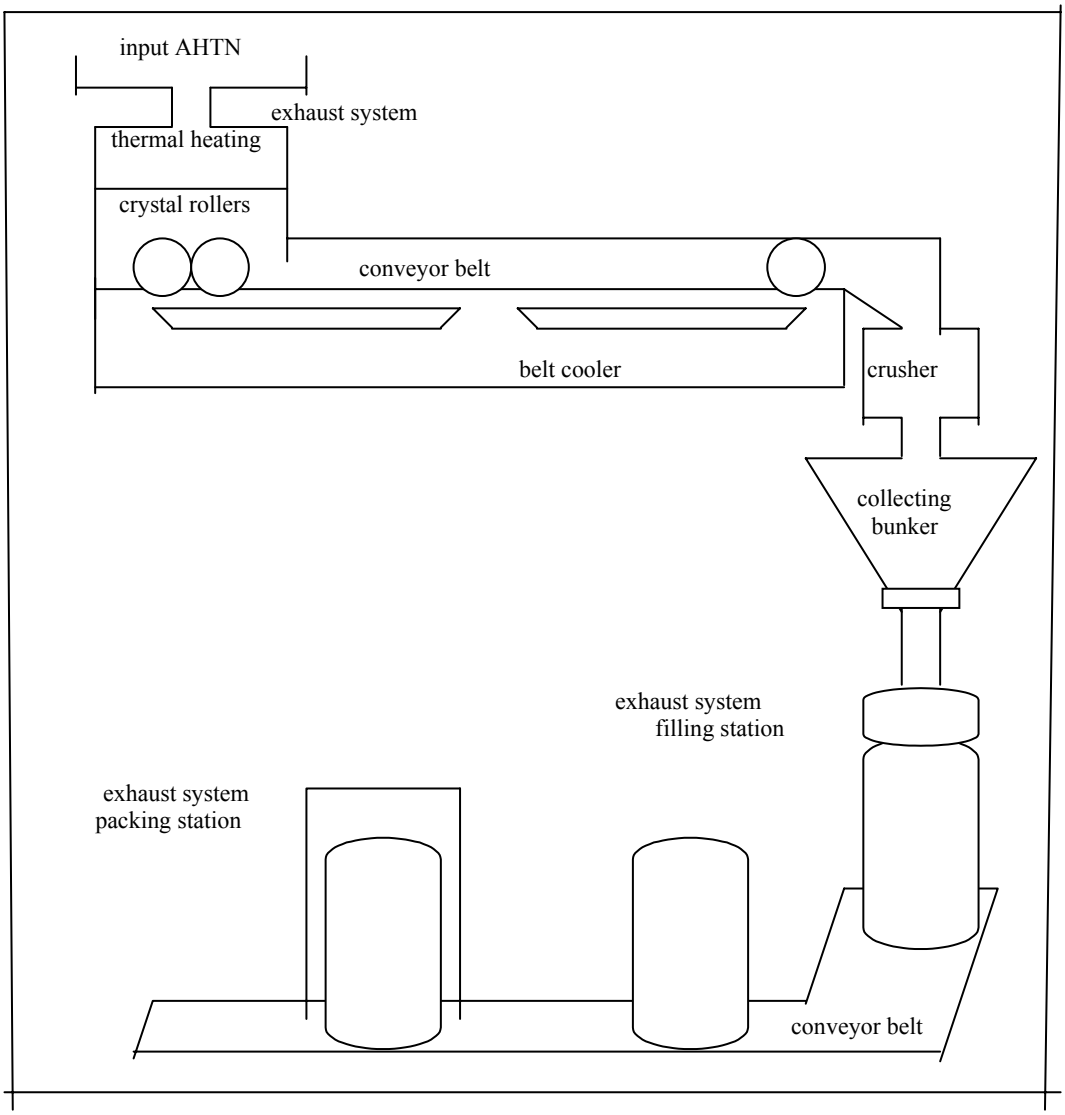


Figure 4.1 Global scheme of the crystallisation process of AHTN

3. Analytical measurements

Activities performed

Two analysts handle together about 2 - 5 samples a day.

Exposure during analytical measurements

Collection of samples is fully automatic. The closed vials are clean on the outside and no contact with the skin takes place.

Exposure of liquid to skin and inhalation are negligible.

4. Odour quality control

Activities performed

The sample bottles are kept dry and clean which is very important in view of contamination disturbing the odour perception. A drop of a 10% solution is absorbed on the tip of a piece of paper and sniffing takes a few seconds.

Exposure during odour quality control

In view of the lack of a good model to estimate exposure in these circumstances, the small quantities that are used and the short time duration, inhalation exposure is estimated to be negligible (expert judgement). The exposure to liquid on the skin is estimated to be negligible.

5. Wastewater treatment

Activities performed

The mixture obtained after acetylation is washed with water. The water phase is pumped into a vessel for storage. The aluminium containing wastewater is pumped into lorries and used for removal in wastewater treatment plants.

Exposure at wastewater treatment

In the whole process no contact with workers takes place.

Quantitative evaluation of exposure at Scenario 1 " Production and crystallisation"

Inhalation exposure

Although the production process is by remote control, it cannot be excluded that the operator will enter the room regularly for a short term and incidentally for maintenance during up to 8 hours. As the equipment for the two reaction steps is completely kept at an under pressure and the process steps are conducted at room temperature and the distillation is conducted at vacuum there will be no losses of vapour to the process room.

At 25 °C the vapour pressure is 0.0682 Pa and with the molecular weight of 258.4 the vapour-saturated air above 100 % AHTN would have a concentration of 7.1 mg/m^3.

Measured data during production

In the storage tanks on the roof, the temperature is 60 °C. The gas directly above the tanks has been sampled on one day over a period of three times in a half hour. Samples were analysed and contained 53, 93 and 203 mg AHTN/m^3. At this place, however, there is no exposure to workers.

Measured data during crystallisation

At the crystallisation and packaging, the workers may be exposed to dust *via* inhalation and *via* the skin. The plastic lined drums are filled automatically and at the end of the conveyor belt the plastic lining is closed manually. On average about six drums are filled per hour. This handling takes only part of the working time. During the rest of the time workers inspect the system and do other things in the same room. The exposure scenario is rather specific because of the character of the crystals such as particle size and dustiness. Therefore monitoring data are preferred over the application of EASE or other models.

The exposure to dust was measured in 2001. A separate report is submitted to the Dutch rapporteur.

The measurements were conducted during three days. The third day represented an exceptional situation. That day the crystallisation rollers jammed and had to be cleaned manually with an extraordinary dust generation. This situation may occur a few times per year. The maintenance workers are instructed to wear dust caps as a personal protection.

Results:

Table 4.1 Exposure measurements during crystallisation

Personal sampling	**AHTN in µg/m³**
Normal day 1	15
Normal day 2	54
Extraordinary, day 3	500
Stationary samples	
Day 1	99, < 6.3, 70, 9.3
Day 2	54, < 5.9, 5.9, 73, <5.8, 3.3
Day 3	500*, 589*, 381*, < 5.2, 33

* During maintenance of the cooling belt - incidental

The data show that the dust concentration in the room is rather variable in time and place. During normal production days some values are in the range between 54 and 99 µg/m^3 other values are in the range between 3.3 and 9.3 µg/m^3.

The monitoring report showed that total inhalable dust contained only a relatively small (10 – 30 %) fraction of respirable dust, whereas the respirable fraction has a relatively low percentage (<1 %) AHTN. This means that the respirable fraction of the dust originates from a different source and AHTN is mainly present in the course crystalline particles.

Total inhalable dust	240	respirable	27	AHTN:	< 0.22 µg/m^3
Total inhalable dust	240	respirable	32	AHTN:	< 0.22 µg/m^3
Total inhalable dust	130	respirable	41	AHTN:	< 0.18 µg/m^3

In a recent study on the particle size distribution of pure (98.6 %, typical production sample) AHTN (Chilworth Technology 8/1/2002) it turns out that the average particle size is 388 µm (median value 479 µm) and less than 10 % has a particle size < 141 µm (= granules).

Modelled data

Exposure during cleaning of tanks is estimated with the EASE model to be 0-0.1 ppm, assuming non-dispersive use direct handling and a vapour pressure of 0.007 Pa. As the partial vapour pressure is lowered by previous rinsing of the tanks, exposure is assumed to be negligible.

With the EASE model, assuming inhalable particles and dry manipulation in the presence of LEV, the estimate is 2-10 mg/m^3. Assuming low dust technique because of the granular nature of the substance, in the presence of LEV, the exposure to dust is estimated to be 0-1 mg/m^3.

Dermal exposure to dust

Measured data at crystallisation

To establish a more realistic quantification of dermal exposure due to the handling of AHTN powder, a measurement was conducted in 2002. AHTN powder is handled by scooping from one vessel to another. The right hand was used and covered by a cotton glove. After scooping

ten times, the cotton glove was removed for extraction of AHTN. This procedure was repeated ten times.

The average amount of dust extracted from the glove was 1.4 mg. The highest value of ten replicates is 5.5 mg.

In the crystallisation room drums and plastic bags are filled and closed automatically. Workers do not actively handle the product but conduct inspections on the equipment. The simulation of the dermal exposure by scooping is in fact representative for the scenario 2 (compounding). An exposure after ten times scooping will, therefore, give an overestimation. For the scenario 1 at the crystallisation the 5.5 mg can be considered as a worst-case estimate.

This value will be used for the risk assessment during packing in the crystallisation room. The exposure time is 4 hours, the duration of the shift.

Modelled data at crystallisation

By using the EASE model (TGD appendix 1 figure 15) for wide dispersive use, direct handling and intermittent exposure, it follows that on a dermal surface of 2000 cm^2 (hands and fore arms) 1-5 mg/cm^2 would deposit. In a similar way for non-dispersive use the value would be 0.1 – 1 mg/cm^2. Total dermal exposure would be in the range between 200 up to 10,000 mg.

Conclusions

None of the above model estimates or similarities is really representative for the specific situation. The production process itself is by remote control, and no drumming or filling, other than incidentally when drumming cleaning liquids, is involved.

The measured data during crystallisation will be used for the risk assessment. Though exposure will be to vapour and to dust, it is assumed that because of the very low vapour pressure of the substance, exposure to vapour will be negligible compared to exposure to dust. As a reasonable worst case it can be assumed that during normal operation the concentration in air will be 0.1 mg AHTN/m^3 at maximum with an exposure time up to 4 hours. This value will be used as a maximum in the risk characterisation for normal operation.

In the incidental situation during maintenance, an AHTN concentration of up to 0.6 mg/m^3 occurred for 2 hours and two times per year.

Exposure of the skin to liquid or dust does not occur under normal operation or during cleaning operation and can be considered negligible for all workers in the production.

Based on the measurements related to scenario 2, the worst case estimate of dermal exposure to dust of AHTN in scenario 1 to be used in risk characterisation is 5.5 mg/day (420 cm^2). This will be an overestimate of exposure because of the process that leads to less interaction with the substance in scenario 1 compared to scenario 2.

4.1.1.2.2 Scenario 2. The compounding of fragrance oils (Occupational exposure from formulation)

To describe the workers scenario for compounding, six out of about 39 large and medium size compounding sites were visited and audited. The six plants together consumed in 2000 an amount equivalent to 187 ton/y undiluted AHTN. If we take into account that 358 ton is

produced for destinations inside EU and 24 % of the EU use volume is directly used by the detergent formulators, and 6 % is used by small size compounders, the use volume of all large and medium size compounders is 70 % of 358 = 250 tonnes. The use volumes of the six large compounders represent 187/250, i.e. 75% of the large and medium size compounding activity.

Based on the six different specific descriptions supplied separately to the rapporteur, a generic scenario is described for the large and medium size compounding facilities.

The small size companies have not been visited. The number of sites in EU is about 140. The description of activities and possible exposure of workers is considered to be completely similar for small size and medium/large size compounders. This view is based on the fact that in both situations the exposure is mainly the result of manual handling procedures for small size batches. The production of large size batches that occurs mainly at the large and medium size facilities is highly automatic and does not contribute significantly to the workers exposure. For the exposure via inhalation, the ventilation rate may be lower or LEV may not be used. As a worst-case approach it is assumed that the inhalation exposure is a factor of five higher than in the observed large size plants.

In these six large compounding sites, fragrance oils are produced in volumes that vary between 1 and 20,000 kg. The operations in these plants are expected to be representative for the operations in all large- and medium size compounding plants.

This scenario for compounding of very complex mixtures does not include the use by detergent and cosmetic industry for bulk products. This type of use represents about 24 % of the AHTN use volume. For these use types the scenario for formulation will be more adequate.

Activities performed

Worker scenarios differ for the following four types of activity:

- Delivery and filling of stock tanks and containers
- Compounding of fragrance oils (Occupational exposure from formulation)
- Analytical determinations
- Odour control

1. Delivery and filling of stock tanks

The amounts delivered to each of the six plants vary between 5 and 40 tonnes of powdery (crystals) AHTN. On one site, AHTN was delivered as a liquid. The total use volume of six plants was 187 tonnes in 2000.

Delivery of crystalline material is between 4 and 13 times a year in units of 25 or 50 kg fibre containers lined with plastic bags. The containers are transferred to stock crystal containers with a top lid. In one plant AHTN is also delivered in molten form with 10 tonnes tanker trucks for about 7 times per year.

Exposure to vapour and dust at delivery and stocking is negligible because containers remain closed.

Exposure to vapour at delivery of the molten form does not occur. There is no direct handling during connecting of valves and tubes.

2. Fragrance compounding

In a typical large compounding facility, about 15 to 60 compounders work in shifts, with a rotation of tasks. The activities include, retrieving of tanks and containers, pouring and weighing, pushing lorries, mixing, sampling and washing.

In general a few thousands (as many as three thousand) of ingredients are mixed in fragrance compounds that contain some 40 ingredients each. About 1/5 of the final products contains AHTN. The average final weight in AHTN containing products is about 2 %.

Batches of fragrance mixtures vary between 0.5 L to 20 m^3, Dosing of AHTN occurs between 2 and 30 times a day on average. Each worker prepares up to one AHTN containing batch per day.

AHTN powder is dosed mainly manually. The plastic inner bag is placed directly in the funnel and emptied with the help of the underpressure. Smaller volumes are dosed by scooping from the stock container. The handling of powdery material takes less than one minute. Due to the internal lining of the containers, limited dust is produced when the container is emptied entirely. When scooping smaller amounts the handling may give temporary and locally somewhat elevated concentrations in the air. Dosing of larger volumes of AHTN powder to larger batches is done mechanically. The vessel has underpressure thus sucking material through a funnel into the vessel. In this way spillages are reduced. The plastic inner bag is placed directly in the funnel and emptied with the help of the underpressure.

In one plant AHTN is dosed as a molten form. The molten AHTN is dosed by automatic systems and therefore contact with the liquid is assumed to take place only rarely.

The worker does not stay at the same place. For the risk assessment it can be assumed that the exposure to dust with AHTN in the air is limited to 15 minutes per day for each worker. Workers have no fixed position in the room but walk around with mobile tanks to different storage tanks, mixing area or washing area or push containers below a series of valves. Sometimes containers are moved automatically.

The room for compounding is centrally vented and several spots are equipped with local ventilation (LEV). Workers wear impermeable gloves in most cases but not 100 % of the time. Gloves are changed and hands are washed 3 to 5 times per day (at each break). As the risk of cross contamination should be eliminated as much as possible the accuracy is high. Paper tissue or cloth may be used to clean incidental spills, hands are usually protected to prevent contamination.

- Bulk volumes are compounded either automatically or by using membrane pumps.
- Vessels for 500 L are on lorries and moved below a series of automatic valves.
- Volumes of 0.2 L are poured manually by cups or volumes of 10 – 20 L poured from buckets into larger size containers.
- Small volumes are taken automatically or by single use pipettes and added to 0.5 L bottles. In a typical example, this occurs by rotation of tasks approximately 8 times per year during one week. In that week, about 10 – 12 samples of AHTN are taken per day.

Mixing of oily liquids is performed by mounting mechanical stirrers on top of the open tanks. Tanks are mixed for about ½ hour, with or without covering. The mixing area has additional LEV. The AHTN containing fragrance oils that are mixed contain on average 2 % AHTN.

This procedure of mechanical mixing is limited to medium size batches between 25 and 500 litres. The amount of AHTN involved in such a batch varies between 0.5 litre and 10 litres. On average each worker does handle up to one AHTN containing batch per day in this way.

Washing of tanks and smaller vessels is by washing machines. The machines are loaded and unloaded by hand. Larger tanks may be cleaned by mobile systems using hot water with detergents. The outside of the tanks is clean. In one plant, vessels are not cleaned by washing, but the tanks are reused to dose dilution ingredients that belong to the same batch and this acts as a cleaning before the next batch. In some cases, the Teflon lined empty tanks are rinsed with alcohol and the few remaining droplets are wiped dry with a cotton rag. During this procedure, gloves are always worn. Tubes and pumps are flushed with alcohol before they are used in a new batch. The alcohol is collected for waste incineration.

Floors are cleaned regularly or continuously by liquid vacuum cleaners.

Exposure by inhalation of dust

There are no measurements of AHTN dust in the working room.

A similarity approach may be used on the basis of the measured AHTN dust concentrations during the crystallisation, filling and packaging. It can be assumed that the concentration in the compounding room is similar to the concentration in the packaging room at the crystallisation plant. The handling of crystalline AHTN during compounding, however, is limited to a few minutes per day per worker.

Exposure by inhalation of vapour

Exposure to vapour will occur during the working day i.e. 8 hours each working day. The concentrations are not regularly monitored. Concentrations can be estimated on the basis of a few studies (see below).

Exposure of skin to dust

The handling of powdery AHTN is conducted by scooping from one stock vessel to a mixing vessel. As each compounder prepares up to one AHTN containing batch per day, this scooping occurs also once per day. To adjust the weight possibly two scoops may be taken. This procedure is simulated for measuring the amount of dust that could stay on the skin.

Exposure of skin to liquid

Exposure of the skin to AHTN containing dilutions may occur incidentally. As the workers are well trained to work neatly and accurately, small spills of liquid are usually prevented. However where automation is not yet 100 % and gloves are not always used, it cannot be guaranteed that there will never be any drop on the hands.

In one plant where the molten form of AHTN is dosed, the exposure due to a small spill of the molten form has to be considered. However, as the stock solution is 70 degrees Celsius personal protection will be used in case the hot liquid has to be handled manually.

Compounders prepare on average one batch per day that contains AHTN. A reasonable worst-case scenario for the Risk Assessment of the frequency of AHTN spills with possible skin contact is once per day. Such contacts will be limited to a maximum of 100 cm^2 (about 50 % of the skin surface of the palm of one hand) and for the duration of less than 2 hours, because of the hand washing frequency. Therefore, the exposure on skin will be highly variable between workers and over time. An estimation of the exposure can be based on a specific study for a similar compounding plant in the US (see below).

3. Analytical measurements

The scenario of analytical work is more or less identical to the description given for the production. The total number of samples per plant may vary by a factor of 10 in relation to the production volume, but the number of AHTN containing samples per analyst will be roughly identical.

4. Odour quality control

The exposure scenario during odour control is more or less identical to the description given for the production. The total number of samples per plant may vary by a factor of 10 in relation to the production volume, but the number of AHTN containing samples per analyst will be roughly identical. In practice, these activities are not conducted for extended periods, because the capacity of odour perception decreases after some time. The worker performs other tasks in between in order to recover the sensitivity of smelling.

Quantification of exposure for compounding in scenario 2

Inhalation exposure

Measured data

In several plants air monitoring in the working area has been conducted. It is assumed that both exposure to dust and to vapour are detected.

Plant 1

Measurements in 1991/1992 for limonene showed a range of 1- 6 mg/m^3. No information is available on the measurement duration and the number of measurements.

The room was vented with 74,000 m^3/hr, which implies an exchange rate of six times per hour. As limonene is used in the same room and often occurs in the same batches as AHTN the concentrations can be estimated on the basis of Limonene measurements and the partial vapour pressures of the two substances in the mixtures.

The vapour pressure for limonene and for AHTN differ by a factor 3,000 (2.1 mbar = 210 Pa / 0.07 Pa) Assuming that AHTN occurs at 2 % and limonene at 10 % in the final solution in the fragrance oils, the partial vapour pressures will differ by a factor of 7500 the corresponding AHTN concentrations would be (1- 6 mg/m^3 divided by 7500) 0.13 – 0.8 $\mu g/m^3$.

It should be remarked here that this assumed proportionality on the basis of Henry's law, does in general not hold for mixtures of a higher concentration.

Applying an empirical ratio between Limonene and AHTN (500:1) on the basis of data of plant 4 results in a concentration of AHTN < 12 μg/m^3.

Plant 2 Extensive air monitoring study in 1988

The air was monitored during 4 – 5 months once per week and over 8 hours per day. The ventilation rate is 50,000 m^3/hr, which means a renewal of five times per hour. Both stationary and personal monitoring was carried out. At the detection level of 2.5 μg/m^3, no AHTN was found. No information is available on activities that were measured, the number of measurements, and the measurement method.

Plant 3 Personal monitoring programme in 1995

Five persons were monitored 8 hr/day during 1 week. No detailed information is available on the number of measurements. The total flow of the air through the building was 300,000 m^3/hr and air renewal rate is 4 times per hour.

The concentration of Limonene was 0.34 – 0.41 ppm.

Taking into account the much lower vapour pressure and assuming proportionality this would correspond to 0.45-0.55 μg AHTN/m^3. By using the empirical ratio between Limonene and AHTN concentration on the basis of data of plant 4, the AHTN concentration would be 3.9-4.6 μg/m^3.

Plant 4 Static measurements in 1999 during 11 working days for 8 hours a day

The various rooms have a common ventilation of altogether 5,100 m^3/hr. Air renewal rates for the various rooms are between 5 and 11 times per hour.

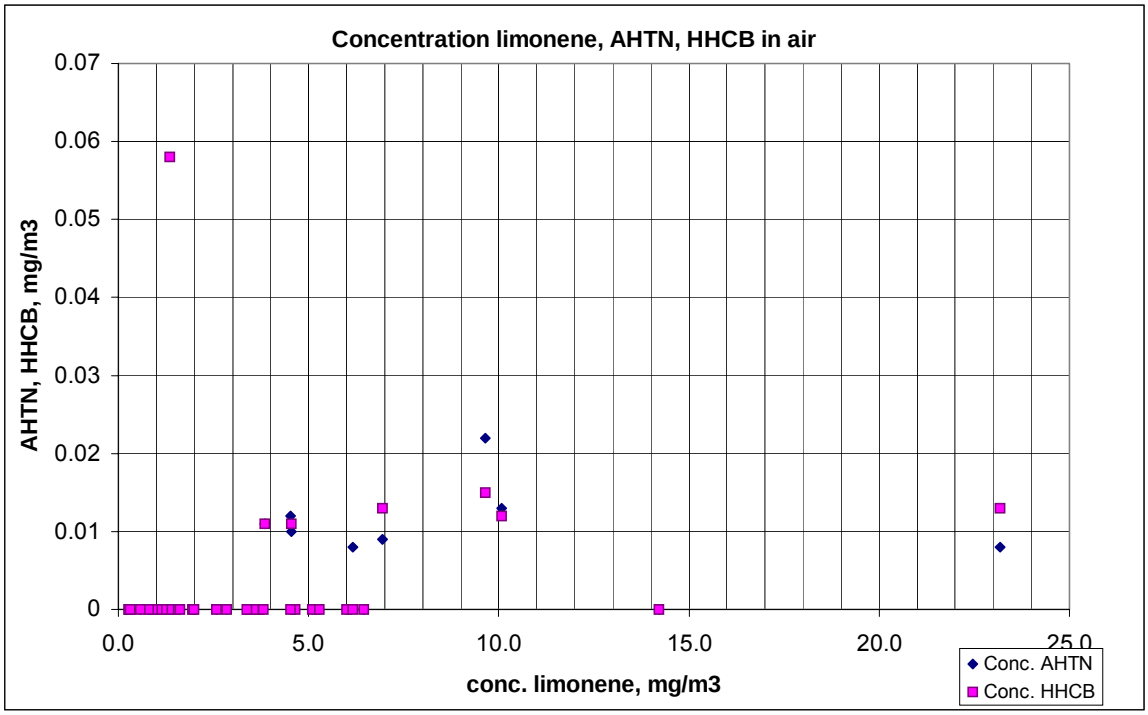
A total of 34 samples from 16 spots in the working room have been analysed.

In the majority of the samples the AHTN concentration was below the detection limit of 5.6 μg/m^3. In 8 samples the AHTN level varied between 8 and 23 μg/m^3. The highest value of 23 μg/m^3 was found in a special mixing room and at the other places the levels were below 13 μg/m^3. No information is available on the measurement method.

Limonene was measured simultaneously in these samples. In 39 samples Limonene varied between 0.3 and 23.2 mg/m^3, with a median value of 2.8 mg/m^3. Limonene constituted 15 – 60 % of the Volatile Organic Carbon in the air. If AHTN was measured above the detection level, the ratio between Limonene and AHTN was approximately 500:1 (when expressed in mg/m3). This ratio is much lower than expected on the basis of proportionality with Vapour pressures and concentrations according to Henry's law.

The relation between the concentration of limonene and AHTN is presented in Figure 4.2**.**

Figure 4.2 Concentrations measured in air, plant 2.



Modelled data

Inhalation exposure to vapour

With a vapour pressure of 0.07 Pa at process temperature (20 °C) exposure to vapour is estimated with EASE 2.0 to be 0-0.1 ppm.

Inhalation exposure to dust

With the EASE model, assuming low dust technique because of the granular nature of the substance, in the presence of LEV, the exposure to dust is estimated to be 0-1 mg/m^3.

Dermal exposure

Measured data for exposure of skin to liquid

In a study by Cohen and Wolff (1998), conducted in a compounding facility in the USA a model substance (isobornyl acetate with vapour pressure13.3 Pa) was used to measure the exposure of the hands. Isobornyl acetate was dispensed and weighed in volumes varying from small (milligram – gram) quantities up to bulk volumes (ten to hundreds of kilograms) using pipettes or pumps. Dispensing procedures are identical to the description given for two of the visited sites

The measurement was based on extraction of the gloves, hand rinsing and extraction of tissues for wiping of the face. The sampling was conducted as a one-day study. A total of 11 workers were monitored and sampled. Nine of the workers were engaged in compounding of fragrances, which included weighing, dispensing and mixing of fragrance ingredients. The remaining 2 workers were material handlers with job duties such as transporting fragrance ingredients in metal drums and periodically collecting aliquots of samples from large drums for QC purposes. The total number of samples was approximately 214 including baseline

samples. Four to six hand rinse samples were taken from each worker at each work-break. Two to six glove samples were collected from each worker in the course of the day and 3 face wipes were obtained from each worker at the end of the work shift.

Cumulative extracted amounts over several pairs of gloves used over the day reached 0.32 – 39 mg, with 9 mg on average. This result supports the concept that incidentally (less than once on a day) one drop of liquid may be spilled on the hands. For the estimation of exposure the frequency per two hours (between washing of the hands) has to be considered. Moreover it has to be taken into account that the spilled liquid contains a low fraction of a specific substance.

The study of Cohen and Wolff (1998) showed that there is no correlation between the exposure and the volume of fragrance oils produced or handled and exposure is not related to a special task of the compounders during the day.

As normally AHTN is not present in liquid form of stock solutions at higher concentrations but on the average at 2 %, the reasonable worst-case exposure to the skin can be set at 1 mg/day (0.02 * 50 µl) at a surface area of 100 cm^2 and at a frequency of once/day.

Quantification of the exposure by handling of cotton rags:

The wiping procedure concerns mixing vessels for compounding. The average concentration of AHTN in the final mixtures is 2 % AHTN. The vessels are Teflon lined to minimize the amount of residue after pouring into larger recipients. After the Teflon lined containers are emptied it can be assumed that less than 0.5 ml of fragrance oil is left. After rinsing with 50 ml alcohol the concentration in the residual 0.5 ml alcohol is less than 1/100 of 2 % and this will be absorbed on the cotton. So the absolute amount is 0.2 mg. In a realistic worst case the cotton rag may be taken in the hand at the wet side, so the 0.1 mg AHTN is spread over a surface of 100 cm^2 to give a dose of 0.001 mg/cm^2.

With the production of one AHTN containing batch per person per day, this may occur not more than once a day and the exposure may hold for a maximum of two hours before the hands are washed.

Measured data for exposure of skin to dust

To establish a more realistic quantification of dermal exposure due to the handling of AHTN powder, a measurement was conducted in 2002. AHTN powder is handled by scooping from one vessel to another. The right hand was used and covered by a cotton glove. After scooping ten times, the cotton glove was removed for extraction of AHTN. This procedure was repeated ten times.

The average amount of dust extracted from the glove was 1.4 mg. The highest value of ten replicates is 5.5 mg.

In a normal scenario the workers scoop only one time with possibly one or more small scoops for correction of the weight. Thus the use of 5.5 mg/day is a realistic worst-case exposure for this scenario.

Modelled data

In the EASE model (figure 15 Appendix I TGD) the incidental handling of liquid drums (non dispersive use) is assumed to result in an exposure of 0 – 0.1 mg/cm^2 per day or based on the surface of hands and forearms (2000 cm^2): 0 – 0.2 g/day.

The assumed exposure of 0 - 0.1 mg/cm^2 applied only on the palms of both hands (420 cm^2) gives 0 - 42 mg/day. In the case of AHTN usually no concentrated liquids are used, so this exposure should refer to the liquid mixture with an average percentage of 2 % AHTN. This corresponds to 0 – 0.8 mg AHTN/day.

Based on the scenario description, the spills on the hand will be limited to a few drops of about 50 µl each. One drop of a compounded fragrance oil will contain on average 2 % AHTN so 1 mg AHTN. If, according to the scenario description, the amount is spread only over the surface of the fingers of one hand (100 cm^2) the concentration on the skin becomes 0.01 mg/cm^2. Due to the discipline of hands washing every two hours, the exposure time will be not more than two hours per day.

At one site AHTN is used as a concentrated stock in molten form. The exposure to liquid will be different in that case. For this site a worst-case scenario can be estimated on the basis of the assumption that one drop of 50 µl concentrated AHTN is spilled. So a maximum exposure is 50 mg/day on 100 cm^2. This theoretical estimate is fairly well in agreement with the study of Cohen and Wolff (see below) where a maximum of 39 mg on the hands was measured. The value of 39 mg will be used for the risk assessment in this special scenario.

This is without considering the use of PPE. As the molten stock solution is 70 ^{0}C this procedure will be automatic or if manual, a personal protection is prescribed for this procedure to prevent serious burning of the skin. For the risk assessment the situation without automatic sampling and without PPE is included as a hypothetical scenario.

Conclusions

The model approach with EASE is not focussed on the specific scenario of fragrance compounding. The high level of automation, the intensive ventilation, the high working accuracy required to prevent any cross contamination are specific for this type of industry. For the measured data no measurement methods are available and it can therefore not be stated with certainty that these values represent both inhalation exposure to dust and vapour. Furthermore there is no information on the types of activities that were measured. However, from the measured data it seems that the EASE model overestimates the exposure during handling of AHTN. There are several sets of measured data. It is assumed that together these sets provide a more or less representative picture of the exposure levels. Therefore the monitoring data are to be preferred.

Monitoring data for limonene are available for three plants. These are indicative and show that the conditions in the various plants are quite similar. In one plant (4) limonene and AHTN were measured simultaneously. Using a ratio of 500 on the basis of these measurements and applying this ratio to the other two plants would result in a concentration of AHTN up to 4.6 and up to 12 µg/m^3. In the second plant AHTN was not measured above the detection limit of 2.5 µg/m^3.

The monitoring program at plant 4 indicated levels between 0.65 and 23 µg/m^3. Only one value of 23 µg/m^3 was observed and this concerns the mixing room. The mixing room is a separate compartment and not representative for the compounding room. As workers do not stay at one place in the area it is a reasonable worst-case scenario for the 8 hours average to use the highest observed level in the compounding room of 13 µg/m^3 in the risk assessment. A reasonable worst case for the short-term exposure will be set at 15 minutes inhalation of 23 µg/m^3. This short-term exposure will also contribute to the daily dose, but this is considered

to be already sufficiently covered by the daily average value of 13 µg/m^3. This level refers to the short-term exposure in the mixing room.

For the small size compounding activities it cannot be assumed that the ventilation rates in the compounding rooms are similar as to large size plants, or the LEV may not be used. Therefore as a worst-case approach for this risk assessment, it is assumed that the 8 hours average exposure via inhalation is a factor of five higher (65 µg/m^3). This is a rather conservative estimate. The ventilation rate in the various plants varied between 4 – 11 per hour for the plants 1, 2, 3 and 4. A normal ventilation rate in a workers room should be 1/hour and if odorous substances are handled this will normally be designed at 3/hour.

For dermal exposure, the model approach with EASE, and the monitoring data from a similar plant in the USA are in fairly good agreement and fit with the observation that small spills of one drop may incidentally occur. As normally only dilutions of 2 % on average are handled, this will give an exposure of 1 mg/day on a surface of 100 cm^2 during two hours.

In the case that hands are in contact with cotton rags for wiping of residues the exposure of 0.1 mg/day on a surface of 100 cm^2 during two hours will be used for risk assessment.

In the hypothetical case where a molten form of AHTN is handled without PPE, the exposure of 39 mg/day on a skin surface of 100 cm^2 during two hours is used.

For the exposure of the skin to dust (of AHTN) the value of 5.5 mg (on 420 cm^2) as based on measured values will be used in the risk assessment.

4.1.1.2.3 Scenario 3. Formulation of consumer products that contain fragrance oils

The drummed liquid fragrance oil is used for production of toiletries, shampoos, soap, and the household cleaning products industry for production of detergents and cleaning agents etc. It is assumed that the production is highly automated with little or no exposure to polycyclic musks. Exposure may be possible during handling of the drums and during cleaning and maintenance of the equipment. Because of the risk of contamination with an odour, it is expected that the procedures limit exposure in a similar way as described for the compounding of fragrance oils. In addition the fragrance oil contains only dissolutions of AHTN.

Quantification of exposure in scenario 3

Inhalation exposure

Measured data

No data available

Modelled data

As the systems are closed and the bulk volume of the products handled will contain not more than 1 % of a 2 % solution of AHTN (0.02% AHTN has a partial vapour pressure of 14 x 10^{-6} Pa) inhalation exposure is estimated with the EASE 2.0 model to be 0-0.1 ppm. both at handling and cleaning/maintenance. Because of the strong dilution of the substance however, exposure is assumed to be negligible.

Dermal exposure

Measured data

No data available

Modelled data

The EASE model estimates dermal exposure for the direct handling of liquids assuming non-dispersive use and incidental contact 0 – 0.1 mg/cm^2/day. With the palms of both hands (420 cm^2) and a maximum concentration of 2 % for the undiluted fragrance oil this gives 0.85 mg AHTN/day.

Based on one site visit, the exposure at cleaning is quantified as follows:

Empty vessels of fragrance oil are rinsed with water that is added to the product. The rinsed vessels are filled with hot water and put aside for some time. Before cleaning the water is drained to the sewer. The two rinsing steps can be considered as a dilution of 0,01 in each step, so in total, a factor of 10^4. With a content of 2% of the substance, total dilution is 2.10^{-6}.

Assuming wide dispersive use, direct and extensive contact, exposure is estimated to be 5 – 15 mg/cm^2/day. With an exposed area of 1300 cm^2 (hands and forearms), exposure is 0,01 – 0,04 mg/day ((5-15) x 1300 x 2.10^{-6}).

Conclusions

Inhalation exposure is considered to be negligible during this activity.

Dermal exposure during handling of drums is estimated to be 0.85 mg/day and during cleaning activities to be 0.04 mg/day.

4.1.1.2.4 Occupational exposure from production (Scenario 4. The use of cleaning agents by professional cleaners)

Professional cleaners may be exposed to AHTN in cleaning products. It is assumed that no special high pressure spraying equipment is used, so that no aerosol formation takes place, and that the products are diluted before use.

Inhalation exposure

Measured data

No data available

Modelled data

For inhalation exposure, with a (partial) vapour pressure < 1 Pa and no aerosol formation, with direct handling and non-dispersive use, the EASE model estimates an exposure of 0-0.1 ppm. Due to the very low vapour pressure of the diluted product, the inhalation exposure is considered to be negligible.

Dermal exposure

Measured data

No data available.

Modelled data

For dermal exposure assuming extensive contact and wide dispersive use the exposure according to EASE ranges from 5 – 15 mg/cm^2/day on both hands (840 cm^2) so 4200 – 12,600 mg water with cleaning agent diluted 1 to 50. To calculate the specific exposure of AHTN the dilution has to be taken into account.

The final professional cleaning products contain 0.1 % fragrance oil which in turn contains on average 2 % AHTN. The (50 times) diluted cleaning agent will contain maximal 0.4 mg AHTN per litre (0.1 % * 2 % * 0.02 = 0.4 mg/l) and the highest exposure is 0.005 mg (12,600 mg liquid with 0.4 mg/l). This exposure is not a single dose, but the outer liquid may be replaced repeatedly if the hands are immersed. Although, these workers will wear rubber gloves for hygienic reasons and to prevent damage to the skin due to a constant exposure to detergents, as a worst case it can be assumed that the liquid on the skin is refreshed every 15 minutes. The external dose after each renewed submersion will be added to the depot in and on the skin to give a daily-accumulated exposure of 32 * 0.005 = 0.16 mg/d.

Summary statement of the dermal exposure level

For dermal exposure, the exposure level from the use of cleaning agents by professional cleaners is 0.005 mg/d (840 cm2) each time the hands are submersed. Over a day this may accumulate to a dose of 0.16 mg/day.

4.1.1.2.5 Summary of occupational exposure

A summary of the occupational exposure assessments is presented in **Table 4.2.**

Table 4.2 Conclusions of the occupational exposure assessment

Workers scenario	**Inhalation**		**Dermal**			
	Vapour	**Dust**	**Liquid**		**Dust**	
	Concentration		**Dose level**	**% AHTN [b]**	**Dose level**	**% AHTN [b]**
Scenario 1						
- production (crystallisation)	Negligible	0.1 mg/m^3 4 hr/day	Negligible	Negligible	5.5 mg on 420 cm^2	100%
maintenance of cooler	Negligible	0.6 mg/m^3 2 hr/day (2 times per year)	Negligible	Negligible	no data	no data
Scenario 2						
- delivery	Negligible	Negligible	Negligible	Negligible	Negligible	Negligible

Workers scenario	Inhalation		Dermal			
	Vapour	Dust	Liquid		Dust	
compounding - large & medium size plants	0.013 mg/m^3 [a] 8 hr/day 0.023 mg/m^3 [a] 15 min/day		1 mg/d on 100 cm^2	2%	5.5 mg on 420 cm^2	100%
Compounding - small size plants	0.065 mg/m^3 [a] 8 hr/day 0.1 mg/m^3 15 min/day		1 mg/d on 100 cm^2	2%	5.5 mg on 420 cm^2	100%
- compounding (molten form)			39 mg/day 100 cm^2	100%		
- analysis	Negligible		Negligible	Negligible		
- odour control	Negligible		Negligible	Negligible		
Scenario 3						
- handling	Negligible		0.85 mg/day 420 cm^2	2%		
-cleaning & maintenance	Negligible		0.04 mg/day 1300 cm^2	0.002%		
Scenario 4						
- handling	Negligible		0.16 mg/day 840 cm^2	0.02%		

If no quantification is given in the field, this means that the route of exposure is not applicable. In one plant dermal exposure to liquid may be 39 mg/day on 100 cm^2.

[a] Exposure is assumed to be a combination of vapour and dust.

[b] Because AHTN is a photosensitiser (e.g., see section 1.4.2 and section 4.1.2.5.3), an effect which is concentration dependent, AHTN exposure is also expressed as '% AHTN'.

4.1.1.3 Consumer exposure

4.1.1.3.1 Introduction

Consumer exposure occurs from consumer products to which AHTN is added intentionally as a component of the fragrance that enhances the product. It is used as an ingredient in commercial preparations (fragrance oils) intended to be used to fragrance a wide variety of consumer products such as perfumes, creams, toiletries, soaps and shampoos (SCCNFP, 24 October 2000).

Two scenarios for direct consumer exposure are discussed. Scenario 1 considers exposure as a result of the use of AHTN in fragrances in cosmetics and scenario 2 considers exposure via other perfumed household products. In both cases it is necessary to know the levels of perfume oil used in the various products; to cover all cases, a worst-case (97.5th percentile AHTN in perfume oil) is used to calculate the amount of AHTN in these products. For understanding the exposure to AHTN, the various patterns of use of these products will be taken into account.

For Scenario 1, cosmetic use, the levels of perfume in the various classes of cosmetics and the 97.5th percentile use level (12%) of AHTN in the perfume were the results of industry surveys (IFRA, survey of use of AHTN in the fragrance industry; private communication via COLIPA, 1996 to the SCCNFP) and are shown in **Table 4.3.** These are the same figures that were used in the report of the SCCNFP (SCCNFP, 24 October 2000).

Table 4.3 .A Use levels (97.5 percentile use) of AHTN in cosmetic products.

Product category	Fragrance oil in product in %	AHTN in fragrance oil (%)	AHTN in product (%)
Body lotion	0.4	12	0.048
Face cream	0.3	12	0.036
Eau de toilette	8.0	12	0.96
Fragrance cream	4.0	12	0.48
Anti-perspirant /deodorant	1.0	12	0.12
Shampoo	0.5	12	0.06
Bath products	2.0	12	0.24
Shower gel	1.2	12	0.144
Toilet soap	1.5	12	0.18
Hair spray	0.5	12	0.06

Scenario 2: AHTN is also used in household and laundry cleaning products and air fresheners (Balk and Ford, 1999). Both the fragrance manufacturing industry and the consumer product industry were surveyed by the International Fragrance Association (IFRA, 2002) to determine the use levels of fragrance oils in product types and the levels of AHTN that are used to formulate these oils. (Table 4.4) Exposure calculations based on use of household products are reported in Scenario 2.

Table 4.4 Use levels of AHTN in household cleaning products. Results of a survey including data from manufacturers of fragrances as well as household cleaning products

Product category	Median use level of fragrance oil in product in %	97.5 percentile use level of AHTN [a]	Level of AHTN in product
Laundry regular powder	0.33	8.7%	0.03%
Laundry liquid	0.80	8.7%	0.07%
Laundry compact (tabs)	0.33	8.7%	0.03%
Laundry compact (powder and other)	0.28	8.7%	0.02%
Laundry liquid concentrate	0.85	8.7%	0.07%
Fabric softener (conditioner)	0.43	8.7%	0.04%
Fabric softener concentrate	0.80	8.7%	0.07%
Laundry additive, powder bleach	0.20	8.7%	0.02%
Laundry additive, liquid bleach	0.20	8.7%	0.02%
Laundry additive, tablet	0.30	8.7%	0.03%

Product category	Median use level of fragrance oil in product in %	97.5 percentile use level of AHTN [a]	Level of AHTN in product
Hand dishwashing liquid	0.23	8.7%	0.02%
Hand dishwashing liquid concentrate	0.45	8.7%	0.04%
Machine dishwashing powder	0.15	8.7%	0.01%
Machine dishwashing liquid	0.15	8.7%	0.01%
Machine dishwashing tablet	0.15	8.7%	0.01%
Surface cleaner liquid	0.60	8.7%	0.05%
Surface cleaner powder	0.25	8.7%	0.02%
Surface cleaner gel	0.75	8.7%	0.07%
Surface cleaner spray	0.13	8.7%	0.01%
Toilet cleaner powder	0.30	8.7%	0.03%
Toilet cleaner liquid	0.35	8.7%	0.03%
Toilet cleaner gel (concentrate)	0.38	8.7%	0.03%
Toilet cleaner tablet	0.30	8.7%	0.03%
Toilet rim block or gel	6.0	8.7%	0.52%

97.5 percentile use level of AHTN in fragrance oils used in household and detergent products

Using any and all of the above products results in some exposure to AHTN, either dermally through direct contact, orally as a result of migrating from dishes and pots into food and drinks or by inhalation of aerosols from cleaning sprays. However, exposure calculations have shown that because of the low contents of AHTN and the use patterns of these products, exposure to AHTN is considered negligible in comparison to the exposure from cosmetic use (HERA draft risk assessment, 2003). Only exposures from laundry detergents and dish washing liquid are shown here. For the estimates of consumer exposure to laundry detergents and dish washing liquids, the consumer exposure models developed by the Association Internationale de la Savonnerie, de la Détergence et des Produits d'Éntretien (International Association for Soaps, Detergents & Maintenance Products) (A.I.S.E) and the European Chemical Industry Council (CEFIC) as part of their program on Human & Environmental Risk Assessment (HERA) of ingredients of household cleaning products and presented in the HERA guidance document are used along with the data presented in the Table of Habits and Practices for Consumer Products in Western Europe, an appendix to that document (HERA, 2002). This table presents use data for cleaning products in grams/task, use frequency, duration of task and other intended uses. While minimum, maximum and typical use frequencies and amounts are given; only the maximum figures are used for the exposure estimations with the understanding that further refinement will be possible if necessary.

In addition, the use of AHTN in fragrance oils in air fresheners and inhalation exposure from such use is discussed.

4.1.1.3.2 Exposure from uses

Inhalation exposure

Measured data: The occurrence of musk fragrances was tested in air samples from 74 kindergartens and in household dust from 30 apartments in Berlin in 2000 and 2001. In indoor air, AHTN levels were above the detection limit (10 ng/m^3) in 73 (out of 74) samples. The median value was 44 ng/m^3 with a maximum concentration found of 107 ng/m^3 and a 95th percentile of 88 ng/m^3. In household dust, AHTN was detected in 83% of the samples (detection limit 0.5 mg/kg) with a median value of 0.9 mg/kg, a maximum of 3.1 mg/kg and a 95th percentile of 2.3 mg AHTN/kg (Fromme et al., 2004).

Synthetic musks were determined in ambient and indoor air by Kallenborn and Gatermann (2004). Five outdoor samples were taken at Kjeller in Norway on different time points in 1998. AHTN concentrations ranged from 41 to 76 pg/m^3. For comparison a few indoor samples were taken. This resulted in AHTN levels of 13.4 ng/m^3 at a hairdresser, 5.8 ng/m^3 at a rest facility, 6.2 ng/m^3 at a toilet, 11.6 ng/m^3 at a cafeteria and 0.6 and 1.9 ng/m^3 at two laboratories.

Synthetic musks were determined in 35 house dust samples from vacuum cleaner bags in Germany (no further specifications). Sample processing consisted of sieving the contents of the vacuum cleaner bags to <63 µm, discarding coarse matter and fiber. AHTN levels ranged from < 0.2 to 77 mg/kg with a median of 0.59 mg/kg (Butte, 2004).

Modelled data

Scenario 1 – Cosmetic products

As is shown below, the largest exposure to AHTN as used in cosmetics is by dermal contact. Also cosmetic exposure via aerosols (perfume sprays) will end up mostly on the skin. The exposure by inhalation from this type of exposure would be expected to be negligible compared to that from dermal exposure to cosmetics. The evaporation from the skin is also considered negligible due in part to its low vapour pressure (0.000682 hPa at 25 ^{o}C) but also because of the relatively small amounts used (see **Table 4.5** below in the dermal exposure section). However, if such evaporation were considered, it would be necessary to lower the dermal exposure by the amount evaporated.

For hairspray, the following calculation can be made, assuming that most of the hairspray will end up on the skin (90%) based on Bremmer et al. (2002) and the exposure time will be short e.g. 5 minutes.

2 x 5000 mg x 0.0006 x 0.1 x 0.083 x 1 x 1 / 60 = 0.0008 mg/kg bw /day

With:

Events per day: 2	
Amount of hairspray per event:	5000 mg
Amount of AHTN in hairspray:	0.06%
Aerosol in air:	10% of hairspray ends up in the air (0.1)
Time of exposure:	0.083 hr (= 5 min)
Inhalation rate (light activity, TGD default):	1 m^3/hr
Emission volume (around the head):	1 m^3
Body weight:	60 kg

Scenario 2 - Household products

Inhalation of AHTN from air freshener aerosols

Air freshener aerosol may contain up to 1% of fragrance. The 95th percentile use level of AHTN in fragrance oils is 8.7% (IFRA, 2002). The estimation of worst-case exposure to AHTN from an aerosol air freshener is 0.065 mg/kg bw/day. This value is calculated on the assumption of 5 g air freshener/event (comparable to hair spray), one event/day, in a living room of 58 m^3, with an exposure period of 16 hr and one event/day in a bedroom of 16 m^3 and an exposure period of 8 hr (Bremmer and van Veen, 2000), combined with an inhalation rate of 20 m^3/day not taking into account deposition and assuming 100% absorption on inhalation. The initial concentrations must be adjusted for a standard ventilation rate of 0.5/hr for the living room and 1/hr for the bedroom (Bremmer and van Veen, 2000).

The ventilation rate of 0.5/h means that every hour half of the amount in the living room will be removed, following the equation

$C = (A / V \times T \times Q) \times (1 - e^{-Q\,t})$

With: C = concentration
A = amount
V = volume room
T = exposure time
Q = ventilation rate

The first hour the exposure will be:

5000 mg * 1% * 8.7 % /58 m^3 * 20 m^3/24 hr * 1 = 0.0625 mg

Every next hour, roughly half of the amount will be removed. After 16h, this results in a total exposure of 0.125 mg per person/day, which will be 0.002 mg/kg bw/day assuming 60 kg bw.

For the bedroom a similar calculation can be made:

5000 mg * 1% * 8.7% /16 m^3 * 20 m^3/24 hr * 1 = 0.23 mg

The next hour roughly the total amount will be removed. This results in a total exposure of 0.0038 mg/kg bw/day assuming 60 kg bw.

These exposures must be considered gross exaggerations because they are based on the assumptions of no deposition, 100% absorption on inhalation and the consumer being only in the bedroom or living room 24 hr/day.

Estimates of long term exposure from a constant diffusion (electric plug-in type) have also been made (Cadby et al., 2002, some details not given). Using maximal observed weight loss data (12 mg/hr) and a number of highly conservative assumptions: (1) A 20 m^3 room with 110 m^3/hr internal airflow and (2) 54.4 m^3/hr external air flow from the SCIES model (Versar Inc., 1991) gives inhalation exposure to the fragrance well below 100 μg fragrance which would be equivalent to 0.0087 mg AHTN/kg bw/day if used at 8.7% in the fragrance oil.

Summary/statement of the inhalation exposure level

Using the highest measured level of 107 ng/m3, the exposure to indoor air would result in a level of 35.7 x 10^{-6} mg/kg bw/day (107 ng/m3 x 24 hr x 20 m3/24 hr (ventilation rate)/ 60 kg). Compared to the calculated data, this is considered to be negligible.

For risk characterisation we used the calculated data, where under realistic worst-case conditions, exposure by inhalation of products containing AHTN results in 0.0046 (= 0.0008 + 0.0038) mg/kg bw/day.

Dermal exposure

Measured data

Reports show that AHTN has been found in certain consumer products at the following concentrations: laundry detergents 1.0 – 550 ppm; shower gels 2 – 520 ppm; fabric softeners 6 – 290 ppm; alcohol-based cosmetics <0.1 – 1.1 ppm (Eschke et al., 1995a). The reported levels in alcohol-based cosmetics are about ten thousand times lower than those used **in Table 4.3** (9600 ppm for eau de toilette) indicating the conservative nature of the modelled exposure.

In a recently performed study, among other chemicals, levels of AHTN were measured in some consumer products. Levels found are presented in Table 4.5. In other tested body care products, perfumes or air fresheners, the levels were lower than the detection limit of 0.5 mg/kg. (Peters, 2003).

Table 4.5 Levels of AHTN in some consumer products.

Product description	AHTN (mg/kg)
A shampoo	60
An eau de toilette spray	20
An eau de toilette	50
An eau de parfum vaporisateur spray	3.2
A baby shampoo	15
A kids shampoo	2.1
An airfreshener (electrical plug-in)	9058

Modelled data

Scenario 1 – Cosmetic products

Because the products containing the highest levels of AHTN, eaux de toilette, fragrance creams, antiperspirants and deodorants, are intended for use on the skin and because of the low volatility of AHTN, the principal route of exposure for consumers to AHTN is considered to be via the skin. Upper use levels in a variety of consumer products have been reported to range from 0.04 to 0.96% (SCCNFP, 24 October 2000). These exposures are based on usage data supplied by COLIPA (1996) to the SCCNFP along with data on the use of fragrance oils in consumer products and a 97.5th percentile use level of AHTN in such oils of 12% (IFRA, survey of use of AHTN in the fragrance industry; private communication via COLIPA, 1996

to the SCCNFP). The resulting exposure to AHTN on the skin from the use of a combination of all classes of consumer products on a daily basis was calculated to result in a "worst case situation" of 0.34 mg/kg bw/day (**Table 4.6**) and this value will be used for the risk characterisation.

The 97.5th percentile was chosen to represent an upper level that could be encountered in cosmetic products. It is highly unlikely that a series of consumer products exist all of which would contain the same fragrance ingredient at the 97.5th percentile use level. Furthermore, it is unreasonable to consider that a consumer could consistently use all of the classes of cosmetic products over their entire lifetime, all of which are perfumed with the upper 97.5th

Table 4.6 Overview of products and uses that can contain AHTN adapted from the SCCNFP (SCCNFP, 24 OCTOBER 2000)

Type of cosmetic product	Application quantity in grams per application	Application frequency per day	Retention factor (%)[5]	AHTN in product (%)	Exposure to AHTN (mg/day)	Exposure to AHTN for 60 kg person (mg/kg/day)
Body lotion[1]	8	0.71	100	0.048	2.7	0.045
Face cream[2]	0.8	2	100	0.036	0.576	0.0096
Eau de toilette[3]	0.75	1	100	0.96	7.2	0.12
Fragrance cream[1]	5	0.29	100	0.48	7.0	0.116
Anti-perspirant /deodorant	0.5	1	100	0.12	0.60	0.010
Shampoo	8	1	1	0.06	0.048	0.0008
Bath products[4]	17	0.29	1	0.24	0.12	0.002
Shower gel[4]	5	1.07	10	0.144	0.77	0.013
Toilet soap	0.8	6	10	0.18	0.86	0.014
Hair spray	5	2	10	0.06	0.6	0.010
				Total	**20.5**	**0.34**

1. Assumes use of conventional body lotion 5 times a week and a fragranced cream twice a week.

2. Including make up and foundation.

3. Including perfume and after shave, but these three products are not used concurrently. The quantity used is inversely proportional to the fragrance concentration so these values include all hydroalcoholic products.

4. Assumes use of bath products twice a week and an average use of shower gel 1.5 times a day, 5 times a week.

5 Proportion of product remaining on the skin.

Scenario 2 - Household products

Direct skin contact from hand-washed laundry

Hand-washed laundry is a common consumer habit. During this procedure, the AHTN-containing laundry solution with an estimated product concentration of 10 mg/ml comes in direct contact with the skin of hands and forearms. A hand-washing task typically takes 10 minutes (Table of Habits and Practices - HERA, 2002). This table also reports a maximum frequency of 18 times per week (3 times/day) when using laundry powder, which seems highly exaggerated but nevertheless is used here as a worst case scenario. The table gives a lower frequency of hand washing with laundry liquid of 10 times per week (1.43 times/day),

which still seems exaggerated. Because the use level of AHTN is different in powder (0.03%) from that in liquid (0.07%) both scenarios are calculated here.

The exposure to AHTN is estimated according to the following algorithm from the HERA guidance document.

$$\mathbf{Exp_{sys} = F_1 \times C \times Kp \times t \times S_{der} \times n / BW}$$

For this exposure estimate, the terms are defined with following values for the calculation considering a worst-case scenario:

F_1	percentage weight fraction of substance in product	**0.03%** (0.0003) or **0.07%** (0.0007) **Table 4.4**
C	product concentration in mg/ml:	**10 mg/ml** (HERA, 2002)
Kp	dermal penetration coefficient	**3.4 x 10^{-5} cm/h***
t	duration of exposure or contact	**10 min (0.167h)** (HERA, 2002)
S_{der}	surface area of exposed skin	**1980cm^2** (TGD, 1996)
n	product use frequency (tasks per day)	**3** or **1.43** (HERA, 2002)
BW	body weight	**60 kg**

* The dermal penetration coefficient was calculated from the dermal flux (10.3 ìg/cm^2) which was determined in an *in vitro* dermal penetration (Green and Brain, 2001) according to the following algorithm: Kp = dermal flux/(exposure time x concentration of test solution); Kp = (0.00815 mg/cm^2)/(24h x 10 mg/cm^3) = 3.4 x 10^{-5} cm/h

For powder use:

Exp$_{sys}$ = [0.0003 x (10 mg/ml) x (3.4 x 10^{-5} cm/h) x (0.167h) x 3 x (1980 cm²)] / 60 kg=
0.0017 µg/kg bw/day

For liquid use:

Exp$_{sys}$ = [0.0007 x (10 mg/ml) x (3.4 x 10^{-5} cm/h) x (0.167h) x 1.43 x (1980 cm²)] / 60 kg=
0.0019 µg/kg bw/day

Direct skin contact from pre-treatment of clothes

Consumers typically spot-treat clothing stains by hand using either a detergent paste (i.e. water/laundry powder = 1:1) or a laundry liquid, which is applied undiluted (i.e. concentration = 1000 mg/ml) directly on the garment. In this exposure scenario, only the skin surface of the hand (~ 840 cm^2) is exposed.

The exposure to AHTN is estimated according to the same algorithm from the HERA guidance document as is used above using 100% liquid detergent since this contains the highest concentration of AHTN.

F_1	percentage weight fraction of substance in product	0.0007 (laundry liquid) (**Table 4.4**)
C	product concentration in mg/ml:	**1000 mg/ml** (100%)
Kp	dermal penetration coefficient	**3.4 x 10^{-5} cm/h** (Green and Brain, 2001)
t	duration of exposure or contact	**10 min (0.167h)** (HERA, 2002)
S_{der}	surface area of exposed skin	**840cm^2** (TGD, 1996)
n	product use frequency (tasks per day)	**0.5** (HERA, 2002)
BW	body weight	**60 kg**

Exp$_{sys}$ = [0.0007 x (1000 mg/ml) x (3.4 x 10^{-5} cm/h) x (0.167h) x (840 cm²) x 0.5]/ 60 kg=
0.028 µg/kg bw/day

This exposure estimate is very conservative in that it does not recognize use of water to dilute the detergent, a common practice and the fact that only a fraction of the two hands' surface skin will actually be exposed.

Direct skin contact from hand dishwashing

The determination of AHTN exposure from hand dishwashing also uses the same algorithm to calculate the dermal exposure to AHTN from hand dishwashing. The following assumptions have been made to address a reasonable worst-case scenario:

F_1	percentage weight fraction of substance in product	**0.02%** (0.0002) (**Table 4.4**
C	product concentration in mg/ml:	**2 mg/ml** (HERA, 2002)
Kp	dermal penetration coefficient	**3.4 x 10^{-5} cm/h** (Green and Brain, 2001)
t	duration of exposure or contact	**45 min** (0.75h) (HERA, 2002)
S_{der}	surface area of exposed skin	**1980 cm^2** (TGD, 1996)
n	product use frequency (tasks per day)	**3** (HERA, 2002)
BW	body weight	**60 kg**

$$\mathbf{Exp_{sys}} = [0.0002 \times (2\ mg/ml) \times (3.4 \times 10^{-5}\ cm/h) \times (0.75h) \times (1980\ cm^2) \times 3] / 60\ kg =$$

0.001 µg/kg bw/day

Summary/statement of the dermal exposure level

Dermal exposure of consumers to products containing AHTN can mainly be attributed to cosmetics (worst-case estimate 0.34 mg/kg bw/day). Compared to this exposure the dermal exposure of consumers to household products is negligible.

Oral exposure

Measured data

No data available.

Modelled data

Fragrance ingredients are not intended for oral ingestion; therefore no data have been modelled.

4.1.1.3.3 Summary of consumer exposure

The worst-case estimate of dermal exposure of consumers to AHTN via cosmetics amounts to 0.34 mg/kg bw/day. The inhalatory exposure of consumers to AHTN via air fresheners and cosmetics is lower, in total 0.0046 mg/kg bw/day. These figures are taken forward to the risk characterisation.

4.1.1.4 Humans exposed via the environment

The daily human intake resulting from indirect exposure via the environment takes into account exposure to AHTN in food, drinking water and inhaled air. Thus the indirect human

exposure is estimated using concentrations in fish, root and leaf crops, meat, milk, drinking water and in air.

In the EUSES model, a log Kow value of 5.4 has been used to assess the distribution in the environment. A measured fish bioconcentration factor of 597 L/kg (see Section 3.1.1.3) has been used in the EUSES model to estimate the concentration in wet fish. For other parts of the food chain, particularly root crops, leaf crops, meat and milk, EUSES estimates the concentrations in these food products using methods that, similar to the fish BCF, rely on log Kow as no equivalent measured accumulation factors exist for these routes.

For the assessment of the indirect exposure to AHTN the scenarios with highest environmental releases will be taken into account. The highest releases found resulted from private use and the generic large/medium compounding site. The daily human intake from food, water and air is calculated for these local scenarios as well as for the regional scale. In **Table 4.7** the estimated concentrations in food and other intake media are shown. The estimated daily human intake using these figures is shown in **Table 4.8**.

Table 4.7 Estimated concentrations of AHTN in human intake media

Lifecycle step	Estimated concentration in human intake media						
	Wet fish (mg/kg)	Root crops (mg/kg)	Leaf crops (mg/kg)	Drinking water (mg/l)	Meat (mg/kg)	Milk (mg/kg)	Air (mg/m³)
Private use, SEU scenario	0.079	0.098	4.45E-4	5.1E-5	2.41E-4	7.63E-5	3.66E-6
Large/Medium Compounding	0.167	0.27	9.1E-4	1.4E-4	5.3E-4	1.7E-4	7.5E-6
Regional, SEU scenario	5.11E-3	6.38E-4	1.54E-5	2.14E-6	7.9E-6	2.5E-6	1.3E-7

Table 4.8 Estimated human daily intake of AHTN via environmental routes

	Estimated human daily intake (mg/kg body weight/day)[1]							
Lifecycle step	Wet fish	Root crops	Leaf crops	Drinking water	Meat	Milk	Air	Total
Private use SEU scenario	1.30E-4	5. 4E-4	7.63E-6	1.46E-6	1.04E-6	6.11E-7	1.05E-6	6.81E-4
Fraction of total daily dose	0.19	0.79	0.01	0.002	0.0015	0.0009	0.0015	
Large/Medium Compounding	2.74E-4	1.48E-3	1.55E-5	4E-6	2.26E-6	1.33E-6	2.13E-6	1.8E-3
Fraction of total daily dose	0.15	0.83	0.009	0.002	0.001	7E-4	0.001	
Regional, SEU scenario	8.4E-6	3.5E-6	2.6E-7	6.11E-8	3.4E-8	2.0E-8	3.6E-8	1.23E-5
Fraction of total daily dose	0.68	0.28	0.02	0.005	0.003	0.002	0.003	

Note 1: Daily intake of: drinking water 2 L/day. fish 0.115 kg/day, leaf crops 1.2 kg/day, root crops 0.384 kg/day, meat 0.301 kg/day, dairy products 0.561 kg/day. Inhalation rate: 20 m^3/day. Bioavailability for oral uptake: 0.5. Bioavailability for inhalation: 1. Body weight of human: 70 kg. SEU= Southern Europe.

Intake in the vicinity of the local sources is mainly via root crops (circa 80%) and fish (15 to 20%). At the regional scale the main contribution comes again from fish and root crops but in a different proportion (68 and 28%). The highest total daily intake for a local site is derived from the large medium compounding site: 1.8 µg/kg bw/day. This value will be taken forwarded to the risk characterization. For the regional scenario a value of 0.012 µg/kg bw/day will be used in the risk characterization.

4.1.1.4.1 Exposure via air

EUSES estimates a local air concentration of 3.66E-6 and 7.5E-6 mg/m^3 or 3.7 and 7.5 ng/m^3. This is above the concentrations measured in ambient air samples taken in the south of Norway (< 0.01 ng/m^3) and over Lake Michigan (0.42 ± 0.20 ng/m^3), see section 3.1.6.3. The measured concentrations are in the same order as the estimated regional air concentration of 0.13 ng/m^3.

4.1.1.4.2 Exposure via food and water

AHTN has not been detected in drinking water (< 0.01 µg/l) based on measurements in several countries, see section 3.1.4.2.2. The EUSES model predicts a concentration in drinking water of 0.05 µg/l from private use and 0.14 µg/l from formulation.

EUSES predicts concentrations in fish of 0.08 and 0.17 mg/kg for the private use and large/medium compounding scenarios, respectively. Monitoring data for AHTN in fish are summarised in 3.1.7.2. As a very conservative approach, the 90th-percentile level of fish from Berlin in the 1990s could be used: 0.57 mg/kg. Thus the assumption is that a citizen would consume only fish caught in high effluent input areas and only the fish with the highest loads. With a daily consumption of 0.115 g fish the intake is 0.94 µg/kg bw/day. This is comparable to the estimated figures in **Table 4.8**.

No data are available on concentrations measured in vegetables cultivated under normal conditionns.

4.1.1.4.3 Exposure via mother's milk

In 1999, a study on synthetic musk fragrance ingredients in human milk was carried out by Sönnichsen et al. From 107 women, milk was taken and analysed for several polycyclic musks and nitromusks. A mean and a median fat content of 3.67 and 3.40%, respectively, were found in the mother's milk. AHTN was detected in 36.4% of the samples. The concentration of AHTN in the milk showed a mean value of 16 µg/kg milk fat with a standard deviation of 35. The minimum and maximum values found were close to zero and 267 µg/kg milk fat, respectively. Arrangements were made to prevent contamination. For more details, see section 4.1.2.1.2.

In 2001, Zehringer and Herrmann published data on 53 milk samples obtained in 1998/1999 from 29 mothers living around the city of Basle. AHTN was found in concentrations ranging from not-detectable (16 samples) to 136 µg AHTN/kg fat. The average fat content was 3.3%.

This resulted in a mean level of 44 µg AHTN/kg fat. No special arrangements were made to prevent contamination of milk samples by AHTN present on the skin.

Other literature reports show levels of 250 and 290 µg/kg fat (only 2 samples measured) (Eschke et al., 1995b) and 11-58 µg/kg fat (5 samples) (Rimkus and Wolf, 1996).

In a case control study for risk factors of early miscarriages in the Uppsala County in Sweden, during the period 1996-2003, women donated milk samples to be analysed for the presence of various polycyclic- and nitromusks. Women were also asked to fill out extensive questionnaires about lifestyle, medical history and dietary habits and a sub-population was also asked to fill out an additional questionnaire about use of perfumes and perfumed deodorants, skin lotions, laundry- and washing detergents. Milk was collected at the beginning and at the end of the breast-feeding sessions The goal was to collect 500 ml from each mother during the 3rd week after delivery. It was not mentioned whether measurements were taken to prevent contamination of the milk samples from musks present on the mother's skin. In total 101 milk samples were analysed. For 42 of these, useful data on use of perfumed products were available. AHTN was found in concentrations ranging from < 3 to 53 µg/kg milk (mean ± SD: 13 ± 12; median 10 µg/kg milk fat). A weak but statistically significant correlation was found between concentrations of AHTN in milk samples and Body Mass Index before pregnancy, but this correlation was not confirmed when the data were analysed with a different statistical method. A significant downward trend in the median concentration was observed during the study period (from *ca.* 14 µg/kg milk fat in 1996 to *ca.* 8 µg/kg milk fat in 2002-2003). The results may indicate that concentrations in milk samples from women using perfumed products were higher that in those collected from non-users, but the differences never reached statistical significance, in this rather small sample population (Lignell et al., 2004).

In a pilot study in the Czech Republic, 59 milk samples were collected from nursing mothers (living but not necessarily born in Prague). The manual sampling (milk expressed from the breast into a clean container) was conducted in accordance with WHO guidelines. Using a detailed questionnaire, relevant information on parameters, such as age, dietary habits (specifically consumption of freshwater/marine fish), use of perfumed cosmetics, frequency of contacts with detergents, etcetera were collated. The lipid content in the human milk samples ranged from 1.5 to 4.2 wt %. The detection limit for the musks was 10 µg/kg fat. AHTN was found in 90% of the samples. Concentrations ranged from not detectable to 565 µg/kg fat, with a median value of 67, a mean value of 112, and a 90th percentile of 304 µg AHTN/kg fat. No correlations were found between the musk levels and personal data of the mothers obtained by the questionnaire (Hajslova and Setkova, 2004). Milk samples were collected in 1999 at Hvidovre Hospital in Denmark from 10 primiparous mothers (25-29 year of age) 14-26 weeks after birth. AHTN was found at levels from 5.58 to 37.9 µ/kg fat. Fat content of the milk samples had an average of 3.5% ±2.5% (2.1%-4.8%). The median level of AHTN was 17.5 µg/kg fat and the average level was 19.5 µg/kg fat (Duedahl-Olesen et al., 2005).

In the recent study from the Czech Republic (Hasjlova and Setkova, 2004), the highest mean (112 µg AHTN/kg fat) and maximum level (565 µg AHTN/kg milk fat) were found, which will be used in risk characterisation.

4.1.1.5 Combined exposure

See under 4.1.3.5 Combined exposure – Risk characterization.

4.1.2 Effects assessment: Hazard identification and dose (concentration)-response (effect) assessment

4.1.2.1 Toxicokinetics, metabolism and distribution

The protocols of all studies in this section, were not specifically designed according to OECD Method 417, however, the basic guidance principles of OECD Method 417 were used for the evaluation of the test results.

4.1.2.1.1 Studies in animals

In vivo studies

All available studies in this section were evaluated for information on the absorption, distribution, excretion and metabolism of AHTN in *in vivo* animal studies. Within the subheadings based on the route of exposure, these sections were further subdivided into these fate processes to assist in the evaluation of AHTN in these studies.

Also, additional sections were added to address the available intravenous animal studies, the animal and human milk studies, and the human fat studies.

Inhalation

No data available.

Dermal

Absorption: In a GLP compliant study, the absorption, distribution and excretion of radioactivity have been determined by topical application (under occlusion) with 4.5 mg/kg bw of ^{14}C-AHTN (uniformly labelled in the aromatic ring – radiochemical purity 98.9%) in 70% aqueous ethanol solution to the shaven backs of 18 male pigmented rats (Lister-Hooded, bodyweight ca 200g, age 5-7 weeks). The application rate was 0.1 mg/cm^2 over an area of 9 cm^2 (200 µl of a solution containing 4.55 mg ^{14}C-AHTN/ml). The solvent was allowed to evaporate for an unreported time before the area was occluded with aluminium foil. (This experiment was conducted for the purpose of obtaining ethical approval for the human simulated exposure experiment (see below) and thus, skin exposure limited to 6 hr). After the 6 hr application the dressing was removed and the remaining dose at the treated area washed off with cotton wool swabs moistened with 70% alcohol. Another occlusive dressing (aluminium foil) was placed on the skin of the animals until sacrifice.

Urine, faeces and expired air were collected for rats killed at 6 hr after start of dosing or later and analysed for radioactivity and metabolite identification. Pairs of rats were killed at 0.5, 1, 3, 6, 12, 24, 48, 72, and 120 hr after start of dosing. Prior to sacrifice, blood was withdrawn for analysis. At sacrifice, all tissues (including untreated and treated skin) as well as the remaining carcass were analysed for radioactivity. Urine was collected at 0-6, 6-12, 12-24, 24-48, 48-72, 72-96 and 96-120 hr, faeces and air (0 – 48 hr only) were collected every 24 hr until 120 hr.

A majority (mean of 69.6%) of the applied material remained on the surface of the skin at the time of washing - 6 hr. Only 9.3% of the applied dose was found in excreta and tissues at that

time point. During the exposure for 6 hr a significant skin reservoir of material (approximately 13%) was formed. Some of this material may be absorbed, since up to 120 hr trace levels in organs are still being present. Based on the amount of radiolabel excreted (see below in Excretion section) combined with what remained in the tissues and carcass, but excluding the amount remaining in the skin at the treatment site at 120 hr (1.5%; see Distribution section below), approximately 18.8% of the applied dose had been absorbed in 120 hr. Analysis of the dressings applied after surface dose removal indicated that about 2% of the 13% of the material in the skin reservoir was lost to the dressing by reverse diffusion and/or desquamation. Average recovery of radiolabel (6 – 120 hr) was 91.2% (Ford et al., 1999; Aikens, 1995).

Distribution: The GLP rat *in vivo* dermal absorption study (radiochemical purity 98.9%), described above (Ford et al., 1999; Aikens, 1995), shows that plasma levels have peaked between 6 hr (time of removal of dose from surface) and 12 hr. This conclusion is based on the similarity of concentrations at both times and the theory of absorption kinetics. Analyses of tissue levels indicate that approximately 1.7% of the absorbed radiolabel was present at 120 hr; at that time, also approximately 1.5% still remained in a skin reservoir at the treatment site. The large majority of the absorbed radiolabel was found in the large and small intestines and their contents (**Table 4.9**). In the large intestine, the highest level was reached after 12 hr, thus at a later time than in the plasma, this behaviour is consistent with biliary excretion. Levels in other organs and fat essentially reflected the plasma levels peaking at 6-12 hr and declining after that. The levels measured in tissues and organs given in **Table 4.9** never exceeded 0.3 µg equivalents/g tissue. Average recovery of radiolabel (6 – 120 hrs) was 91.2%.

Table 4.9 istribution of radioactivity in selected tissues during 0.5 to 120 hours after dermal application of 14C-AHTN to male rats at a dose of 4.5 mg/kg bw over an area of 9 cm^2 (as µg equivalents/g of tissue)

Tissues	**Time (hr after initial application)**								
	0.5	**1**	**3**	**6**	**12**	**24**	**48**	**72**	**120**
LI* + contents	0.014	0.053	0.35	2.28	6.70	3.36	2.48	0.91	0.26
SI* + contents	0.041	0.36	2.45	4.49	4.13	2.02	1.36	0.67	0.24
stomach + contents	0.027	0.035	0.29	0.99	0.67	0.36	0.14	0.077	0.067
Liver	0.039	0.17	0.48	1.01	1.35	0.86	1.01	0.84	0.54
Fat	0.006	0.035	0.16	0.32	0.35	0.27	0.30	0.22	0.12
Plasma	0.009	0.044	0.13	0.21	0.22	0.12	0.10	0.077	0.037
Adrenal glands	0.067	0.19	0.25	0.28	0.15	0.071	0.085	0.053	0.036
Kidneys	0.026	0.12	0.25	0.28	0.16	0.11	0.11	0.095	0.072
Thyroid	Nd	0.14	0.44	0.30	0.22	0.084	0.14	0.078	0.049
Untreated Skin									
non-pigmented	0.010	0.044	0.14	0.23	0.20	0.13	0.067	0.047	0.029
pigmented	0.007	0.04	0.09	0.14	0.25	0.11	0.11	0.081	0.063

*LI = Large intestine

*SI = Small intestine

Excretion: In the *in vivo* dermal absorption study (GLP) in rats (6 hr application under occlusion) described above, after 120 hr, 17.1% of the applied dose had been excreted. This was primarily in the faeces (14.5%), with the remainder in the urine plus cage washing (2.6%). There was no radioactivity in the expired air. The majority was excreted within 48 hr (10.1%). No attempt was made to characterize possible metabolites. Average recovery of radiolabel (6 – 120 hrs) was 91.2% (Ford et al., 1999; Aikens, 1995)

Metabolism: No data available.

Oral

Absorption: In a GLP compliant study designed to explore the coloured organs seen in some studies (see Repeated Dose Toxicity, section 4.1.2.6 below), AHTN (purity 98.8%) was administered in corn oil to groups of 5 male or 5 female Sprague-Dawley (Crl:CD BR; ~ 7 weeks old, individually housed) in daily doses of 15 or 100 mg/kg bw/day for 14 days followed by 2 daily doses of 15 or 100 mg/kg bw/day radiolabelled AHTN (uniformly labelled in the aromatic ring – radiochemical purity 97%). A third group (one animal per sex) was treated orally with a single dose of vehicle (Mazola corn oil) for 16 days and served as the control. Urine was collected 0-8, 8-24 and 24-48 hr after the first radiolabel dose and faeces were collected daily. Cage rinse was collected daily for 2 days post first radioactive dose. Twenty-four hours after the 2nd radioactive dose, animals were sacrificed by CO_2. Blood, liver, kidneys, lachrymal glands, mesenteric lymph nodes and any other tissues showing abnormal coloration due to the chemical treatment were collected (Wu, 2002).

Distribution: In the Wu (2002) study (GLP; purity unlabelled AHTN > 98%, radiochemical purity 98.9%), liver, kidney, mesenteric lymph nodes and lachrymal glands showed a dark green discolouration to almost black colour in the high dose group (100 mg/kg bw/day). In addition, these animals showed a discolouration of the urine. Only slight discolouration of the internal organs was seen in the low dose (15 mg/kg bw/day) animals. The levels measured in different tissues were given as percentage of dose in **Table 4.10.**

Table 4.10 Distribution into livers, kidneys, carcasses and spleens (females only) of AHTN after oral dosing in the rat (as % dose)

Group	Liver	Kidney	Carcass	Spleen
Male low dose	6.35 ± 0.83	0.09 ± 0.01	20.21 ± 7.61	-
Female low dose	4.52 ± 0.71	0.11 ± 0.03	23.90 ± 10.96	0.02 ± 0.01
Male high dose	4.46 ± 0.61	0.10 ± 0.02	15.07 ± 6.64	-
Female high dose	3.71 ± 0.48	0.11 ± 0.03	14.97 ± 7.49	0.02 ± 0.0

Animals in the high dose group either lost weight or had a slower growth rate. Some animals had alopecia starting around 4 – 5 days of treatment.

Excretion: After oral administration of 14 daily doses of unlabelled AHTN followed by 2 days of oral dosing of radiolabelled material to rats (see Wu, 2002 above), All samples were processed for radioanalysis but only those listed below were reported. Pooled urine, faeces and liver/kidney samples from both dose groups were analysed per sex and collection time for metabolite radioprofiles by HPLC radiochromatography. Radioactivity levels in samples were

determined by liquid scintillation counting. The results are shown in **Table 4.11.** Within 48 hours 66-67% (low dose) vs. 74-76% (high dose) radiolabel was excreted. In the low dose, the majority was in the faeces while in the high dose approximately equal amounts were found in faeces and urine plus cage rinse.

Table 4.11 Total radioactivity recovered from urine, cage rinse, faeces and tissues after oral dosing with AHTN in rat expressed as percentage of dose.

Group ID	Sex	% Dose in Urine	% Dose in Cage Rinse	% Dose in Faeces	% Dose in Tissues	% Dose Recovered
1 (15 mg/kg bw/day)	M	11.04	2.92	52.18	26.66	92.80
	F	14.16	7.97	44.76	28.55	95.44
2 (100 mg/kg bw/day)	M	35.06	6.27	35.00	19.63	95.95
	F	28.20	3.98	41.74	18.82	92.74

Metabolism: In the two-week oral study (Wu, 2002) discussed above, the metabolic profile of [^{14}C]AHTN in urine, faeces and liver samples was evaluated by HPLC radiochromatography which revealed the formation of numerous and complex metabolites however, none of these were characterised. No unchanged AHTN was detected in the urine and liver however; extensive levels of AHTN were seen in the faeces (especially in females at the high dose), possibly as a result of lack of absorption. Similar complex metabolites were seen in the liver but these were also not characterised. In the liver 63-73% of metabolites were not readily extractable but irreversibly bound. Attempts to relate metabolites to discolouration of organs were unsuccessful.

Intravenous

Distribution: In a GLP study, a single intravenous dose of 2 mg/kg bw ^{14}C-AHTN (uniformly labelled in the aromatic ring – radiochemical purity 98.4%) in 0.4 mg/ml ethanol/ Emulphor EL 620/ Isotonic saline (1:1:7) solution was administrated into the tail vein to groups of four female Sprague-Dawley CD rats (bodyweight range 216-233 g). The animals were sacrificed after 5, 15, 30 minutes and 1, 2, 4, 6, 12, 24 and 48 hr and 7, 14 and 28 days. Tissues (fat, kidney, liver) were weighed and blood was collected by cardiac puncture. Urine, faeces and air were collected every 24 hr from the four animals sacrificed at day 7 (air only up to 48 hrs). The mean recovery of total radioactivity in these four animals represented 93.9% of the dose administered: 88.9% in excreta + cage washings, 3.6% in carcass and 1.4% in liver.

Maximum concentrations of total radioactivity were observed in all tissues at 5 min (earliest time of measurement) except for the fat where the maximum was reached at 2 hr **(Table 4.12**). Between 48 hr and 16 days, total radioactivity in plasma and fat decreased with half-lives of 1.9 days and 2.5 days, respectively; after 16 days the concentrations declined more slowly. In fat, up to 85% was associated with parent compound and in plasma only 6% or less. Levels of radioactivity declined between 7 and 28 days with half-lives of 15.2 days in whole blood, at 5.4 days in the liver and 11.2 days in the kidneys (Hawkins, 1997).

Table 4.12 Concentrations of radioactivity in tissues after an intravenous dose of ^{14}C-AHTN to rats at a dose level of 2 mg/kg bw (in µg equivalents/g tissue).

Time	Tissues				
	Plasma	Whole Blood	Liver	Kidney	Fat
5 min	2.55	1.47	9.64	4.64	0.912
15 min	1.88	1.11	6.13	3.29	1.95
30 min	1.55	0.934	4.78	2.62	3.22
1 hr	1.66	1.02	4.94	1.81	3.20
2 hr	1.63	1.05	4.68	1.32	5.13
4 hr	1.83	1.18	5.10	1.02	4.36
6 hr	1.81	1.15	4.74	1.08	3.52
12 hr	1.47	1.05	4.54	0.803	3.52
24 hr	0.823	0.681	2.22	0.532	2.89
2 days	0.532	0.531	1.94	0.385	1.75
7 days	0.0929	0.225	0.655	0.176	0.356
16 days	0.00327	0.133	0.132	0.0807	0.0382
28 days	0.00110	0.0857	0.0423	0.0470	0.0132

In a GLP study, a nominal dose of 0.1 mg/kg bw ^{14}C-AHTN (actual dose 0.091 mg/kg bw; uniformly labelled in the aromatic ring – radiochemical purity >97%) in ethanol/ Emulphor EL 620/isotonic saline (1:1:7) solution was administered by intravenous injection into the ear vein of one male domestic pig (*Sus scrofa* of Large White Hybrid strain – age 8-12 weeks, bodyweight 33 kg). Urine was collected at 0-6 hr and 6-24 hr and every 24 hr up to 14 days and faeces were collected at 24-hr intervals up to 14 days. Blood was collected at 10, 20, 40 minutes and 1, 2, 4, 8, 12, 24 hr and 2, 3, 5, 7, 14, 21, and 28 days. Biopsies of skin and underlying fat tissue were taken at day 9, 16 and 28. The pig was sacrificed at 28 days. The recovery of total radioactivity (assessed in excreta only) was 98.4% of the administered dose.

The maximum concentrations of total radioactivity in whole blood and plasma were observed at 10 minutes (earliest collection) (see **Table 4.13**). Total radioactivity decreased rapidly in blood and plasma with apparent half-lives of about 0.9 hr during the first two hr. Thereafter the concentrations declined at a slower rate, with half–lives of 190 hr in plasma and 270 hr in whole–blood during 48–672 hr. There was no obvious accumulation of radioactivity in blood cells. In underlying fat of the skin, the maximal concentration (earliest collection) was at 9 days (7.57 ng equiv./g). After that, the fat concentration decreased and it was <5.6 ng equiv./g 16 days after injection and <0.8 ng equiv./g after 28 days. In skin, 1.08 ng equiv./g was found at 9 days and <0.8 ng equiv./g at 16 and 28 days (Girkin, 1997).

Table 4.13 Concentrations of radioactivity in blood and plasma after an intravenous dose of ^{14}C- AHTN to a pig at a dose level of 0.1 mg/kg bw (in ng equivalents/g).

Time (hr)	Whole blood	Plasma
10 min	70.7	108
20 min	64.1	100
40 min	48.9	74.0
1	33.9	52.8
2	17.5	25.9
4	9.8	13.7
8	8.5	11.6
12	7.4	9.6
24	6.1	7.3
48	5.0	6.1
72	4.5	5.3
120	4.1	4.4
168	3.3	3.4
336	1.1	1.6
504	1.4	1.2
672	1.0	0.6

Metabolism: The urine data collected at the two (rat and pig) intravenous studies (GLP) described above were analysed for metabolites. Extraction of pig urine (collected at 6-48 hrs) and rat urine (collected at 0-24 hrs) with solvent E (chloroform/methanol/water/formic acid, 75/25/3/3 by volume) revealed at least 13 metabolites in pigs and 16 in rats (**Table 4.14**). Extraction with solvent H (chloroform/methanol/ammonia, 80/20/1 by volume) revealed at least 9 metabolites in pigs and 8 in rats (

Table 4.15). None of the metabolites were characterized other than by retention times (R_f). Treatment with glucuronidase or aryl sulphatase had little effect on the proportions of the main metabolites in rat and pig urine, but solvent H had somewhat more conjugates than solvent E. The parent compound was extracted from urinary radioactivity with solvent A (100% chloroform), but neither pig nor rat urine contained unchanged AHTN (Girkin, 1998). In addition, it should be noted that the dose excreted in rat urine (21.5%), was considerably less than in the pig (86.2%), so the percentage of most rat urinary metabolites detected are much lower.

Table 4.14 Proportion of urinary metabolites as a percentage of dose after intravenous injection in rats or a pig – solvent E

R_f value	Pig urine 6-48 hr		Rat urine 0-24 hr	
	Untreated	Enzyme treated	Untreated	Enzyme treated
0.06	0.81	0.29	0.31	0.19

R_f value	Pig urine 6-48 hr		Rat urine 0-24 hr	
	Untreated	Enzyme treated	Untreated	Enzyme treated
0.20	*	*	0.22	0.17
0.26	0.12	*	0.86	0.75
0.31	0.10	*	0.64	0.43
0.36	0.16	0.06	4.0	3.86
0.43	*	*	0.13	0.09
0.47	*	*	0.29	0.60
0.52	0.19	*	0.50	
0.62	0.87	0.89	0.29	0.43
0.65	5.0	5.79	1.25	1.34
0.69	6.22	5.45	2.47	2.42
0.74	7.46	8.7	1.32	1.28
0.79	13.84	13.67	0.67	0.89
0.84	33.85	33.98	0.40	0.78
0.91	7.13	5.31	0.21	0.61
0.97	1.41	1.30	0.18	0.23
others	0.38	2.10	0.38	0.02

*Not detected

Table 4.15 Proportion of urinary metabolites as a percentage of dose after intravenous injection in rats or a pig – solvent H

R_f value	Pig urine 6-48 hr		Rat urine 0-24 hr	
	Untreated	Enzyme treated	Untreated	Enzyme treated
0.03	3.44	3.16	1.85	1.14
0.12	4.34	2.75	3.44	3.44
0.21	0.12	1.79	*	*
0.25	1.97	1.14	3.02	2.81
0.34	5.49	5.19	3.07	3.00
0.50	*	0.27	-	-
0.60	-	-	1.03	0.93
0.62	6.05	4.06	0.46	0.49
0.69	8.24	9.04	0.17	1.11
0.75	16.63	15.35	0.34	
0.82	20.36	19.02	*	
0.88	6.47	7.84	*	0.20
0.92	*	1.23	*	*
0.99	*	1.89	*	*
Others	4.46	4.81	0.73	0.99

*Not detected

Excretion: In the rat intravenous study (GLP) described above (Hawkins, 1997) the main route of excretion after 7 days was by faeces (67% of the administered dose) and excretion in the urine was 21.5% of the administered dose. Most excretion took place in the first 48-72 hrs. An absence of excretion of radioactivity to the air was noted.

In the pig intravenous study (GLP; radiochemical purity >97%) described above (Girkin, 1997) the main route of excretion was urinary (86.2% of total dose after 14 days) and total radioactivity recovered in faeces after 14 days represented 12.2 % of the administered dose. The process of elimination was rapid and mainly occurred during the first 48hr (78.9% in urine and 7.4% in faeces).

Animal milk studies

In a GLP compliant study, designed to measure plasma and milk levels that would be reached as a result of oral dosing, ^{14}C-AHTN (uniformly labelled in the aromatic ring – radiolabel purity >97%) was administered by gavage to pregnant Charles River CD rats (n=18/group – bodyweights *ca* 250-400 g – age 10-15 wks) at 2.0 or 20 mg/kg bw as a solution in corn oil, daily from day 14 of gestation up to 7 days post-parturition. The dosing regimen was designed to achieve steady state prior to parturition but not to have exposure during organogenesis, which takes place mainly before day 14. The sponsor was aware that some organogenesis occurs after day 14. Milk samples of *ca*. 0.5 ml (after administration of oxytocin) and blood samples of about 4 ml were obtained from 3 dams per dose level per time point at 4, 8 and 24 hr after dosing with AHTN, on days 3 and 7 post-parturition. Milk and blood samples were analysed for radiolabel. The highest mean levels of radiolabel were found in the 4 hr plasma samples, declining to about 35% of that level at 24 hr after dosing (see **Table 4.16**). Lower levels were consistently seen after 7 days as opposed to 3 days indicating no significant accumulation. Plasma levels were roughly proportional to dose with levels at 20 mg/kg bw/day approximately 10 fold higher than those at 2 mg/kg bw/day.

Table 4.16 Analysis of total radioactivity in plasma after daily oral administration of 2 or 20 mg/kg bw/day ^{14}C-AHTN in µg equivalents AHTN/ml plasma

Time after parturition	Time after oral administration (hours)	Mean level after oral dose of 2 mg/kg bw/day	Mean level after oral dose of 20 mg/kg bw/day
Day 3	4	3.13 ± 0.40	25.1 ± 3.5
	8	1.72 ± 0.29	24.3 ± 6.4
	24	1.10 ± 0.18	9.98 ± 1.6
Day 7	4	2.41 ± 0.46	21.0 ± 2.2
	8	2.20 ± 1.02	17.3 ± 1.9
	24	0.86 ± 0.10	6.53 ± 0.9

Levels of total residue found in the milk (**Table 4.17**) were also highest at 4 hr after dosing declining 5 to 10 fold by 24 hr. Similar levels were generally seen after 7 days dosing as compared to those after 3 days dosing (except for 2 mg/kg at 4 and 8 hrs) also consistent with no significant accumulation. Additionally, the major residues in the milk were not associated with the peak assigned as AHTN based on retention time. Although not fully characterised, the AHTN peak in the milk extracts is considered authentic as the metabolites are expected to be more polar and would therefore precede AHTN on the polar column. About 66-85% and

57-81% of the radioactivity was associated with other materials (metabolites) at the low and high dose, respectively. (Hawkins et al., 1996).

Table 4.17 Analysis of total radioactivity and unchanged AHTN in milk after daily oral administration of 2 or 20 mg/kg bw/day ^{14}C-AHTN in µg equivalents AHTN/ml milk (ppm)

Milk collection after parturition	Time after oral administration (hours)	After oral dose of 2 mg/kg bw/day			After oral dose of 20 mg/kg bw/day		
		Total radiolabel Mean	AHTN Mean	Ratio AHTN/total residue	Total radiolabel Mean	AHTN Mean	Ratio AHTN/total residue
Day 3	4	1.45 ± 0.43	0.35 ± 0.25	0.22 ± 0.10	25.0 ± 11.1	9.43 ± 2.57	0.41 ± 0.10
	8	0.66 ± 0.12	0.10 ± 0.02	0.15 ± 0.05	10.5 ± 3.35	2.14 ± 1.39	0.19 ± 0.10
	24	0.31 ± 0.07	Na	-	2.89 ± 0.71	na	-
Day 7	4	1.89 ± 0.57	0.63 ± 0.20	0.34 ± 0.02	18.0 ± 1.1	7.73 ± 0.71	0.43 ± 0.02
	8	0.87 ± 0.21	0.14 ± 0.05	0.15 ± 0.03	8.76 ± 2.40	2.95 ± 1.65	0.32 ± 0.09
	24	0.21 ± 0.06	Na	-	1.55 ± 0.30	na	-

na : not analysed due to low radioactivity levels

In vitro studies

Absorption: The *in vitro* absorption of ^{14}C-AHTN (place of labelling not given) was measured (non-GLP, purity unknown) using full thickness dorsal skin (male F344 rat) in flow-through diffusion cells. A receptor fluid containing 50% v/v aqueous ethanol to enhance absorption flowed across the underside at a rate of 1.5 ml/hr. Dose solutions of 0.1% and 0.5% in an ethanol/DEP (75:25) vehicle (15 and 78 µg/cm^2, respectively) were applied to occluded (Teflon caps) and non-occluded systems. Receptor fluid was collected every 2 h for up to 72 h. At the end of the experiment, the skin surface was washed and swabbed, after which the skin was digested in methanolic sodium hydroxide. Radioactivity in receptor fluid, skin washes and skin was determined by liquid scintillation spectrometry. AHTN was poorly absorbed through non-occluded skin after 24 hr (0.28% of applied dose). Occlusion enhanced AHTN absorption at 24 hours to 3.0%. Significant amounts of radioactivity were recovered from within the skin (at 24 h, 55% in both unoccluded and occluded skin). Over 48 h, AHTN continued to be absorbed into the receptor fluid and the total absorption at 48 h was enhanced by occlusion. No data were presented on the 48-72 hr time-period. Total recovery of radioactivity was not presented but was stated to be generally >80% (Ashcroft and Hotchkiss, 1996). Based on the use of a non-physiologically receptor fluid, no report of testing the integrity of the skin (the data were taken from a poster presentation) and poor recovery of radiolabel, these results cannot be used as determinates of dermal absorption.

Radiolabelled (position and radiochemical purity not reported) AHTN, dissolved in methylcarbitol at concentrations of 1, 3 and 10 %, was applied to an intact excised minipig skin (strain and source not reported) area of 5 cm^2 at a dose of 12 µl/ cm^2 (= 120, 360 or 1200 µg/ cm^2). The receptor fluid was physiological saline in which the solubility of AHTN is not reported. Unabsorbed material (92.3%, 89.1% and 86.2% at 10, 3 and 1%, respectively) from the skin surface was removed after 16 hr. The stratum corneum was then removed by successive tape stripping, and the stratum corneum and the remaining stripped skin were then analysed for radiolabel. Applying doses of 1, 3 and 10% the amounts of labelled material in the horny layer were 5.5, 5.3, 4.4 % and 8.2, 5.6, 3.3 % in stripped skin. Penetration into the receptor fluid was undetectable after 16 hr (Klecak, 1983). This study was not conducted under GLP. Whilst demonstrating that AHTN is only absorbed to a small extent, the study does not provide quantitative data because of the limited solubility of AHTN in the receptor fluid. The solubility of AHTN in physiological saline would be too low to allow penetration in the absence of skin metabolism.

4.1.2.1.2 Human data

In vivo studies

Human adipose tissue studies

In a study (non-GLP, purity unknown) to measure residues of AHTN, two human fat samples (origin not specified) were extracted with hexane and the extracts analysed by selective ion trap GC/MS/MS technique for residues of AHTN. Residues were found in both samples at levels of 56 and 72 µg/kg fat (Eschke et al., 1995b).

In a similar study (non-GLP, purity unknown), human adipose samples were obtained from 8 females and 6 males in Germany between 1993 and 1995. These samples were extracted with a mixture of water/acetone/petroleum ether and analysed for AHTN residues by GC/MS.

AHTN was found in all 14 samples at concentrations ranging from 8 to 33 µg/kg fat (mean 19 µg/kg). Although the small number of samples and wide range of data preclude meaningful statistical evaluation, a visual inspection of the data reveals no clear correlation with sex or age (Rimkus and Wolf, 1996).

In a non-GLP study (purity unknown), 15 human fat samples obtained over the years 1983/4 and 1994 in Switzerland from corpses of 10 females and 5 males (age group 3-100 years) were analysed for residues of AHTN by homogenisation followed by extraction with cyclohexane/ethyl acetate (1:1) and analysed by GC/MS. AHTN was detected in all samples with a range of 1.0 – 23 µg/kg fat (mean 9 µg/kg) (Müller et al., 1996).

Human blood studies

Blood samples from 413 German subjects (85 men and 328 women) were extracted (details not reported) and analysed by GC-MS for levels of AHTN as well as for musk ketone and musk xylene for comparison. The average level of AHTN (Tonalid) was 274 ng/l with a range of <100 (37 samples) to >1600 ng/l (1 sample). The 95-percentile level was 545 ng/l (Bauer and Frössl, 1999).

Blood samples from 100 Austrian volunteers (55% woman, median age 23, average age 25.5, range from 19 to 43 years) were analysed by GC-MS for the determination of levels of musk fragrance substances. A questionnaire on the use of cosmetics, household products and food was completed by these volunteers. Blood samples were analysed using GC-MS analysis after extensive sample extraction and clean-up. AHTN was not detected in 83% of the samples, with a detection limit of ±30 ng/l. The average level of AHTN found in 17 samples was 138.3 ng/l with a maximum level of 800 ng/l. A weak correlation was found between the levels of musk fragrances in blood and the use of cosmetics (Sattelberger et al., 2003; Hutter et al., 2005).

Blood samples from 91 Dutch volunteers (48 males and 43 females, age ranging from 19 - 78 years) were analyzed by GC-MS for the determination of levels of musk fragrance substances after solvent extraction. AHTN was found in 88 of the 91 samples. The median level of AHTN was 0.4 ng/g serum with a range of <0.1 to 11 ng/g serum. The 95-percentile level was 1.3 ng/g serum (Peters, 2004b).

Human milk studies

In a study (non-GLP, purity unknown) to determine residues of AHTN, two human breast milk samples (origin not specified) were extracted with hexane and the extracts analysed by selective ion trap GC/MS/MS technique for residues of AHTN. Residues were found in both samples at levels of 290 and 250 µg/kg fat or 3.1 and 1.0 µg/kg whole milk based on measured fat contents of 1.06 and 0.41%, respectively (Eschke et al., 1995b).

In a similar study (non-GLP, purity unknown), five breast milk samples were obtained from 4 nursing mothers in Germany and were extracted according to an AOAC method (Helrich, 1990) and analysed for AHTN residues by GC/MS. All samples contained some AHTN at concentrations ranging from 11 – 58 µg/kg milk fat (Rimkus and Wolf, 1996).

In another larger study (non-GLP, purity unknown) of AHTN residues, breast milk samples (mean 34 g) were obtained from 107 nursing mothers in Germany (mean age 31.5 years, mean body mass index 24.5 kg/m^2 at time of birth and 23.2 kg/m^2 at time of milk sampling). The protocol was designed to minimize contamination (all equipment was carefully cleaned and the breast area was cleaned 3 times with cotton swabs immersed in propylene glycol). All

mothers were asked to report on their use of various household products including soaps, detergents and cosmetics as well as their consumption of fish products. As established in a separate study, the back-ground level of AHTN in cyclohexane extracts of cotton swabs was 0.7 ng/ml before these swabs were used for skin cleaning purposes. After the first, second, third and fourth cleaning steps of breast surface with cotton swabs soaked in propylene glycol, AHTN levels were 76, 112, 73 and 54 ng/ml respectively. Because of the decline after 3 cleanings and in consideration with the mothers, the study authors decided to take milk samples after 3 cleaning steps. AHTN was detected in 36.4% of all samples. Levels were reported to be zero up to maximal 267 µg/kg milk fat and the mean concentration was 16 µg/kg of milk fat. Based on the reported mean milk fat level of 3.67%, this corresponds to a maximum level in the whole milk of 9.8 µg/kg whole milk with a mean of 0.59 µg/kg whole milk. (Sönnichsen et al., 1999).

In 2001, Zehringer and Herrmann published data on 53 milk samples obtained in 1998/1999 from 29 mothers living around the city of Basle. AHTN was found in concentrations ranging from not-detectable (16 samples) to 136 µg AHTN/kg fat. The average fat content was 3.3%. This resulted in a mean level of 44 µg AHTN/kg fat. No special arrangements were made to prevent contamination of milk samples by AHTN present on the skin.

In a case control study for risk factors of early miscarriages in the Uppsala County in Sweden, during the period 1996-2003, women donated milk samples to be analysed for the presence of various polycyclic- and nitromusks. Women were also asked to fill out extensive questionnaires about lifestyle, medical history and dietary habits and a sub-population was also asked to fill out an additional questionnaire about use of perfumes and perfumed deodorants, skin lotions, laundry- and washing detergents. Milk was collected at the beginning and at the end of the breast-feeding sessions The goal was to collect 500 ml from each mother during the 3rd week after delivery. It was not mentioned whether measurements were taken to prevent contamination of the milk samples from musks present on the mother's skin. In total 101 milk samples were analysed. AHTN was found in concentrations ranging from < 3 to 53 µg/kg milk (mean ± SD: 13 ± 12; median 10 µg/kg milk fat) (Lignell et al., 2004).In a pilot study in the Czech Republic, 59 milk samples were collected from nursing mothers (living but not necessarily born in Prague). The manual sampling (milk expressed from the breast into a clean container) was conducted in accordance with WHO guidelines. Using a detailed questionnaire, relevant information on parameters, such as age, dietary habits (specifically consumption of freshwater/marine fish), use of perfumed cosmetics, frequency of contacts with detergents, etceera were collated. The lipid content in the milk ranged from 1.5 to 4.2 wt %. The detection limit for the musks was 10 µg/kg fat. AHTN was found in 90% of the samples. Concentrations ranged from not detectable to 565 µg/kg fat, with a median value of 67, a mean value of 112, and a 90th percentile of 304 µg AHTN/kg fat (Hajslova and Setkova, 2004).

Milk samples were collected in 1999 at Hvidovre Hospital in Denmark from 10 primiparous mothers (25-29 year of age) 14-26 weeks after birth. AHTN was found at levels from 5.58 to 37.9 µ/kg fat with a median level of 17.5 and an average of 19.5 µg/kg fat (Duedahl-Olesen et al., 2005).

Inhalation

No data available.

Dermal

In a GLP compliant study, the absorption and excretion of total radioactivity was determined in 3 human male volunteers (age 33-47 years, weight 73-81 kg). ^{14}C-AHTN (uniformly labelled in the aromatic ring – radiochemical purity >98%) in 70 % ethanol was applied to the skin of human volunteers under conditions intended to simulate a typically high exposure from the use of alcohol-based products such as perfumes or eau de toilette, i.e. 0.24% in 70% ethanol. A nominal volume of 0.5 ml of the solution containing 2.4 mg ^{14}C-AHTN/ml (actual mean 1.09 mg ^{14}C-AHTN) was applied to 100 cm^2 (0.011 mg/cm^2) area of skin on the upper back. After 30 min to allow the ethanol to evaporate, the area was covered with light gauze dressing. Six hr after application, the dressing was removed and the treated area washed with cotton wool swabs moistened with 70% aqueous ethanol. An area of 6.25 cm^2 was stripped by 5 successive applications of adhesive tape to determine the amount of total radioactivity in the upper level of the horny layer. The treated site was again covered with fresh dressings up to 120 hr after compound application at which time the dressings were taken off and another skin area of 6.25 cm^2 was stripped to determine the remaining total radioactivity in the stratum corneum. Samples of blood (at 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 36, 48, 72, 96 and 120 hr) and excreta (urine, at 0-2, 2-4, 4-6, 6-12, 12-24, 24-48, 48-72, 72-96 and 96-120 hr intervals, and faeces at 24 hr intervals) were collected during the five-day period.

The majority of the applied material (~ 67%) was still on the surface of the skin at the time of washing - 6 hr. The first tape stripping at time of removal of the dose indicated that approximately 4.2% of the applied radioactivity (AR) remained in the upper layers of the stratum corneum. Recovery in the faeces was below the limits of accurate detection (<0.1 % applied radioactivity, AR) in two subjects and 1.1% in the third. A mean of 0.5% (range 0.2-0.8%) was excreted in the urine. Concentrations in whole blood and plasma were also below the limits of accurate measurement (*ca* 6 ng/ml and 1.04 ng/ml, respectively) at all sampling times. A further 14.5% AR was detected in dressings at the 120 hr time showing that considerable radioactivity remained in the skin after washing, which would subsequently evaporate from skin. From the recoveries in faeces and urine a mean total absorption of 0.9% (range 0.2-1.9%) could be calculated. Tape stripping at 120 hr indicated that only trace amounts (0.064%) remained in the upper layers of the stratum corneum at that time. The mean total recovery from excreta, dressings, swabs and skin strips was 86.4% AR. A separate evaporation experiment indicated that approximately 24.2% of the AHTN would be expected to evaporate under conditions of the experiment (Girkin, 1996; Ford et al., 1999).

Oral

No data available.

<u>In vitro studies</u>

The dermal absorption (non-GLP, but with QA-statement) of AHTN was determined over a 24-hr period according to the methodology of the SCCNFP. Radiolabeled ^{14}C-AHTN (uniformly labelled in the aromatic ring – radiochemical purity 99.6%) was applied in 1% (w/v) solution in ethanol (96% v/v) to human epidermal membranes (prepared from full-thickness female breast or abdominal skin and assayed for integrity with tritiated water)

supported on a piece of filter paper (for strength) in glass diffusion cells (n=12). There were two control cells. The area of the membrane available for absorption was approximately 1 cm^2 and the applied dose was a mean of (n=12) 20±0.4 µL/cm^2 (hence, 200 µg AHTN/cm^2). Receptor fluid (6% Volpo N20 (to enhance solubility) in pH 7.4 phosphate buffered saline) was sampled at 1, 2, 6, 12 and 24 hrs. After 24 hrs the epidermal membranes were wiped and stripped. The amount of material absorbed into the receptor phase after 24 hr was 0.38±0.06% of the applied dose. The majority of applied AHTN (85±2% of the applied dose) was found in the 24-hr surface wipe and donor chamber wash plus wipe. The stratum corneum tape strips contained 3.8±0.3% of the applied dose and the remaining stratum corneum plus epidermis 3.5±0.3% of the applied dose. As per SCCNFP guidelines, levels of AHTN in the remaining stratum corneum plus epidermis, filter paper (on which the epidermis samples rested; 0.19±0.03% of the applied dose) and permeated AHTN were combined to produce a total absorbed dose value of 4.1±0.4% of the applied dose. The evaporative loss (assessed in a separate experiment under the same conditions) over 24 hrs was 2.9% of the applied dose. Overall-recovery of radioactivity at 24 hrs was 92.5±0.7% (Green and Brain, 2001).

4.1.2.1.3 Summary of toxicokinetics, metabolism and distribution

There are no data available on the toxicokinetics of AHTN after inhalation exposure. For inhalation exposure, an assumption of 100% absorption as a worst-case will be used in the risk characterization. The latter assumption would probably over estimate exposure from dust because absorption in the lung is likely to occur only for dissolved AHTN and AHTN is poorly soluble in water (1.25 mg/L).

The quality of the available oral absorption data is not sufficient to establish an absorption percentage. For oral exposure, a default of 50% absorption will be used in the risk characterization.As a support, based on urine, cage washing and tissue levels in the study by Wu (2002), absorption of about average 50% can be concluded. In the *in vivo* human study a 6 hr unoccluded exposure with 0.24% AHTN in 70% ethanol, intended to simulate a typically high exposure from the use of alcohol-based products such as perfumes or eaux de toilette, resulted in absorption *into* the skin (at least 15%). However, most of the material in this skin reservoir was not absorbed, but was recovered from dressings over the site of exposure over a 120 hr period, presumably from reverse diffusion and/or desquamation. Based on amounts excreted, primarily in the urine, maximally 2% in total was actually absorbed under the conditions of this experiment. An *in vivo* study in rats supports the assumption that a good indication of the amount absorbed is the amount excreted. Although approximately 14% of the applied radioactivity was not recovered, from a separate evaporation, study it appears that under non-occlusive conditions about 24% of an applied dose may evaporate from human skin.

A similar picture (although, as expected, with considerably higher absorption) was seen *in vivo* with rats where the material was applied for 6 hr under occlusion in 70% alcohol. Here again, a reservoir in the skin of about 13% of the applied dose was formed after the 6-hr application with about 2 of this 13% reservoir being lost presumably from reverse diffusion and/or desquamation to the dressing after dose removal. Based on the amount remaining in the tissues (excluding that at the site of dosing, i.e. 1.5%) at sacrifice (1.7%) and the amount excreted (17.1%), almost all (14.5%) of which was in the faeces, a total absorption under the conditions of this experiment of 18.8% can be concluded. The principal differences with the human study were the much larger absorption because of the application under occlusion and the well-known fact that rat skin is more permeable than human. For risk characterisation a

value of 20% will be used for dermal absorption via rat skin. This value also accounts for a continued absorption of (part of) the skin reservoir still present at 120 hr (1.5%).

Recently, an *in vitro* 24-hr absorption study using 1% AHTN in 96% alcohol with human epidermal membranes was performed according to the recommendations of the SCCNFP. In this study, 0.38% of the applied dose was found in the receptor fluid after 24 hr under non-occlusive conditions, however, 3.5% of the applied dose remained in the epidermis. Adding these amounts to the small amount remaining on the filter paper used to support the membranes leads to the calculation of total absorption of 4.1% of the applied dose. From a separate experiment, it appears that under the same conditions about 2.9% of an applied dose may evaporate from human skin *in vitro*. Correcting for evaporation loss would bring the skin permeation close to the results of the human *in vivo* skin absorption study. This study is the only 24-hr application and shows linear skin permeation, thus all demands on such a skin permeation study are fulfilled. As the other skin absorption studies have limitations such as 6 hr exposure time or a small number of samples, for risk characterization 4.1% as value for skin absorption will be used as a conservative estimate.

Intravenous administration of AHTN to rats and the pig results in rapid distribution. Excretion in the rat is primarily via the faeces as was seen in the dermal study (~ 76% of total excretion compared to ~84% after dermal exposure) but in the pig the principle route of excretion is via urine similar to what was seen in the human study. In neither of these studies was any evidence of accumulation seen. However, clearance from the fat was slower than from other organs. It is noteworthy that in the intravenous studies, no unmetabolised AHTN is present in the urinary radioactivity. This means that all AHTN present in urine is metabolised (for rat 21.5%, and for pig, 86.2%). The faeces (the major excretion route of the rat) were not analysed for metabolites or parent.

An oral study with pregnant and later lactating rats shows that orally dosed AHTN and its metabolites can end up in the mother's milk. The levels seen in the milk of the lactating dams can aid in the interpretation of the study (see 4.1.2.1.I above) where neonate rats were exposed to AHTN and its metabolites through nursing.

AHTN is also found in human milk at a maximum level of 565 µg/kg milk fat and a mean level of 112 µg/kg milk fat (equivalent to levels of 23.7 and 4.7 µg/kg whole milk based on the highest measured fat content of 4.2%) and in adipose tissue at levels ranging from 1 – 72 µg/kg fat.

In summary, for the purpose of risk characterization, 50% absorption for oral exposure and 100% for inhalation will be used. For dermal absorption of AHTN in rats and humans, values of 20% and 4.1% respectively, are taken forward to the risk characterisation.

4.1.2.2 Acute toxicity

4.1.2.2.1 Studies in animals

In vivo studies

Table 4.18 Acute toxicity studies for AHTN

Study	Solvent	LD50 (in mg/kg bw)	OECD	GLP	Reference
Rat gavage	isopropyl myristate	920	401*	Yes	Spanjers and Til, 1985a
Rat gavage	isopropyl myristate	1150	401*	Yes	Spanjers and Til, 1985b
Rat gavage	50/50 Ethanol/ Polyethyleneglycol	1377	401	No	Meisel, 1982
Rat gavage	Ethanol	825	No	No	Minner and Foster, 1977
Rat gavage	Unknown	570	No	No	Moreno, 1975
Rat dermal	Ethanol	7940	No	No	Minner and Foster, 1977
Rabbit dermal	Unknown	>5000	No	No	Moreno, 1975
Rat IP	Ethanol	79.4	Na	No	Minner and Foster, 1977
Rat IP	DMSO	> 100 no death	Na	No	Steinberg et al., 1999
Mouse IP	Corn oil	~2000	Na	Yes	Gudi and Ritter, 1997

*Except for variation in volume of dose, not part of that guideline at that time; Na – not applicable; No OECD guideline.

Inhalation

No data available.

Dermal

AHTN (Fixolide; purity >98%) was administered by inunction to the shaved skin (area not reported) of groups of five female Charles River Sprague Dawley rats (initial bodyweight 108–197 g) at doses of 464, 1000, 2150 (all 10% solutions in ethanol), 4640 (20% solution in ethanol) or 10,000 (40% solution in ethanol) mg/kg bw that were then observed for 7 days. Toxic signs, mainly seen at the highest dose, consisted of depression, hunching, hind limb weakness and prostration. There were no deaths at any dose on the first day but 0, 1, 0, 1 and 5 deaths were seen at the low to high doses, respectively, during days 2-7, resulting in a calculated dermal LD_{50} of 7940 mg/kg bw. There were no remarkable findings upon gross necropsy (Minner and Foster, 1977). This study was conducted prior to GLP and OECD guidelines but was according to acceptable procedures at the time.

In an incompletely reported study, it was reported that dermal administration of AHTN (solvent and purity not reported) to 10 rabbits at a dose of 5000 mg/kg bw resulted in no deaths after 14 days of observation (Moreno, 1975). Two animals showed anorexia. Signs of skin irritation (slight redness in 3/10 and moderate redness in 7/10 animals, slight oedema in 6/10 and moderate oedema in 3/10 animals) were observed in all animals. The LD_{50} was

>5000 mg/kg bw. This study was conducted prior to GLP and OECD guidelines and specific details regarding the study are not available. However, the study was reported by the Research Institute for Fragrance Materials (RIFM) and was conducted by a standard protocol that was state of the art at the time.

Oral

Groups of five male (weight 245-283 g) and five female (weight 162-187 g) young adult SPF-bred albino rats (Cpb:Wu;Wistar random) were administered by gavage 4.00, 4.80, 5.76, 6.91 or 8.29 ml/kg bw of a 17.5% (w/v) solution of AHTN (Tonalid®; purity =98%; equivalent to doses of 700, 840, 1008, 1209 and 1451 mg/kg bw AHTN) in isopropyl myristate and observed for 14 days. Within a few hr, signs of sluggishness and piloerection were seen. Later on haematuria, encrustrations around eyes and nostrils and signs of emaciation were observed. Death occurred between 6 hr and 8 days of dosing at which time survivors recovered gradually looking healthy at the end of the observation period. Macroscopic examination at autopsy of the rats that died in the first few days revealed blood stained urine in the bladder. No other treatment-related gross alterations were seen in these or other animals. An LD_{50} of 920 mg/kg bw was calculated (Spanjers and Til, 1985a). This study complied with OECD Guideline 401 except for the variation in volume of dosing; that recommendation was not part of that guideline.

Groups of five male (bodyweights 242-279 g) and five female (bodyweights 162-186 g) young adult SPF-bred albino rats (Cpb:Wu;Wistar random) were administered by gavage 6.0, 7.2, 8.64, 10.37 or 12.44 ml/kg bw (equivalent to 1050, 1260, 1512, 1815 or 2177 mg/kg bw) of AHTN (GLP; Tonalid®; purity >98%) as a 17.5% (w/v) solution in isopropyl myristate and observed for 14 days. Within a few hr, signs of sluggishness and piloerection were seen. Later on haematuria, encrustrations around eyes and nostrils and signs of emaciation were observed. Death occurred between 6 hr and 8 days of dosing at which time survivors recovered gradually looking healthy at the end of the observation period. Macroscopic examination at autopsy of the rats that died in the first few days revealed blood stained urine in the bladder. No other treatment-related gross alterations were seen in these or other animals. An LD_{50} of 1150 mg/kg bw was calculated (Spanjers and Til, 1985b). This study complied with OECD Guideline 401 except for the variation in volume of dosing.

Groups of five male (weight 119-133 g) and five female (weight 116-129 g) Sprague Dawley rats were administered by gavage doses of 10 ml AHTN (Non-GLP; Fixolide; >98%) in 50% ethanol/polyethylene glycol solution with concentrations varied so as to result in doses of 1260, 1588, 2000 or 2520 mg/kg bw and observed for 14 days. The majority of animals showed lethargy, piloerection, hunched position, oscillated movements, shaggy coat and emaciation. Other occasional signs included green urine, hypothermia, half-closed eyes, difficult breathing and increased breathing, prostration and lacrimation. Upon gross examination, alterations were seen in stomach and forestomach, liver and kidneys (discoloration), testes (atrophy) and bladder. Deaths occurred on days 5 to 9. LD_{50} values of 1331 (males), 1468 (females) and 1377 (combined males and females) mg/kg bw were calculated. This study complied with OECD Guideline 401. (Meisel, 1982).

AHTN (Non-GLP; Fixolide; purity >98%) as a 10% solution in ethanol was administered undiluted (hence, there was variation in volume of dosing) by oral intubation at doses of 0.10, 0.215, 0.464, 1.0 or 2.15 g/kg bw to groups of 5 female Charles River Sprague Dawley rats (initial bodyweight 100–166 grams) that were then observed for mortality and signs of effects for 7 days. Deaths occurred in the high dose group in the first day while in all others occurred during days 2-7. Toxic signs mainly consisted of depression and lethargy. Upon gross

necropsy no specific findings were observed, except for three animals in the 1.0 g/kg bw group exhibiting a noticeable yellow coloration of the small and large intestines. An LD_{50} of 0.825 g/kg bw was calculated (Minner and Foster, 1977). This study was conducted prior to GLP and OECD guidelines but was conducted according to acceptable procedures at the time.

In an incompletely reported non-GLP study, it was reported that oral administration of AHTN (solvent and purity not reported) to groups of ten rats at doses of 0.34, 0.67, 1.31, 2.56 or 5.0 g/kg bw resulted in 100% mortality at 1.31 g/kg bw and higher by day 3. There was one death at the lowest dose and seven at 0.67 g/kg bw. Ataxia was observed from 0.67 mg/kg bw. An LD_{50} of 0.57 g/kg after 14 days of observation was calculated (Moreno, 1975). This study was conducted prior to GLP and OECD guidelines and specific details regarding the study are not available. However, the study was reported by the Research Institute for Fragrance Materials (RIFM).

Intraperitoneal

Groups of five female rats (Charles River Sprague Dawley – weighing 104-150 g) were dosed with a 10% solution of AHTN (non-GLP; Fixolide; purity >98%) in ethanol by intraperitoneal injection at doses of 0.0464, 0.10 or 0.215 g/kg bw, and observed for 7 days. In the high dose group 4/5 exhibited depression within 2 hr, 1/5 had died at the end of the first day and all had died by day 3. At the middle dose, animals appeared normal at 2 hr after dosing but slightly depressed at 24 hr. Two were found dead on the 3rd day and 1 more died by day 6. At the low dose, one animal appeared depressed at 2 hr but returned to normal at 24hr; there were no mortalities at this dose. There were no remarkable findings upon gross necropsy. A calculated IP LD_{50} of 0.0794 g/kg bw was reported (Minner and Foster, 1977).

In preliminary studies (GLP; purity >98%) for a mouse micronucleus study (see section 4.1.2.7 below) groups of 5 male (weight 30-38 g) and 5 female (weight 24.5-29 g) ICR mice were dosed with 26, 100, 300 or 500 (study 1) and 1500, 2500 or 5000 (study 2) mg/kg bw AHTN (in corn oil - purity not reported) by intraperitoneal injection at a constant volume of 20 ml/kg bw and observed for 3 days. In study 1, the mice appeared lethargic at 300 and 500 mg/kg, but there were no deaths. Based on the lack of mortality in study 1, the second study at higher doses was undertaken. In study 2 all mice died within 3 days at the 2 higher doses and signs of lethargy and diarrhoea, but no mortality, were seen in the low dose animals. Based on these findings an IP LD_{50} of approximately 2000 mg/kg was reported (Gudi and Ritter, 1997).

In a study (non-GLP; purity >98%) designed to test the hepatotoxicity of AHTN in rats 6 male Sprague-Dawley rats were treated i.p. once with AHTN in DMSO at 100 mg/kg and sacrificed 7 days later. No death occurred during the 7-day observation period. There was no increase in absolute liver weights but a decreased bodyweight gain compared to untreated controls resulted in increased relative liver weights. Examination of the livers revealed single cell necrosis accompanied by lymphocyte infiltration and Kupffer cell activation. Electron microscopic examination of the livers revealed a strong disruption of the rough endoplasmic reticulum and decreased associated ribosomes (Steinberg et al., 1999).

<u>In vitro studies</u>

No data available.

4.1.2.2.2 Human data

No data available.

4.1.2.2.3 Summary of acute toxicity

The data provided are considered sufficient to meet base set requirements for acute toxicity. Based on the oral LD_{50} values of 570-1377 mg/kg bw, AHTN should be classified as harmful if swallowed (Xn, R 22). The dermal LD_{50}-values are >5000 mg/kg bw, so there is no need to classify AHTN for acute dermal toxicity.

Data for acute inhalation toxicity are not available.

4.1.2.3 Irritation

It is standard operating procedure for in vivo tests for irritation and photoirritation to test more than one substance on the same animal or human subject. That is the case in most of the studies reported in this section. Rarely are the identities of these additional substances revealed. They are not mentioned here unless they affect in some way the results with the reported substance.

4.1.2.3.1 Skin

Skin irritation

Studies in animals

AHTN has been tested in several skin tests for skin irritation in rabbits. A summary of the results is shown in **Table 4.19** for skin irritation.

Table 4.19 .A *In vivo* rabbit studies of the dermal irritation of AHTN

Species	Number Tested	Concentration of AHTN	Results – Primary irritation	Reference
Rabbit	3	0.5 g AHTN moistened with water	No erythema or oedema	Haynes, 1984
Rabbit	6	50% AHTN in DEP	Avg. erythema = 0.7, Avg. oedema = 0.3,	Haynes, 1985

In a GLP compliant study according to directive 79/831/EEC, 3 New Zealand White female rabbits were dermally exposed to 0.5 g AHTN (Tonalid®; purity>98%; moistened with 0.5 ml water) under a 6.25 cm^2 semi-occlusive patch for 4 hr on the dorsal skin (clipped free of fur). Four hours later, the treated skin was cleaned by gentle swabbing with cotton wool soaked in warm water. Skin observations were at 1, 24, 48, 72, and 168 hr after patch removal. Controls were not used. No erythema or oedema was seen in any rabbit at any time point (Haynes, 1984).

In another GLP compliant study according to directive 79/831/EEC, 6 New Zealand White female rabbits were treated dermally with 0.5 ml of a 50% solution of AHTN (Tonalid®; purity >98%) in diethyl phthalate (DEP) or with 100% DEP alone (only 4 rabbits). The

substances were applied under a 6.25 cm^2 semi-occlusive patch on the dorsal skin (clipped free of fur). Four hours later, the treated skin was cleaned by gentle swabbing with cotton wool soaked in warm water. Skin observations were at 1, 24, 48, 72, and 168 hr after patch removal. For AHTN, after one-hour post exposure, very slight erythema in 4/6 and well-defined erythema in 1/6 was observed. At 24 hr, well-defined erythema and slight oedema was seen in 1 rabbit, very slight oedema and erythema in another and very slight erythema in a third animal. Well-defined erythema and slight oedema remained in one animal at 168 hr at which time marked desquamation of the test site was seen. At this time point, desquamation was also observed in another animal in which only slight erythema was observed at the 1 hr and 24 hr observations. The average irritation score calculated from the numerical scores given at 24, 48 and 72 hour observations for erythema was 0.7 for AHTN treated animals, and 0.2 for DEP treated animals while average scores for oedema were 0.3 for animals with AHTN and 0.0 with DEP (Haynes, 1985).

In a subchronic dermal study (see 4.1.2.6 Repeated dose toxicity) 100 mg AHTN/kg bw/day was rubbed into the shaved skin of female Wistar rats (Til and Kuper, 1978). Due to strong irritation, the dose was reduced to 10 mg/kg bw/day. In another subchronic dermal study, a similar dose of 100 mg AHTN/kg bw/day was used for 13 weeks. No irritation was reported (Gressel et al., 1980).

Human data

Irritation

All observations of skin irritation stem from HRIPT studies, no specific test with humans for skin irritation have been performed.

During the induction phase of a Human Repeat Insult Patch Test (HRIPT; non-GLP, purity > 98%) for sensitisation (see below), an occlusive patch (Hilltop chamber 25 mm) with 0.3 ml AHTN (purity not reported) at a concentration of 10% in ethanol/diethyl phthalate (DEP) (75/25) was applied on the backs of 111 subjects for 24 hr three times per week for three weeks. Reactions were scored at 24 and 72 hr after patch removal. Macular erythema was observed at AHTN-treated as well as at vehicle-treated sites at about the same frequencies. Therefore, no irritation was observed after repeated occlusive applications as a result of treatment with AHTN (Berger, 1998).

During the induction phase of an HRIPT, 0.5 ml of 2% AHTN (non-GLP; Tonalid®; purity >98%) in dimethyl phthalate was applied on the skin of 52 subjects in patches occluded with impervious tape (area not reported) first on the inner surface of the right deltoid area, then on the inner surface of the left deltoid area, then on the upper portion of the right forearm and then on the upper portion of the left forearm. This cycle was repeated for 10 applications throughout the 23-day induction period. Patches were left on for 48 hr; except for the weekend when they were left in place for 72 hr. Reactions were scored immediately after patch removal. No irritation was observed after repeated occlusive applications (Edelson, 1964). This study is poorly reported.

Photoirritation

Studies in animals

Because AHTN absorbs in the UV region, several studies to detect a possible photoirritation hazard have been conducted (see **Table 4.20**). Several of these were designed for method development. Up to now, there are no validated *in vivo* tests for phototoxicity. However, draft

testing guidelines for photoirritation have been circulated by OECD both for *in vivo* as well as *in vitro* tests. These draft guidelines have been used to facilitate the interpretation of the studies cited below. The test described in the draft guideline for *in vitro* testing has also been discussed and adopted by the EU-SCCNFP. In addition, the (USA) Cosmetic, Toiletry, and Fragrance Association (CTFA) have developed a guideline for photoirritation studies. One test has been performed according to this CTFA guideline. The available data for this test indicate that the CTFA guideline is less comprehensive than the draft OECD guideline for *in vivo* testing.

Table 4.20 *In vivo* animal studies of the photoirritancy of AHTN

Study Type	GLP	Method	Results	Reference
Photoirritancy in guinea pigs	Yes	None	Positive reactions observed at ≥0.5%	Stern, 1994
Photoirritancy in guinea pigs	No	None	Positive reactions observed at 1.0%	Klecak et al., 1982
Photoirritancy in guinea pigs	No	CTFA	Slight reactions observed at ≥0.3%	Klecak and Ciullo, 1983a
Photoirritancy in guinea pigs	No	None	Positive reactions observed at 10%	Guillot et al., 1985
Photoirritancy – rabbits and guinea pigs	No	None	Reactions at ≥ 5%	Ogoshi et al., 1980, 1981
Photoirritancy - rabbit	Yes	None	Negative at 1%	Prinsen and van Beek, 1985b
Photoirritancy - rabbit	Yes	None	Slight reactions at 2 & 4%	Prinsen and van Beek, 1985a
Photoirritancy –guinea pigs	No	None	Positive reactions observed at ≥1.0%	Sato et al., 1978
Photoirritancy – hairless mouse	No	None	Negative at 1%	Forbes et al., 1978a,b

In a GLP compliant study, designed to determine the minimal photoirritation concentration and maximum concentration not producing photoirritation for use in the photosensitisation study (see below), photoirritation was assessed by means of visual inspection and by monitoring concentration of extravasal (radio-labelled) albumin in skin biopsies. Groups of 20 female Hartley guinea pigs were i.p injected with 1.0 ml of ^{125}I–bovine serum albumin (^{125}I–BSA; 1 μCi) in a radioisotopic assay before application of 100 μL of a solution of AHTN (according to the study foreword a 1:1 mixture of Tonalid® and Fixolide – purity >98%) in absolute ethanol at concentrations of 1.0, 2.0, 4.0 or 8.0% to the shaved and lightly abraded skin (area approximately 1 cm^2). The substance was only applied to one side of the body. The other side of the body was also shaved and lightly abraded, but not treated with AHTN. Approximately 20-30 min after application, for 10 animals per group, the treated and untreated sites were irradiated for 1 hr with broad–band UV–A (7 W/cm^2) and some UV–B light (25-35 μW/cm^2 at 2 cm). Wavelength ranges were not specified. At 4-5 hr after the application of the test substance or vehicle the animals were given a second i.p. injection of 1 μCi of radioactive BSA. In a follow-up study, two extra groups of 10 animals were treated with 0.25 or 0.5% solutions in a similar way, but for these dose levels no non-irradiated AHTN-treated controls were used, because no signs of primary dermal irritation was observed in animals treated with 1 to 8% AHTN in absence of irradiation. In each animal, photoirritancy was evaluated approximately 24 hr after application by comparing the irritation

scores for irradiation + AHTN-treated vs. irradiation-only sites. It was stated that no dermal irritation was observed at any site only irradiated. At the sites irradiated and treated with the solvent some irritation was visually seen. Irritation of AHTN-treated sites was seen visually (scores higher than vehicle controls) at concentrations as low as 0.5% but not statistically significant. Irritancy scores at the irradiated sites increased with concentration and became statistically significant at 1% and higher. Using the radio isotopic assay for extravasation (of radioactive BSA), the irritancy response of AHTN after irradiation was higher than controls (vehicle + irradiation) at all concentrations tested and also when compared to the AHTN- or vehicle-treated non-irradiated sites. But the response expressed as the differences between AHTN + irradiation vs. AHTN only was essentially identical at all concentrations. 8-Methoxypsoralen **(**8-MOP) was used as the positive control. According to the foreword of the study, with 8-MOP, photoirritation was observed at concentrations as low as 0.001% or 0.01%, but no further study details or results were presented (Stern, 1994).

In a limitedly reported non-GLP study, AHTN (Fixolide – purity >98%) in ethanol (0.3 or 1%) was applied (amount not reported) to the skin (animal pre-treatment, area size, and patch description not reported) of both flanks of albino guinea pigs for 48 hr. Four hr after patch removal application sites were irradiated with UVA using a Westinghouse FS–40 "Black Lamp" (10^4 erg/cm^2/sec, wavelength: 320-400 nm) or with UVB using Westinghouse FS 40 "Sunlamp" (10^4 erg/cm^2/sec, wavelength: 280-370 nm; for < 15 minutes i.e. less than the minimal erythematous dose). Reactions were measured at 4, 24 and 48 hr after radiation. Per concentration the following treatment groups were used (each consisting of 10 animals): A: AHTN-treated and irradiated on the right flank with UVA, B: AHTN-treated and irradiated on the left flank with UVB, C: AHTN-treated but not irradiated, D: not treated with AHTN, but irradiated with UVA and UVB. At 0.3 %, no dermal irritation was observed in any animal, irrespective of treatment with substance or irradiation. At 1 %, all animals of group A showed slight dermal irritation (score 1 in all animals). No signs of dermal irritation were seen in any of the animals in groups B-D (Klecak et al., 1982).

In another limitedly reported non-GLP study, the photoirritant potential of AHTN (Fixolide NP purity >98%) was tested according to a CTFA safety testing guideline. AHTN in ethanol at concentrations of 0.3%, 1% or 3% (with 2% DMSO added to enhance skin penetration), was applied to the shaved skin in doses of 25 µL /2 cm^2 area of male or female Himalayan white spotted guinea pigs (4 per test group). Test substances were applied on both flanks of the animals, probably without occlusion. 30 Min after application, the right flanks of the animals were irradiated with a non–erythemogenic dose of UV–A light (20 J/cm^2 over 7 hr, spectrum 320-400 nm). 8-MOP (0.1%) was used as a positive control. No skin reactions were observed on non-irradiated sites treated with test material or positive control. At 0.3% AHTN, slight dermal reactions (score 1) in 2/4 and in 4/4 animals were observed at 24 and 48 hrs after application, respectively. Both at 1 and 3%, all animals showed slight photoirritant reactions (score 1) at all observation times. With the positive control, slight dermal reactions (score 1) were seen in 4/4 animals at 4 hr and well-defined dermal reactions (score 2) was observed at 24 and 48 hr (Klecak and Ciullo, 1983a).

In a study (non-GLP; purity unknown) conducted to develop a new method for screening for photoirritancy, the photoirritant potential of AHTN (purity unspecified) was evaluated in a group (group 2) of 10 male and 10 female young adult albino Dunkin-Hartley guinea pigs. The animal's fur on back and flanks was clipped and depilated 24 hr prior to dosing. A single application of a 10% solution of AHTN in ethanol (0.5 ml) on a gauze pad of 2 cm^2, was applied to the skin on the back for 1.3 hr under an occlusive aluminium foil sheet of 5 × 5 cm. Another (non-treated) part of the dorsal skin was also covered with aluminium foil to protect

it from unwanted irradiation. Five guinea pigs were maintained as a control group (group 1) and were treated with the same solution of AHTN but were not irradiated. The treated patches were irradiated using a system of 2 fluorescent lamps with continuous UV-A spectral emission of 310-400 nm (peak at 360 nm) and 285-350 nm (UV-B; peak at 310 nm) delivering energy of 12.5 J/cm^2 (99% UV-A; 1% UV-B), as dosimetrically determined. This amount of irradiation was the minimal erythematous dose. Readings of erythema and oedema as well as histopathological examinations were carried out 6 and 24 hr after irradiation. The readings were performed in a blinded way. Erythema and oedema were scored on a 5-unit scale (no effect – very pronounced effect). Erythema and oedema scores for the group 2 animals were considered positive if it was 2 units greater than the one attributed to the group 1 animals. Negative and doubtful scores were equal to that or slightly higher than that of group 1, respectively. Positive histopathological readings were those representing "skin burned" type of lesions. The final score for each treated and irradiated animal was made on the basis of both macroscopic and histopathological examinations. Macroscopically, in 2/20 and in 8/20 animals, positive or doubtful responses were observed, which were confirmed as positive by histopathology (Guillot et al., 1985).

(The following was taken from a symposium paper presented in 1980 and from the published paper, in Japanese, presumably reflecting the same data). A solution of AHTN at a concentration of 1, 5, 10 or 20% in either Vaseline or 99.5 % ethanol was tested in rabbit and guinea pig. The number of animals used in the study is not included in the study summary. A 50 µg sample of the test mixtures were applied to 4 cm^2 shaved skin area for 2 hr and then the animals were exposed to Toshiba FL20sBLB fluorescent lamps, 300-430 nm. Non-irradiated sample patches were used as control sites. The total irradiation dose was 1.6 -7.6 J / cm^2. Skin reactions were assessed 3 days after irradiation. There were no photoirritant effects observed at 1 %. Photoirritancy was reported at all higher doses in both species. No data were given at longer periods after irradiation nor were any quantitative data given on strength of the reactions (only -, ± or +) therefore dose response was impossible to determine (Ogoshi et al., 1980, 1981).

In a GLP compliant study, 0.1 ml of a 1% solution of AHTN (Tonalid®; purity >98%) in absolute ethanol was applied to the clipped skin (area not specified) of 6 male New Zealand White albino rabbits and the site irradiated with UV light (Philips TL 40W/08 – wave length about 365 nm) for 60 min at 8 inches from the skin surface. A solvent control and a positive control 8-MOP (1/128% in ethanol) were included in the test. Skin reactions were evaluated at 24, 48, 72 and 96 hr after application and compared to reactions of treated but non-irradiated sites on the opposite flank of the body. In one animal very slight erythema (score 1 at 24 hr, only) was seen after treatment with AHTN, but with and without irradiation. Moderate to severe reactions were seen with 8-MOP with but not without irradiation (Prinsen and van Beek, 1985b).

The above test was then repeated (still GLP) with 2 and 4% solutions in ethanol again with 8-MOP as the positive control. Severe reactions were seen with 8-MOP with irradiation but no reactions without. No significant reactions were seen at either concentration of AHTN without irradiation but both concentrations produced very slight to moderate oedema and very slight erythema with irradiation Primary irritation scores were 1.5 and 1.8 at 24 hr for the 2% and 4% solutions, respectively. The skin reactions decreased in severity over the next 72 hr. The difference in the strength of the reactions seen at 2 and 4% was not considered significant (Prinsen and van Beek., 1985a).

In tests for photoirritation, 0.02 ml aliquots of AHTN (non-GLP; purity unknown) in ethanol or diethyl phthalate (DEP) were applied evenly to 1.5 x 1.5cm areas on both sides of the

shaved, depilated backs of rabbits or guinea pigs. One side of the animal's back was used as a control side and covered with aluminium foil. Three rabbits and 3 guinea pigs (strains not reported) were treated with 0.5, 1.0, 2.5 % AHTN in ethanol, 5% AHTN in ethanol or DEP, 20% AHTN in DEP and 50 % AHTN in DEP or ethanol (the guinea pigs were not treated with 0.5 and 2.5% and rabbits were not treated with the 50% concentration), followed (time after dosing not specified) by glass-filtered UV-A irradiation for 110 min from Toshiba 40 WFL BLB lamps (300-400 nm; peak at 360 nm) at a distance of 10 cm. Readings were taken at 24, 48 and 72 hr after irradiation. The degree of difference between the average score of irradiated and non-irradiated site was evaluated to determine photoirritancy. With rabbits no significant effects were observed at the 0.5% concentration. At 5% in ethanol (2.0-3.3) compared to 0.0 in controls) and at 20% in DEP (1.3-3.7 compared to 0.0 in controls) clear photoirritancy was seen. Positive reactions (average scores of 1.7 to 2.0 at 24 hr) were seen in guinea pigs from 1 up to 20% compared to scores for the non-irradiated control sites of 0.0. At 50% scores were 2.3-3.0 compared to controls with 1.3-2.3 for the non-irradiated controls over the entire observation period. According to the study report, the differences in scores should be considered as an indication of moderate photoirritancy. The average irritation scores declined during the 72 hr period following the irradiation. No positive control was tested (Sato et al., 1978).

An aliquot of 20 µl of a 1% solution of AHTN (non-GLP; purity unknown) in methanol was applied to 5 cm^2 of normal skin in SKF Hairless-1 mouse (number of animals not given). At 30 min after the application, the centre 1 cm diameter circle of the application site was irradiated for 30 min with simulated sun light using a filtered (Schott WG 320) Osram XBF 6000 w Xenon lamp to produce a very slight erythema, or was irradiated for 1 hr with a bank of F40T12BL fluorescent black lights, (glass-filtered to eliminate "sunburning UV light" (< 320 nm)). Skin reactions were assessed at 2, 4, 24, 48 and 72 hr. No reactions were observed, but irritation scores were not provided (Forbes et al., 1978a).

Forbes et al. (1978b) have also studied photoirritancy of AHTN after four applications of 20 µl of 1% AHTN on the skin of hairless mice. This study was stated to be negative, but the report was too incomplete to be taken into account.

Human data

AHTN has been tested in several skin tests for photoirritation. A summary of the results is shown in **Table 4.21.**

Table 4.21 *In vivo* human studies of the photoirritancy of AHTN

Study Type	GLP	Method	Results	Reference
Photoirritancy-human	No	None	Negative at 10%	Mark and Gabriel, 1987
Photoirritancy-human	No	None	Negative at 10%	Cuthbert and D'Arcy-Burt, 1983
Photoirritancy-human	No	None	Inconclusive	Folk and Dammers, 1987

There is no standardized protocol for conducting photoirritancy screening in humans. Because of this, several tests were conducted in different laboratories using their standard protocol and standard operating procedures.

AHTN (Tonalid®; non-GLP, purity >98% - 10% dilution in ethanol/DEP (3:1)) was tested for photoirritancy on 10 female human volunteers (white Caucasian; ages 18-39). Before

application of the preparation for each of the subjects, the Minimal Erythemal Dose (MED) was determined using UV light irradiation from a xenon arc solar light simulator. Subsequently, areas on the back were stripped 3 times to remove the superficial stratum corneum; followed by application of 20 µL of the test solution to 3 designated test sites, each approximately 1.5 cm in diameter. One of the sites was used as the non-irradiated control site and one site was irradiated but was not treated with AHTN. In addition, three of the 10 subjects were randomly selected and treated with 20 µL of a 0.2 mg/ml solution of 8-methoxypsoralen in ethanol as a positive control. After 30 min of contact, one AHTN-treated site and the site not treated with AHTN were irradiated with 10 MED of UV-A followed by 0.5 MED of UVA + UVB The sites were scored 5 min after irradiation and again at 3, 24, 48 and 73 hr after irradiation. There were no significant reactions to the AHTN solution but all three subjects exposed to 8-MOP showed clear positive reactions (Mark and Gabriel, 1987).

Twenty five microliters of 1%, 3% or 10% solutions of AHTN (Fixolid; purity >98%) dissolved in ethanol/acetone 1/1 were applied to 6 sites (2 cm^2) per concentration on the back of 6 female volunteers (aged 20-40 yr), after 30 min followed by irradiation with UVA light at doses of 0, 1, 2.5, 5, 10 or 20 J/cm^2. Skin reactions were compared with those elicited by a positive control (8-MOP 0.005 or 0.01 %, irradiated with 1 and 2.5 J/cm^2) using black light fluorescent tubes having a wavelength of 320–400 nm. Examination of test sites at 4, 24, 48 and 72 hr after application revealed no photoirritant reactions at any concentration of AHTN but slight to severe reactions with 8-MOP (Cuthbert and D'Arcy-Burt, 1983).

In another photoirritancy study, 26 volunteers (male and female Caucasians) received single applications in duplicate of ~0.3 ml AHTN (10% Tonalid® in unspecified solvent; non-GLP) under occlusive (Parke-Davis with Webril) patches along with 4 other substances and a blank control patch. The test material was applied on the back for 24 hr. The test site was irradiated with 16-20 J/cm^2 of UVA from a filtered xenon arc solar simulator within 10 min after patch removal. Prior to irradiation, any excess test material remaining on the skin was wiped off with a wet towel. All sites were evaluated 1, 24, 48, and 72 hr after irradiation. One test material, Tagetes absolute, produced strong photoirritant reactions, which spilled over onto other sites including the blank control making interpretation difficult (Folk and Dammers, 1987). In three volunteers low score responses were observed in non-irradiated patches. In one of these the response increased after irradiation, while in another one, the response did not change after irradiation. In the third one the irradiated site did not show a reaction, but in three additional volunteers low score reactions were observed after irradiation, but not without irradiation. Because of lack of information with respect to the scoring system, these results are difficult to interpret. Data on reactions to the solvent control were not provided. The test is considered inconclusive (Folk and Dammers, 1987).

Studies in vitro

Table 4.22 *In vitro* studies of photoirritancy of AHTN

Study Type	GLP	Method	Results	Reference
Mouse Fibroblasts 3T3 Assay	Yes	EC/COLIPA	Negative	Harbell et al., 2001
Yeast *Saccharomyces*	No	None	Positive	Forbes et al., 1978a

In a GLP compliant study according to the draft OECD 432 guideline (draft adopted) EU-SCCNFP guideline mentioned above, Balb/c 3T3 mouse fibroblasts were exposed to 50 µL aliquots of AHTN (purity >98%) in Hank's Balanced Salt Solution (HBSS) containing 0.5%

ethanol (concentrations of 0.992 – 56.2 µg/ml for irradiated cells or 1.77-100 µg/ml for non-irradiated cells both spaced at a ratio of 1.78) for 1 hr followed by irradiation with UV-A light for 50 minutes for a total irradiation dose of 5 J/cm^2. Duplicate slides were kept in the dark for the 50-minute period. After the irradiation period, the test solutions were decanted from the plates and the cells were washed with HBSS. Assay medium was then added to the cells and the cells were incubated for 24 hr at which time the assay medium was decanted from the cells and 100 µL of filtered Neutral Red solution added. After a 3 hr incubation, the cells were washed, scored for Neutral Red uptake and the IC_{50}, the Mean Photo Effect (MPE) and Photo-Irritation Factor (PIF) were calculated. The average (2 runs) IC_{50} for AHTN was 5.43 µg/ml with irradiation and 6.89 without. The MPE was 0.002 and 0.014 (<0.1 is considered non-photoirritant) for each run and the PIF was 0.949 and 1.233 (>5.0 is considered photoirritant). Chlorpromazine was tested as a positive control. The average IC_{50} was 31.5 µg/ml with irradiation and 2.0 without. The MPE was 0.627 and 0.642 for each run and the PIF was 17.48 and 13.62 (Harbell et al., 2001). In conclusion, AHTN is not photoirritant when tested with the Neutral Red Uptake Phototoxicity Assay.

A 1% solution of AHTN (Tonalid® – non-GLP; purity >98%) in methanol was applied to filter paper disks, allowed to dry and placed on culture media that had been seeded with yeast (*Saccharomyces*). The plates were then incubated for 24 hr under black light (10 W/m^2) and then cultures were examined for growth at 24-hr intervals for 4 days. Growth was compared to that observed in substance-treated plates that did not receive irradiation. Growth inhibition was seen in the area adjacent to the plate and considered as evidence for photoirritancy (Forbes et al., 1978a).

4.1.2.3.2 Eye

Table 4.23 *In vivo* rabbit studies of the eye irritation of AHTN

Species	Number Tested	Concentration of AHTN	Results – Primary irritation	Reference
Rabbit OECD Guideline 405	3	pure AHTN	Slight effects seen No classification	Prinsen and van Beek, 1985c
Rabbit OECD Guideline 405	3	pure AHTN	Slight effects seen No classification	Prinsen and van Beek, 1985d

Studies in animals

In a GLP compliant study according to OECD Guideline 405, AHTN (Tonalid®; purity >98%) was applied undiluted as a finely ground powder (0.1 g) to the eyes of 3 male New Zealand White albino rabbits. The test substance remained in the eyes for at least 24 hr (no further details). Eye irritation was evaluated at 1, 24, 48, 72 and 168 hr post application. For results, see **Table 4.24.** One rabbit developed a slight corneal opacity (score 1 at 24 and 48 hr, only). Moderate redness and slight to moderate chemosis of the conjunctivae was seen in all three animals after one-hour post application. After 24 hours, this was reduced to slight (1/3 animals) to moderate redness (2/3 animals) and slight chemosis in all three. Complete recovery occurred by 7 days. (Prinsen and van Beek, 1985c). Based on these results and the EU classification rules AHTN does not need to be classified as an eye irritant.

Table 4.24 Individual scores for 3 animals from an eye irritation test with AHTN.

timepoint	Cornea opacity	Iris	Conjunctivae Redness	Conjunctivae chemosis
After 1 hour	0-0-0	0-0-0	2-2-2	2-2-1
After 24 hours	0-1-0	0-0-0	1-2-2	1-1-1
After 48 hours	0-1-0	0-0-0	1-1-1	1-1-1
After 72 hours	0-0-0	0-0-0	1-1-1	1-1-1
After 7 days	0-0-0	0-0-0	0-0-0	0-0-0

In a second GLP compliant study according to OECD Guideline 405, AHTN (Tonalid®; purity >98%) was applied undiluted as a finely ground powder (0.1 g) to the eyes of 3 New Zealand White albino rabbits. The test substance remained in the eyes for at least 24 hr (no further details). Eye irritation was evaluated at 1, 24, 48, 72 and 168 hr post application. For results, see **Table 4.25**. Two rabbits had slight corneal opacity at 24-72 hr. Slight iritis was observed in one animal at the 1 hr observation and in another animal at the 24 and 48 hr observations. A slight (1/3 animals) to moderate (2/3 animals) redness and slight (2/3) to moderate (1/3 animals) swelling of the conjunctivae was seen after 24-48 hr. After 7 days a slight redness of the conjunctivae without any other effects was still seen in two animals. All effects had cleared by 29 days (Prinsen and van Beek, 1985d). Based on these results and the EU classification rules AHTN does not need to be classified as an eye irritant.

Table 4.25 Individual scores for 3 animals from an eye irritation test with AHTN.

timepoint	Cornea opacity	iris	Conjunctivae redness	Conjunctivae chemosis
After 1 hour	0-0-0	0-0-1	2-1-2	2-1-2
After 24 hours	1-1-0	1-0-0	2-2-1	2-1-1
After 48 hours	1-1-0	1-0-0	2-2-1	2-1-1
After 72 hours	1-1-0	0-0-0	1-1-1	1-1-1
After 7 days	0-0-0	0-0-0	0-1-1	0-0-0

Human data

No cases reported

4.1.2.3.3 Respiratory tract

Studies in animals

No data available

Human data

No cases reported

4.1.2.3.4 Summary of irritation

AHTN has been tested in two dermal irritation studies in animals. In one study, no dermal effects were observed. In the other study as a 50% solution in diethyl phthalate (DEP), slight dermal irritation was observed with the solution as well as DEP although the score for DEP was less. Based on recommended studies for hazard classification in rabbits, with the undiluted substance, AHTN does not need to be classified as a skin irritant. Dermal effects observed after topical application of AHTN in repeated dose toxicity studies may reflect (photo)-sensitisation, rather than irritation (see section 4.1.2.5).
Several sensitisation studies in humans showed no signs of dermal irritation by AHTN.

The photoirritation studies in animals indicate that AHTN is more irritating to the skin after irradiation with UV light. The results in human tests do not indicate a photoirritating effect in humans. Also, an *in vitro* phototoxicity test (in compliance with test guideline B.41 (EU/COLIPA Test)) was negative. No criteria for the classification of substances for photoirritation are available in Annex VI of Directive 67/548.

AHTN has been tested for ocular irritation in rabbits in two studies. In both studies, slight ocular irritation was observed. However, the magnitude of the effects is not high enough to require classification according to the EU guidelines.

No data on respiratory tract irritation are available.

4.1.2.4 Corrosivity

In two skin irritation studies in rabbits (see under 4.1.2.3) no corrosivity was observed. (Haynes, 1984, 1985).

AHTN does not need to be classified as corrosive.

4.1.2.5 Sensitisation

4.1.2.5.1 Studies in animals

Skin

Skin sensitisation

In vivo studies

A summary of the tests are presented in **Table 4.26**.

Table 4.26 .A *In vivo* studies of skin sensitisation of AHTN

Species	Number of animals	GLP	Results	Reference
Guinea pig	6	No	No sensitisation at 30% induction, 1% challenge	Geleick et al., 1978
Guinea pig	8	No	No sensitisation at 5% induction, 5% challenge (lower volume)	Klecak et al, 1978

Neither of these studies was conducted in compliance with GLP.

Solutions of 3, 10 or 30% (all of them clearly irritating concentrations under the conditions of repeated application) AHTN (Fixolid; purity >98%) in ethanol/acetone (50/50) were applied in 0.1 ml doses to 8 cm^2 clipped flank skin of 6 guinea pigs each daily for 3 weeks in an open epicutaneous test. Because of cumulative irritation at the site of application especially at the high dose, the application site was changed after 2 wks. Challenge doses of 0.025 ml of a 1% solution (the maximal non-irritating dose in a pre-test) were then applied to a 2 cm^2 naïve site and again 2 weeks later. Reactions were read 24, 48 and 72 hr after each challenge dose, and compared to reactions in non-induced control animals. No sensitisation reactions were observed (Geleick et al., 1978).

In an incompletely reported study, 0.1 ml of a 5% emulsion of Fixolide in Freund's complete adjuvant (FCA) injected intradermally into the necks of 8 guinea pigs on days 0, 2, 4, 7 and 9. Challenge tests of 0.025 ml on a 2 cm^2 skin area (preparation not reported) were then applied 3 and 5 wks after the first intradermal dose and reactions read at 24, 48 and 72 hr. It was concluded that Fixolide was a weak intradermal sensitiser under these conditions but it was not clear that the reactions reported, 6/8 at day 21 and 8/8 at day 35 were reactions on test or control animals nor were any reaction scores reported (Klecak et al., 1978).

Photosensitisation

Table 4.27 Photosensitisation studies with AHTN on animals

Species	Number of animals	GLP	Results	Reference
Guinea pig	10	No	Positive reactions at 1% induction, 0.3 % challenge	Klecak and Ciullo, 1984a
Guinea pig	10	No	Positive reactions at 10% induction, 0.3% challenge	Klecak and Ciullo, 1984b
Guinea pig	10	No	Positive reactions at 5% induction, 1% challenge, also with sensitisation reactions to photodegradation products	Klecak and Ciullo, 1985
Guinea pig	9	Yes	Positive at 10% induction, 1 and 0.3% challenge	Klecak, 1990
Guinea pig	30	Yes	Positive at 1% induction (slightly abraded skin), 0.3, 0.6 and 1% challenge	Stern, 1994
Guinea pig	10	Yes	Negative at 2% induction, 2% challenge	Prinsen and Til, 1985a
Guinea pig	10	Yes	Negative at 2% induction, 2% challenge	Prinsen and Til, 1985b

In a photosensitisation assay according to CTFA guidelines, the shaved nuchal area of 10 Himalayan white spotted guinea pigs was treated with 4 intradermal injections of Freund's Complete Adjuvant (FCA) and with AHTN (Fixolid, purity >98%) at a concentration of 10% in ethanol in a dose of 0.1 ml over an area of 8 cm^2 (0.0125 ml/cm^2) and the site irradiated after 30 min with 10 J/cm^2 UVA from a bank of 4 black light florescence tubes. Application of AHTN and exposure to UV light was repeated for a total of 5 times over a 10-day period. At day 21 and 35, the guinea pigs were freshly shaven on both flanks and challenged with applications of 25 µl of 0.3 % AHTN in ethanol on 8 patches of 2 cm^2 followed by irradiation in the same manner on one site only. Reactions were recorded 24 and 48 hr later and compared to those obtained from a control group of untreated animals. A 3% solution of 3,3',4',5-tetrachlorasalicylanilide (TCSA) in acetone was included as a positive control. Defined erythema (score 1) was seen in 9/10 animals with irradiation but none without irradiation at 24 hr after the challenge. At 28 hr, still in 6 out of 10 animals defined erythema

was observed. Six of the ten guinea pigs were sensitised under these conditions (Klecak and Ciullo, 1984b).

In another photoirritancy study, apparently similar to the above, a group of 10 guinea pigs (strain not reported) was treated with AHTN (non-GLP; Fixolid; purity >98%) as a 1% solution in ethanol for induction as above and challenged with 0.3% in ethanol at day 21 and 35. A 3% solution of 3,3',4',5-tetrachlorasalicylanilide (TCSA) in acetone was included as a positive control. It was concluded that under these conditions all animals showed photosensitisation (Klecak and Ciullo, 1984a).

A group of 10 guinea pigs were photosensitised with a 5% ethanolic solution of AHTN (non-GLP; Fixolid; purity >98%) by the same procedure (with adjuvant) described in the previous study (Klecak and Ciullo, 1984b). Photosensitisation to AHTN for 10/10 animals was confirmed. These 10 photosensitised guinea pigs were then rechallenged with 1.0, 0.3, 0.1 or 0.03% ethanolic solutions of AHTN and each of its 4 photodegradation products with no UV-A irradiation in order to detect whether the degradation products were responsible for the photosensitisation effect. Positive skin reactions were seen in 10/10 animals with AHTN and 2 of the photodegradation products. In a separate experiment, another group of 10 guinea pigs was tested for photosensitisation with a 5% ethanolic solution of AHTN in the presence of Parsol 1789 (a UV-A filter) and the rate of photosensitisation was reportedly decreased to 6/10 animals (no details presented). When these 10 sensitised animals were rechallenged with the photodegradation products, one caused sensitisation reactions in 6/10 and another in 2/10 (again detail not given) (Klecak and Ciullo, 1985).

In a GLP compliant study, the nuchal area of 5 female and 4 male albino guinea pigs was shaved and injected with 4 doses of 0.1 ml of FCA followed by application of 0.1 ml/8 cm^2 of a 10% solution of AHTN (Fixolid; purity >98%) which in turn was followed by irradiation with UVB (=1.8J) and UVA (= 20 J UVA). This dosing, but without the FCA, was repeated for a total of 5 applications plus irradiation in 2 weeks. Twelve days later, challenge was with 1, 0.3 or 0.1% solutions (0.025 ml/2 cm^2) on each of the flanks followed by 20 J UVA/cm^2 on one flank only. Reactions were read at 24 and 48 hr after challenge. There were 9/9 reactions at 1%; 8/9 at 0.3 % and 1/9 at 0.1 % with irradiation but no reactions without (Klecak, 1990).

In a GLP compliant photosensitisation assay, 3 groups of 10 female Hartley guinea pigs were treated five consecutive days each week for 3 weeks on shaved and slightly abraded (every other day) skin with 100 µL (area approximately 1 cm^2) of 1% AHTN (1:1 mixture of Tonalid® and Fixolid; purity >98%) in absolute ethanol followed approximately 20-30 min after application by irradiation with broad–band UVA (7 W/cm^2) and some UV–B light (25-35 µW/cm^2 at 2 cm) (group designation: AHTN + irradiation). The site of application was the left dorsal area. The 1% induction concentration was chosen based on the observation (see above) that this dose produced mild photoirritation. In addition, one group of 10 animals was treated with 1% AHTN in a similar way, but not irradiated (AHTN-irr; to detect non-photosensitisation) and also four groups were treated with vehicle + irradiation (VH+irr) but not with AHTN for comparison purpose. Two weeks after the induction phase, the animals in the four VH+irr groups were treated 5 times at 1 hr intervals with 100 µl of 0, 0.3%, 0.6% or 1% AHTN solutions followed by UV irradiation 20-30 min after the last challenge dose. Similarly, the three AHTN+irr groups received 0.3, 0.6 and 1% AHTN challenges with irradiation and the AHTN-irr received a 1% AHTN challenge without irradiation. 6-Methylcoumarin (6-MC; induction 1%, challenge 0.3%), a known photosensitiser, was administered to 5 guinea pigs in the same manner to serve as a positive control. Challenge sites were then scored for erythema and oedema 24 and 48 hrs after the challenge application by visual evaluation and by measuring the size of the erythematous areas.

In the uninduced animals (VH+irr), the challenges with 0.3, 0.6 or 1% AHTN followed by irradiation showed with visual evaluation a challenge dose-related response of photoirritancy. In the induced (AHTN+irr) animals a more pronounced response was observed after the challenge, but the magnitude of this response was similar in all challenge groups. After the challenge with 1% AHTN, the photoirritation response (in the VH+irr group) and the photo-sensitisation response (in the AHTN+irr group) were essentially the same. The differences of response between the VH+irr and AHTN+irr groups were maintained for up to two days after challenge (end of the observation period). No hypersensitivity was observed in the AHTN-irr group.

In contrast to the visual scoring, a clear challenge dose-related response was observed in the size of the erythematous area in the AHTN+irr induced animals, in any case this response was larger than that observed in the VH+ir animals which were challenged with 1% AHTN+irr. The magnitude of the visual scoring with 6-MC was about similar to that of the 1% AHTN challenge group, but the size of the erythematous area with any challenge of AHTN was larger than that with 6-MC.

Induced and uninduced animals were held for rechallenge with 0.3% AHTN at 5 and 18 wks after the initial challenge. A clear photosensitising response was obtained with mean scores essentially the same as at the original challenge. In this rechallenge study the challenge dose was applied either dissolved in ethanol or dissolved in dimethyl phthalate (DMP). With DMP a much less severe response was obtained than with ethanol. In addition with DMP no signs of photoirritation were observed in the uninduced animals, while with 0.3% in ethanol an indication of photoirritation was obtained (Stern, 1994).

In a GLP compliant photosensitisation assay 0.2 ml of a 2% solution of AHTN (Tonalid®; purity >98%) in ethanol was applied once daily for 5 days to the shaved dorsal neck region (area 6 cm^2) of 10 male guinea pigs (Cpb:GpHi 65) followed by irradiation for 30 min using a Philips UV TL fluorescent lamp (40w/08, F40 Tl2 BLB; 310-420 nm distance to lamp 37 cm). At the mid-dorsal region of the back an area was treated with solvent only. During the induction phase slight erythema was observed at the AHTN-treated sites at days 3-5 after the irradiation in 2 to 4 animals. Ten days after the last topical induction a 2% solution was again applied to the shaved skin of induced and control animals followed by exposure to UV irradiation in the same manner as the induction doses. Skin readings were made after 24, 48 and 72 hr. No significant reactions were seen with the test solutions or with the solvent control (5 animals only) after the challenge at any time point (Prinsen and Til, 1985a).

The above test (also GLP) was repeated exactly with a second sample of AHTN (Tonalid®; purity >98%) and in one or two animals very slight erythema on the AHTN-treated sites was seen at days 3 to 5 of the induction phase. Again, no indications for photosensitisation were obtained after the challenge at any time point (Prinsen and Til, 1985b).

In vitro studies

Na data available

Respiratory tract

No data available

4.1.2.5.2 Human data

Skin

Skin sensitisation

In vivo studies

A summary of the tests are presented in **Table 4.28**.

Table 4.28 Sensitisation studies with AHTN on humans

species	GLP*	Results	Reference
HRIPT	Yes	Negative at 10%	Berger, 1998
HRIPT	No	Negative at 2%	Edelson, 1964
HRIPT	No	Negative at 2%	Epstein, 1975
Human Patch Test	No	No conclusion possible	Meynadier et al., 1986
Human Patch Test	No	Negative at 1 and 5%	Frosch et al., 1995

*Good Clinical Practice Guidelines

A Human Repeated Insult Patch Test (HRIPT) was performed with a 10% solution of AHTN (purity not specified) in ethanol/DEP (75/25) on 111 subjects. Nine induction applications on the same site on the back were made with occlusive patches (Hill Top Chambers, 25 mm) containing 0.3 ml of the preparation for 24 hr-periods, 3 times a week for 3 weeks. After a rest period of about two weeks, a challenge application of the same type and duration was made on a site previously not exposed. Reactions were scored 48 and 96 hr after the application of the challenge dose. A mild skin reaction (score 1) was observed in 9 persons after the challenge with AHTN, while in 7 persons a similar mild skin reaction was observed with the vehicle. It was concluded that no evidence of allergic potential was seen (Berger, 1998). This test was conducted in accordance with Good Clinical Practice guidelines.

A HRIPT (non-GLP; Tonalid; purity >98%) with a 2% solution of AHTN in dimethyl phthalate was conducted on 52 subjects (age 16-77 yr – 21 M and 31 F) with application of 0.5 ml on patches occluded with impervious tape (area not reported) first on the inner surface of the right deltoid area, then on the inner surface of the left deltoid area, then on the upper portion of the right forearm and then on the upper portion of the left forearm. This cycle was repeated for 10 applications throughout the 23-day induction period. Patches were left on for 48 hr, except for the weekend when they were left in place for 72 hr. After a 2-week rest period, another 48 hr patch of test solution was applied to a naïve site and scoring of reactions was on patch removal and 72 hr later. No irritation or sensitisation reactions were observed on any of the 52 subjects. The study was poorly reported (Edelson, 1964).

A human maximization test was performed with AHTN (2% Tonalid®; presumably in petrolatum) of 25 subjects. AHTN was applied (volume not reported) on the volar aspects of the forearm under occlusion (patch not described) of volunteers for 5 alternate day 48 hr periods. Patch sites were pre-treated with 5% sodium lauryl sulfate (SLS) under occlusion for 24 hr to enhance penetration prior to the initial patch only. Challenge patches were applied on the back under occlusion (sites pre-treated with 2% SLS for 30 min) for 48 hr after a 2-week rest period and the sites scored for reactions 24 and 72 hr after application. No sensitisation reactions were seen. The study was poorly reported (Epstein, 1975).

Twenty-one patients allergic to perfumes and sweet smelling constituents were studied by patch testing with 21 substances recommended by the International Contact Dermatitis Research Group and with 57 perfume components. Fixolide (purity not reported) was tested at 3% in petrolatum. Two sensitisation reactions were observed however, the methodology was questionable and the results cannot be interpreted (Meynadier et al., 1986).

A multicentre study with patch tests with 48 fragrance materials was reported. Tonalid (purity not reported) was tested in either a 1 or 5% solution in petrolatum on 313 patients. The material was applied to the back for 2 days using Finn Chambers on Scanpor tape, and the reactions were evaluated on days 2 and 3 or on days 2 and 4. No sensitisation was observed with Tonalid (Frosch et al., 1995).

Photosensitisation

In vivo studies

A summary of the tests are presented in **Table 4.29**.

Table 4.29 Photosensitisation studies with AHTN on humans

Species	GLP	Results	Reference
Human photo-RIPT	No	Negative at 1 and 5%	Frentzko and Shanahan, 1989
Human photo-RIPT	Yes*	Negative at 10%	Mills, 1997
Human photo-RIPT	Yes*	Negative at 10%	Mills, 1997

*Good Clinical Practice Guidelines

A non-GLP repeated insult patch test with irradiation (photo-RIPT) with 1% and 5% AHTN (Fixolide/Tonalid® 1:1; purity 98%) each in ethanol and dimethyl phthalate (4 separate dosing solutions) was carried out on each inner forearm (one for irradiation and one for control) of 25 human subjects with and without a non–erythemogenic UV–A radiation of 10–15 J/cm^2 (320-400 nm; peak at 365 nm). On the site to be irradiated, 0.01 ml of test material was applied directly to the site followed by irradiation for 45 min to 1 hr. These sites and the non-irradiated sites were then covered with Parke-Davis Ready-Bandage occlusive patches to which had been applied approximately 0.2 ml additional test material and the patch left in place for 24 hr. This was repeated 3 times per week for 3 weeks. Patched sites were scored 48 hr after application. After 14 days rest, challenge applications were made in the same manner as the induction applications but on previously unpatched sites. The sites were scored 24 and 48 hr after application/irradiation. Barely perceptible to mild transient responses where seen throughout the induction with the 5% ethanol solutions in 5/25 persons, sometimes on irradiated and sometimes on non-irradiated sites (scores are given for the 5% solution applications only). On challenge, two subjects (12 and 24) showed similar reactions at 24 hr with irradiation but not without and no reactions at 48 hr. Two subjects (7 and 21) showed similar reactions on both irradiated and non-irradiated sites at 24 hr but not at 48 hr. One subject exhibited a minimal barely perceptible reaction with papular eruption at 48 hr but not at 24 hr and only with irradiation. This subject also had similar reactions with and without irradiation following the first induction patch. Because of the marginal nature of these reactions, follow-up tests were undertaken. Subject 20 was rechallenged with 5% in ethanol as above 2 weeks later and exhibited moderate erythema plus mild oedema with irradiation at 24 and 48 hr but also had a moderate erythema without oedema at 24 hr without irradiation, however in this case the reaction subsided to mild erythema at 48 hr. The subject was again

rechallenged approximately 2 months later as above but this time with a 1% solution in ethanol applied on both a naïve site and a previously "reactive" site. With irradiation, a moderate level reaction was seen at the reactive site and a mild reaction at the naïve site at 24 hr with both subsiding to barely perceptible reactions at 48 hr. A barely perceptible reaction was also seen with solvent control at both 24 and 48 hr. Without irradiation, a mild reaction was seen on both the reactive site and the naïve site at 24 hr with both subsiding to barely perceptible at 48 hr. The solvent gave a barely perceptible reaction at 24 hr that disappeared by 48 hr. Subject 20, along with subjects 12 and 21 (subject 24 was no longer available) were again challenged approximately 3 months later with 1 and 5% solutions AHTN, ethanol solvent and dimethyl phthalate all without irradiation to determine skin reactivities to these materials. No reactions were seen on any subject. A final follow-up occurred with subject 20 about 1 month later (approximately 7 months from the original test conclusion). This time the challenge was as in the original test with 1 and 5% solutions in ethanol and in dimethyl phthalate (6 applications including the 2 solvent controls) with and without irradiation. This time there were no reactions with any application with or without irradiation at any time point. Based on the original marginal results and the extensive follow-ups, the authors concluded that there was no significant evidence of photo sensitisation with AHTN in ethanol or dimethyl phthalate at concentrations up to 5% (Frentzko and Shanahan, 1989).

Two human allergenicity and photoallergenicity studies using a modified repeated insult patch test (photo-RIPT) according to the procedure of Kaidbey and Kligman (1980) were conducted in accordance with applicable Good Clinical Practice guidelines. These two tests were with 0.2 ml of a 10% solution of AHTN (purity not stated) in ethanol/DEP (75/25) applied over an area of 4 cm^2 under occlusive patches (Webril covered by an occlusive hypoallergenic tape) on 29 humans with two different methods of irradiation using a 1000 watt Xenon Arc Solar Simulator with UV-A/UV-B filters. Prior to induction, the minimal erythema dose (MED) for the radiation to be used was determined for each subject. Test solution was applied to 2 sites (one for irradiation and one for control) 2 times a week for 3 successive weeks. At 24 hrs after application of the induction dose, patches were removed and half of the sites were exposed to UVA/UVB (2 × MED of UVB with 5% UVA)(time period not given). After a 2-week rest period, challenge doses were applied in triplicate in the same manner as the induction doses and the sites scored at 1, 24, 48 and 72 hr after patch removal. To monitor contact sensitisation one application site was not irradiated, one site was irradiated after patch removal at 24 hr post application and one site was treated with AHTN and irradiated at 10 minutes after application. This latter site was not further covered with an occlusive patch. Patch sites exposed to radiation during challenge received a dose of 16 J/cm^2 of UVA over the entire area of the patch site and UVB (0.75x MED. During the challenges, at least one additional test article (2 photodegradation products of AHTN: "product 4001"; 0.1% and "product 4002"; 1%, and a 3% solution of AHTN in 75/25 ethanol/DEP irradiated sample with 10 J UV-A) was included in the test, and these were not irradiated after the application.

Slight to mild signs of dermal irritation were obtained during the induction period. However, these reactions were observed in both AHTN-treated and vehicle-treated sites at about the same rate. There was no clear increase in the severity of the dermal irritation during the progress of the induction phase. After the challenge, slight irritation was observed in irradiated sites more often than in non-irradiated sites. However, upon comparison, differences between AHTN-treated and vehicle-control sites were not observed, neither with respect to severity nor with respect to incidence. No cross-reactions to the photodegradation products of AHTN were seen, either (Mills, 1997).

In vitro studies

No data available.

Respiratory tract

No cases reported.

In vivo studies

No data available.

In vitro studies

No data available.

4.1.2.5.3 Summary of sensitisation

The available data include sensitisation and photosensitisation studies in both animals and humans. The sensitisation studies with animals indicate some potential for sensitisation but the studies are only limitedly reported and were not done according to guidelines. Sensitisation studies in humans were negative.

In animal studies investigating photosensitising effects, mostly positive results were reported for AHTN, whereas negative results were reported in human studies on photosensitisation. It is to be noted that for this endpoint there is no validated test method available. The negative human data do not overrule the positive animal data, in line with 3.1.1 of Annex VI of Directive 67/548, which states that tests on man (human volunteers) should be discouraged and should not normally be used to negate positive animal data. Hence, it is concluded that AHTN is a photosensitiser. This may be due to photosensitising effects of AHTN itself, or to sensitising effects from photodegradation products arising from the interaction of AHTN and UV light. Evidence for the latter was obtained from the Klecak and Ciullo (1985) study, where two of the four photodegradation products of AHTN reacted positive. This phenomenon may also explain the dermal effects observed in a dermal repeated dose toxicity study (see 4.1.2.6.1).
There are no criteria for classification for photosensitisation present in Annex VI of Directive 67/548. However, given the need to communicate the photosensitising potential of AHTN to users, the TC-C&L meeting of November 2005 concluded that the proper way to deal with it would be by way of additional safety phrases and a Note, rather than applying R43 (see 1.4.2). However, in the October 2006 meeting of the TC-C&L, the Commission stated that such a note will not be developed under the current legislation.

4.1.2.6 Repeated dose toxicity

4.1.2.6.1 Studies in animals

In vivo studies

Inhalation

No data available

Dermal

Two subchronic dermal non-GLP studies have been conducted but limitedly reported. In the 13 week study, groups of 15 female rats (Crl:COBS CD (SD) BR strain; weight 156-232 g) were exposed topically unoccluded (gentle inunction to the anterior dorsal shaven skin) to dose levels of 1, 10 and 100 mg AHTN (purity >99%) /kg bw/day as a 1% (w/v) solution in ethanol. In the 26 week study (with one 13 week interim sacrifice), groups of 20 female rats (Crl:COBS CD (SD) BR strain; weight 156-232 g) were similarly exposed to dose levels of 0, 9, 18 and 36 mg AHTN /kg bw/day as a 1% solution in ethanol (area of application not reported in either study). Untreated controls and ethanol controls were included. Observations included mortality, clinical signs, behavioural and motor function and (limited) haematology, serum chemistry, organ weights, macroscopy and histopathology. These studies were designed primarily to screen for possible neurotoxicity, because another closely related synthetic musk, Acetyl Ethyl Tetramethyl Tetralin (AETT), appeared to be neurotoxic. Therefore, special neuropathological examination of brain, spinal cord, and peripheral nerves was included for 2 animals per dose group and AETT was included as a positive control.

A significant depression in body weight gain was seen in the 100 mg/kg/day, 13-week group and “a similar effect” was reported at interim and terminal sacrifice in the 26-week study at 36 mg/kg bw/day. A significant depression in haemoglobin, haematocrit and red blood cell count was reported for animals given 100 mg/kg bw/day in the 13-week study and a depressed haemoglobin level and red blood cell count at 36 mg/kg bw/day in the 26-week study. Elevated serum alkaline phosphatase was also seen at 100 mg/kg bw/day. Liver discoloration and prominent liver lobular patterns were observed in animals treated with 100 mg/kg bw /day and “similar effects” were reported at 36 mg/kg bw/day after 26 weeks. Relative and absolute liver weight increases were seen at 100 mg/kg bw/day. After 26 weeks, animals treated with AHTN at 36 and 18 mg/kg bw/day also demonstrated elevated liver weights but it is not stated whether these were relative, absolute or even statistically significant. In the 100 mg/kg/day, 13-week group moderate degrees of hepatocytomegaly and minimal to moderate deposition of an iron positive pigment were observed and “a similar, but less pronounced effect” was reported for the 36 mg/kg bw/day group. For none of these effects is any quantitative data given; thus, it is impossible to determine the severity of the effects or any dose response.

However, given the consistency of effects at 100 mg/kg bw/day at 13 weeks and 36 mg/kg bw/day at 26 weeks, along with what appears to be related to the dose, these treatments must be considered to cause adverse effects. The problem is that the studies were limitedly reported and the severity of these effects cannot be judged. Additionally, no caution was taken to prevent oral intake so the exact dose cannot be determined. The most serious omission is data on the increased liver weight at 18 mg/kg for 26 weeks. If this is a mild adaptive effect not

associated with any histopathology as it appears, then it could be argued that 18 mg/kg is the NOAEL. There is still the problem, however, of not being able to accurately determine the dose of exposure and the route of intake (dermal or oral).

Clear evidence of neurotoxicity, both clinically and pathologically, was seen with the positive control but no such evidence for AHTN was seen in either study at any dose level (Gressel et al., 1980).

Because of 1) the uncertainties in the severity of the effects reported, 2) the study was conducted without collar or occlusion to prevent oral intake of compound making it impossible to determine actual exposures and 3) the area of application was not reported, this study should not be used to determine a dermal NOAEL (Gressel et al., 1980; Ford, 1998).

In a 14-week dermal study of another fragrance material (information on identity deleted), AHTN (10% in ethanol) was included "for comparison" at a dose initially of 100 mg/kg bw/day to 15 female Wistar rats. The alcoholic AHTN solution was rubbed into the shaved skin daily with no collars or occlusion to prevent oral intake. This produced such strong irritation within a few days of initiation, that growth was completely stunted. Because of this, the dose was reduced to 10 mg/kg bw/day on day 8 and onwards. The skin gradually recovered and weight gain became normal one week after the lowering of the dose, however, "erythema and inflammations" were still seen at the end of the study. At week 15, 10 of 15 animals were killed for pathological examination, and 5 of 15 remained without treatment for another 6 weeks recovery period. After 14 weeks roughness and scaliness of the treated skin-area was seen in 1/10 animals at the dose level (reversible). Slight to moderate skin lesions, hyperkeratosis, parakeratosis and acanthosis, occurred too. 3/10 animals showed dark-colouration of kidneys (reversible). The reported growth inhibition and other effects can be attributed to the initial severe irritation (Til and Kuper, 1978).

Because, 1) the effects reported appear to be a consequence of the dermal irritation, 2) the dose was changed during the conduct of the study, 3) there was no NOAEL, and 4) because the methodology was conducted without collar or occlusion to prevent oral intake, this study should not be used to determine an NOAEL. Given that in skin irritation studies (see 4.1.2.3.1) AHTN was not irritating, the dermal effects observed in this repeated dose toxicity study may indicate (photo)sensitisation.

Oral

A GLP compliant 28-day oral study with HanIbm:Wistar (SPF) rats according to OECD Guideline No. 407 was conducted by gavage dosing groups of five males (weights 175-195 g) and five females (weights 150-167 g) with 0 (control), 1, 3 or 10 mg/kg bw/day AHTN (Fixolide; purity 98.9%) in Oleum maydis germinis (total dose volume 10 ml/kg bw). There were no mortalities, compound-related incompatibility reactions, ophthalmological changes, food consumption changes, body weight changes, effects on haematological or clinical chemical parameters, necropsy or histopathology findings (Dotti et al., 1993). There were no adverse effects at the highest dose tested, 10 mg/kg bw/day.

As described in a 13-week dietary toxicity study (see study by Lambert and Hopkins (1996) below) the doses to be used in this 13-week study were determined in a 2-week oral (dietary) range finding and palatability study. In the 2-week study, groups of 5 male and female Crl:CD(SD)BR rats were administered AHTN (GLP; purity not given in report but confirmed to be >98%) by dietary admixture at achieved doses of 0, 33, 88 and 169 mg/kg bw/day for males and 0, 32, 91 and 150 mg/kg bw/day for females. All animals in the high dose group

were sacrificed prematurely on day 5 due to marked reduction in food consumption and bodyweight losses. Food consumption and body weight losses were slightly reduced at the mid-dose. A slightly higher absolute liver weight was noted in the females at the 2 lower doses and relative liver/bodyweights and kidney/bodyweights were increased in males and females at the mid and high dose. Relative liver weight was also increased in females at the low dose. Upon histopathology, hepatocyte fine vacuolization was observed in all groups, including controls, but with increased severity in mid dose males and high dose males and females. Based on these findings, the high dose for the 90-day study was selected to be 50 mg/kg bw/day.

In a GLP compliant study according to OECD Guideline 408, 5 groups of 15 male (weight 224-301 g) and 15 female (weight 157-214 g) Crl:CD(SD)BR rats received by dietary admixture nominal doses of 0 (control), 1.5, 5, 15 and 50 mg AHTN/kg bw/day for 13 weeks. AHTN (purity 99.3%) . Analyses of diet indicated that desired homogeneity was reached. The concentrations of AHTN in the test diets were adjusted weekly. The mean achieved daily intakes of AHTN were 1.6, 5.0, 15.2 and 50.9 mg/kg bw for males and 1.5, 5.1, 15.1 and 50.8 mg/kg bw for females. After the treatment period, 3 females and 3 males from the control and the high dose groups were maintained for a treatment-free period of 4 weeks.

Observations included mortality and clinical signs (daily), body weight and food consumption (weekly), ophthalmoscopy (at week 13 and at the end of the treatment-free period, only controls and high dose animals), urinalysis (at weeks 6 and 12 of treatment and at the end of the treatment-free period), haematology and clinical chemistry (at weeks 7 and 13 of treatment and at the end of the recovery period), macroscopy, organ weights and histopathology (on all tissues from controls and high dose animals, on all gross lesions, and on lungs, liver, kidneys and male and female reproductive and accessory organs from all animals).

There were no mortalities during the study. No clinical signs or ophthalmological abnormalities attributable to the administration of AHTN were noted during the study. During treatment, the mean body weight gain of males and females given 50 mg/kg bw/day was statistically significantly lower than that of controls (78 and 88% of controls, respectively). Body weight at week 13 was only statistically significant decreased with 15% in the male high dose group. This improved upon cessation of treatment. Treatment with AHTN did not affect food consumption.

A reduction in red cell count, haemoglobin concentration and packed cell volume were observed at weeks 7 and 13 for males and females at 50 mg/kg bw/day (see **Table 4.30**). At 7 weeks but not at 13 weeks the animals at that dose showed polychromasia and anisocytosis. These findings were also observed in some animals of the two mid dose groups. A small but statistically significant prolonged prothrombin time was noted at week 13 for males and females given 5, 15 and 50 mg/kg bw/day but the values were all within the standard deviation (except for the high dose males) of controls, were well within the range of historical controls, and not clearly related to dose. Males at 50 mg/kg bw/day had statistically significantly higher white blood cell counts (WBC) at weeks 7 and 13. After the treatment-free period no statistically significant differences were observed, but females and males at the high dose group still had slightly prolonged prothrombin time and higher WBC, respectively.

Table 4.30 Selected haematological and blood chemistry parameters in rats at week 7 or 13 after oral exposure to AHTN

Dose	PT (sec)	PT (sec)	Chol	Chol	Chol	Chol	Trigs	Trigs	Trigs	Trigs	Gluc	Gluc	Gluc	Gluc	A/G	A/G	A/G	A/G
	Male W13	Female W13	Male W 7	Female W 7	Male W13	Female W13	Male W7	Female W7	Male W13	Female W13	Male W7	Female W7	Male W13	Female W13	Male W7	Female W7	Male W13	Female W13
0	15.3±0.78	16.4±0.72	69±13.8	79±12.2	69±15.3	69±15.4	66±29.5	53±11.1	88±44.0	57±16.8	113±8.8	105±14.3	137±14.6	113±11.5	1.3±0.10	1.4±0.11	1.2±0.11	1.3±0.10
1.5	15.6±0.93	16.7±0.75	69±16.6	70±11.2	68±17.9	63±11.8	61±14.1	46±9.5a	86±18.8	54±14.1	112±11.5	111±8.9	128±14.2a	120±11.4	1.4±0.12	1.4±0.11	1.3±0.12	1.3±0.11
5	15.9±1.08a	17.0±0.70a	64±14.3	73±13.0	62±12.9	62±9.1	54±8.4a	42±8.3c	69±18.7	48±8.1a	120±12.0	109±7.6	125±17.2a	110±11.8	1.3±0.13	1.4±0.13	1.3±0.13	1.2±0.10
15	15.9±0.66a	16.9±0.79a	57±11.6b	69±17.2a	53±10.8c	59±13.7a	48±13.9b	41±5.4c	59±16.5b	48±9.8a	102±13.4b	98±12.6	119±12.2c	114±15.4	1.3±0.10	1.4±0.10	1.3±0.14b	1.4±0.11a
50	16.7±0.73c	17.0±0.87a	44±9.2c	57±18.1c	41±9.9c	49±12.8c	37±7.5c	40±6.3c	41±8.5c	41±6.3c	100±10.4b	100±13.1	113±11.2c	107±10.5	1.5±0.15c	1.6±0.09c	1.6±0.12c	1.4±0.12c
HH	17.1	18.1	112	105	99	101	118	104	118	94	127	127	136	137	1.5	1.6	1.3	1.3
HM	15.2	16.2	76	75	66	73	78	57	85	54	97	102	111	111	1.2	1.3	1.1	1.1
HL	13.3	14.3	41	46	32	46	38	10	52	14	67	78	85	85	0.9	1.0	0.9	0.9

	RBC	RBC	Hb (g%)	Hb (g%)	PCV	PCV	PCV	PCV	MCHC	MCHC	MCHC	MCHC	Tprot	Tprot	Tprot	Tprot
	Male W13	Female W 13	Male W13	Female W13	Male W7	Female W7	Male W 13	Female W13	Male W7	Female W7	Male W13	Female W13	Male W7	Female W7	Male W13	Female W13
0	9.1±0.35	8.5±0.31	16.9±0.59	16.5±0.66	45.1±1.73	44.5±1.85	46.9±2.28	45.7±2.10	36.5±0.61	35.5±1.04	36.0±0.87	36.2±0.73	7.1±0.33	7.0±0.31	7.2±0.37	7.1±0.42
1.5	8.9±0.38	8.8±0.34	16.6±0.73	16.8±0.53	44.7±1.40	44.2±1.45	45.3±1.82	46.2±1.85	37.0±0.41	36.6±0.44c	36.7±0.37	36.4±0.68	6.9±0.30	6.9±0.45	7.0±0.30a	7.2±0.47
5	9.1±0.41	8.6±0.34	16.7±0.68	16.6±0.62	45.2±1.67	42.7±1.91b	46.1±1.81	45.9±1.88	36.4±0.59	36.8±0.27c	36.2±0.59	36.2±0.69	6.9±0.30	6.8±0.31	7.0±0.27a	7.2±0.26
15	9.0±0.28	8.2±0.36a	16.6±0.39	16.3±0.61	44.7±1.30	43.1±1.44b	46.1±1.40	44.3±1.56a	36.3±0.73	36.2±0.65c	35.9±0.68	36.7±0.38a	6.9±0.30a	7.0±0.33	6.9±0.37a	7.2±0.42
50	8.3±0.35c	8.0±0.41c	15.1±0.45c	15.5±0.57c	41.6±1.62c	41.8±1.83c	41.3±1.27c	42.4±1.46c	36.4±0.58	36.4±0.44c	36.6±0.37	36.6±0.46a	6.8±0.46b	6.7±0.28	6.8±0.41c	7.1±0.32
HH	9.8	9.2	17.6	17.4	49.7	48.1	50.7	49.3	37.5	37.9	37.4	37.5	7.4	7.2	7.9	8.4
HM	9.0	8.4	16.4	16.0	44.5	43.7	46.9	45.2	35.1	35.6	35.2	35.3	6.6	6.5	7.1	7.5
HL:	8.2	7.6	15.2	14.6	39.3	39.3	43.1	41.1	32.7	33.3	33.0	33.1	5.8	5.8	6.3	6.6

M: male rats; F: female rats; W7: week 7 observations; W13: week 13 observations HH: historical high values; HM: historical mean values; HL: historical low values

(for measurements at W7, values from historical animals 0-3 months of age are used and for measurements at W13, values from animals 4-6 months are used.)

PT: prothrombin time (sec) Chol: cholesterol (g%) Trigs: Triglycerides (mg%)) Gluc: Glucose (mg%)

A/G: albumin/globulin ratio RBC: Red blood cells count (*10^6/l) Hb: Hemoglobine (g%) PCV: Packed cell volume (%)

MCHC: Mean cell haemoglobin conc. (g%) Tprot: Total protein a, b, c: significantly different from control, $p<0.05$, $p<0.01$, $p<0.001$

At weeks 7 and 13, in the male high dose group higher alkaline phosphatase (19% (week 7) and 38% (week 13) increase) and alanine aminotransferase (17% (week 7) and 36% (week 13) increase) activities were found. A higher A/G ratio was noted at week 13 for males and females given 15 mg/kg bw/day and at weeks 7 and 13 for males and females given 50 mg/kg bw/day, in males related to a small but statistically significant reduction in total protein at the two highest doses after 7 weeks and at all doses after 13 weeks No significant differences were seen for these parameters after the treatment-free period. A lower plasma glucose concentration was seen at week 7 in males given 15 and 50 mg/kg bw/day, and in males of all dose groups at week 13. A reduction in plasma cholesterol was observed at weeks 7 and 13 for males and females given 15 and 50 mg/kg bw/day. Plasma triglyceride concentration was dose-relatedly reduced in all dose groups in week 7 and at 5, 15 and 50 mg/kg bw/day in week 13, at least in the two highest dose groups, a clear dose response was seen. No values were significantly different from controls after the treatment-free period.

A brown colouration of the urine was observed at weeks 6 and 12 for 5/15 males given 50 mg/kg bw/day (not seen after the treatment-free period). There were no effects on urine composition or cellularity.

The only organ weight changes attributable to AHTN were the increased absolute and relative liver weights in males and females. The absolute liver weight was at the highest dose increased with 14 and 7%, respectively in males and females (not statistically significant). The relative liver weight was significantly increased with 32% (males) and 13% (females) after exposure to 50 mg/kg bw/day. The liver changes were not seen after the treatment-free period and there was no increase in liver weight relative to brain weight. There were no histopathological changes in the liver. This indicates that the difference in liver/body weight could be due to the decreased body weight gains rather than an effect on the liver.

Upon macroscopy, abnormal green to dark brown coloured livers were observed in 11/12 males and 4/12 females given 50 mg/kg bw/day. Similar findings were noted in the mesenteric lymph nodes of 10/12 males and 3/12 females in the same group. No such discolouration was seen in the lower dose groups. A green colouration was seen in the lachrymal glands of females only at 50 mg/kg bw/day (8/12), 15 mg/kg bw/day (4/12), and 5 mg/kg bw/day (1/12). There were no associated histopathological findings in these organs. In addition, in liver there was no porphyrin accumulation. At the end of the treatment-free period only 1/3 males in the high dose group showed green coloured mesenteric lymph nodes, and 2/3 females in the same dose group showed green coloured lachrymal glands.

Another finding upon macroscopy was uterine distension in females from all treated groups in a dose-related manner (see **Table 4.31**). Due to the incidence of distended lumen for the uterus noted macroscopically, histopathological trackdown was performed on the reproductive and accessory organs of all animals in the dosed groups. The ovaries demonstrated normal cyclical activity as evidenced by large numbers of corpora lutea and lesser numbers of graafian follicles. Vaginal examination demonstrated that the females were in various stages of the oestrus cycle, suggesting normal cyclical activity (see **Table 4.32**). Distension of the uterine lumen was observed in all groups, including controls, with slightly increased incidence and grade in the treated animals compared with controls (see **Table 4.31**). Uterine distension mostly occurred during pro-oestrus (see **Table 4.33**). The incidence of animals in pro-oestrus was also higher in the treated groups. No evidence of any effect was found in the very extensive histopathology on both male and female reproductive organs. It is considered that the finding of uterine distension, particularly in the absence of any other reproductive organ (male and female) effects, reflects normal cyclical change and should not

be considered as an adverse effect in this case. The absence of any other effect in the reproductive organs in either males or females may indicate that endocrine disruptor activity *in vivo* is limited if not absent (see also section 4.1.2.9.3).

Table 4.31 Summary incidence table of pathology in the uterus in rats at week 13 after oral exposure to AHTN (n=15 animals per group)

Dose (mg/kg bw/day)	**0**	**1.5**	**5**	**15**	**50**	**Total**
Uterine distension (macroscopy)	0	2	3	5	7	17
Uterus: distension of lumen (histopathology):						
- minimal	0	1	0	0	0	
- slight	1	0	1	3	2	
- moderate	2	1	4	4	5	
Total	**3**	**2**	**5**	**7**	**7**	**24**

Table 4.32 Phase of oestrus cycle in rats at week 13 after oral exposure to AHTN (n=15 animals per group)

Dose (mg/kg bw/day)	**0**	**1.5**	**5**	**15**	**50**	**Total**
Di-oestrus	1	1	1	1	1	5
Pro-oestrus	2	2	4	5	7	20
Oestrus	4	3	3	4	2	16
Met-oestrus	8	9	7	5	5	34

Table 4.33 Animals without/with uterine distension vs phase of oestrus cycle

Phase of oestrus cycle	**Total no.**	**No. Animals without uterine distension**	**No. Animals with uterine distension**
Di-oestrus	5	3	2 (40%)
Pro-oestrus	20	0	20 (100%)
Oestrus	16	15	1 (6%)
Met-oestrus	34	33	1 (3%)

In summary, the highest dose level of AHTN of 50 mg/kg bw/day clearly elicited adverse effects. The observations at this dose level point to reduced weight gain, signs of haematotoxicity and pigmentation of livers, mesenteric lymph nodes and, in females only, lachcrymal glands, but there were no histopathological findings in these, or in any other organs, including reproductive organs. At the end of the treatment-free period, the haematological and biochemistry parameters had returned to control values and the green to dark brown colouration in the livers and mesenteric lymph nodes was almost completely reversed. The green colouration of the lachcrymal glands, however, was still present.

The evidence of toxicity is less consistent at lower doses. Some of the changes observed in the highest dose group in the haematological and biochemistry parameters were also observed at lower doses. Although all values for these parameters were within the range of historical

controls, for some a dose-response was seen (mainly at 15 and 50 mg/kg bw/day). At lower doses the pigmentation of livers and mesenteric lymph nodes was not observed, whereas the green coloured lachrymal glands were also seen in females given 15 and 5 mg/kg bw/day, but again without associated histopathological findings (Lambert and Hopkins, 1996; Ford, 1998; Api *et al*., 2004).

Further work was carried out to elucidate the nature of the tissue discolouration. In a cell-free system, AHTN was submitted to UV-light. Three chromogenic products were detected, which showed reactivity to glycine or albumin, giving rise first to a blue colour, followed by pink and, finally, a dark green colour. The absorption spectrum associated with the late green colour showed an increased absorbance in the long wavelength region of the spectrum, similar to the green colour extracted from the liver of AHTN–treated animals. When the photo-oxidation of AHTN was carried out in the presence of liver homogenate, the chromogenic derivative of AHTN was tightly bound to the protein pellet and resistant to acidic pH, unlike the colour from the ninhydrin reaction. The dark green colour produced in the liver by feeding AHTN to rats was also tightly bound to proteins and stable to acidic pH. The exact chemical nature of the chromogenic substance was not elucidated. No coloured product, either free or bound to protein could be detected in microsomal incubations from normal and phenobarbital-pretreated rats, carried out in the dark (Pipino *et al*, 2004).

Although the tissue discolouration observed at levels down to 5 mg/kg bw/day is clearly a treatment-related and undesirable effect, this effect is not considered adverse for the following reasons:

- in none of the tissues where the colouration was observed (livers, mesenteric lymph nodes and lachcrymal glands) tissue damage was seen upon histopathology, not even at the highest dose of 50 mg/kg bw/day;
- the colouration was reversible, at least in livers and mesenteric lymph nodes;
- from the studies with microsomes by Pipino *et al*. (2004) it is clear that the pigment does not originate from a direct reaction of metabolites with proteins. It seems plausible that the pigment results from the reaction of photo-oxidation products with proteins and therefore is likely to be formed in tissues other than those in which the colouration was seen, and where this pigment is apparently merely sequestered.

Therefore, based on the small but dose-related changes in some of the haematological and biochemistry parameters at 15 and 50 mg/kg bw/day, the NOAEL in this study is set at 5 mg/kg bw/day.

Intraperitoneal

In order to study whether AHTN had any phenobarbital (PB)- and/or 3-methylcholanthrene (3MC)-like inducing properties, groups of 4 male Sprague-Dawley rats (weight 130-150 g) were treated i.p. with 0, 15 or 100 mg/kg bw/day AHTN (purity >99%) in DMSO for 3 consecutive days. At 24 hrs after the last injection liver microsomes were prepared from these animals and the activities of 7-ethoxyresorufin-O-deethylase (inducible by 3MC) and 7-pentoxyresorufin-O-deethylase (inducible by PB) were measured. At both dose levels tested, AHTN did not enhance the activities of the two liver enzymes (100 mg/kg bw even resulted in a strong decrease in activities of both enzymes). There was also no induction when the animals were sacrificed 4, 7 or 10 days after the last injection (Steinberg et al., 1999).

The same authors also studied the peroxisome-proliferating potential of AHTN, using the same dosing regimen as described above, with sacrifice of the animals at 5 days after the last i.p. injection. Nor at 15 nor at 100 mg/kg bw/day did AHTN enhance hepatic peroxisomal palmitoyl-CoA β-oxidation activity (Steinberg et al., 1999).

AHTN (Tonalid) was administered to 5 female rats (CRL:COBS CD) in ethanol via the intraperitoneal route at a dose of 36 mg/kg bw/day for a period of 4 days. The purpose of this study was to determine if AHTN caused blue coloration similar to acetyl ethyl tetramethyl tetralin (AETT) a polycyclic musk identified as a neurotoxic ingredient and which induced blue coloration to the internal organs of dosed animals. AHTN did not induce blue coloration of the internal organs in the treated animals (Minner and Foster, 1976b).

An additional comparative study was conducted in groups of 5 female rats (CRL:COBS CD(SD)BR). Each animal was administered a daily intraperitoneally injection of 0.6 ml/kg bw of a 6% solution in absolute ethanol of AHTN daily (5/wk) for a at least a 10-day period. AHTN was not found to induce blue coloring in this test, however similar administration of Versalide (AETT) did. Two animals were found dead, one was sacrificed within the 10-day period (Minner and Foster, 1976a).

In vitro studies

No data available.

4.1.2.6.2 Human data

No data available.

4.1.2.6.3 Summary of repeated dose toxicity

In a 28-day oral gavage study, no effects of AHTN were seen at doses up to and including 10 mg/kg bw/day.

In an adequate 90-day oral study, clear mild haematological effects were seen at the highest dose administered, 50 mg/kg bw/day. These effects may be associated with observations of dark discolouration of the liver and mesenteric lymph nodes seen in most high dose animals but not in animals at lower doses. Observations in animals maintained on a treatment-free regime for 28 days following the 90-day treatment period indicate that the effects are reversible. Although the differences from controls were small and generally within historical ranges seen for rats in this laboratory, the overall pattern is such that it cannot be excluded that these effects are of adverse nature. At the lower doses, some statistically significant differences from controls in blood biochemistry and haematology were found, but these differences were small and within the values for historical controls. Some of these, however, showed a dose-response relationship at 15 and 50 mg/kg bw/day. The green colouration of the lachrymal gland was clearly dose-related but not associated with any histopathology at any dose in any animal. The most likely explanation for this observation is accumulation of a pigment resulting from reaction of a photo-oxidation product of AHTN with proteins, and this finding, albeit undesirable, is not considered an adverse effect. Based on the marginal effects observed at 15 mg/kg bw/day, the NOAEL is set at 5 mg/kg bw/day, which will be used in the risk characterization.

Three subchronic dermal studies of AHTN are available. In two of these, 13-weeks at 1, 10 and 100 mg/kg bw/day and 26-weeks at 0, 9, 18 and 36 mg/kg bw/day both applied unoccluded, the purpose was to screen AHTN for neurotoxicity against AETT as positive control. While clear evidence of neurotoxicity, both clinically and pathologically, was seen with the positive control AETT no such evidence for AHTN was found in either study at any dose level. Clear evidence of haematological and hepatotoxicity were seen at 100 mg/kg bw/day for 13 weeks and at 36 mg/kg bw/day for 26 weeks, however, because of the limited nature of the report, it is not possible to judge the severity of these effects. In the third study, AHTN was included only for comparison purposes at one dose level, which proved to be so irritating (possibly resulting from (photo)sensitisation) that the results with respect to systemic effects were confounded. In none of these studies is it possible to determine the actual doses received and because of the lack of collars the real route of exposure and no NOAEL could be established. Therefore, these studies are not used in the risk characterisation.

In a sub-acute study with i.p. administration, AHTN did not show peroxisomal and PB/3MC-like inducing properties.

Repeated dose toxicity studies after inhalation exposures were not available for AHTN.

4.1.2.7 Mutagenicity

Table 4.34 Mutagenicity studies available for AHTN

Type	Activation	Doses	Results	GLP	OECD	Reference
in vitro Bacterial (*S. typhimurium and E. coli*) Reverse Mutation Assay	with and without S-9	8, 40, 200, 1000, 5000 µg/plate	negative	Yes	471	Gocke, 1993; Api and San, 1999
in vitro Bacterial (*S. typhimurium)* Reverse Mutation Assay	with and without S-9	5, 16.6, 50, 166.6, 500 µg/plate	negative	No	OECD-like	Mersch-Sunderman et al., 1998a
in vitro SOS Induction with *E. coli*	with and without S-9	0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50 µg/assay	negative	No	other	Mersch-Sunderman et al., 1998b
in vitro Cytogenetic Assay with Chinese Hamster ovary	with and without S-9	5.9, 11.7 or 23.4 µg/ml w/o S-9 and 17.8, 20, 25 µg/ml & 3.9, 7.8 and 15.6 µg/ml w S-9	negative	Yes	473	Curry, 1995; Api and San, 1999
in vitro Micronucleus Test (human lymphocyte cells)	with and without S-9	00.05, 0.49, 4.85, 48.5, 97, 194 µM	negative	No	other	Kevekordes et al., 1997
in vitro Micronucleus Test (human hepatoma cells)	none added, some inherent	0.1, 0.97, 9.7, 97, 194 and 387 µM	negative	No	other	Kevekordes et al., 1997
in vitro Sister Chromatid Exchange Assay with human lymphocytes	with and without S-9	2.5-40 µg/ml	negative	No	OECD-like	Steinberg et al., 1999
in vitro Sister Chromatid	with and	0.025, 0.25,	negative	No	OECD-	Kevekordes et al., 1998

Type	Activation	Doses	Results	GLP	OECD	Reference
Exchange Assay with human lymphocytes	without S-9	2.43, 24.25, 48.5, 97 µM			like	
in vitro Unscheduled DNA synthesis with rat hepatocytes	inherent	0.15, 0.50, 1.5, 5.0, 15 µg/ml	negative	Yes	482	San and Sly, 1994; Api and San, 1999
in vivo Mouse Micronucleus Assay	na	400, 800, or 1600 mg/kg	negative	Yes	474	Gudi and Ritter, 1997; Api and San, 1999

4.1.2.7.1 Studies in vitro

AHTN (GLP; Fixolid; purity >98%) in DMSO was tested in the Ames test according to OECD guideline 471 using seven strains (*Salmonella typhimurium* TA 1535, TA 1537, TA 97, TA 98, TA 100, TA 102 and *Escherichia coli* WP2 uvrA) and appropriate positive controls. Two versions of the Ames test, the standard plate incorporation method and the preincubation method, were used. In both procedures the substance was tested in absence and presence of S9 mix (from phenobarbital/β-naphthoflavone treated rats) at doses of 8, 40, 200, 1000 and 5000 µg/plate. No significant cytotoxicity was seen at any dose but at the three highest doses signs of limit of solubility were seen (milky appearance). No significant increase in the number of revertant colonies was observed for any of the seven test strains with AHTN by either procedure. All positive controls gave acceptable responses (Gocke, 1993; Api and San, 1999).

An Ames test (standard plate incorporation assay) was conducted with AHTN (non-GLP; purity unknown) using *Salmonella typhimurium* strains TA97, TA98, TA100 and TA102 with and without rat liver S-9 (Aroclor 1254-induced) metabolic activation and with appropriate positive controls. The method used resembled OECD guideline 471. The vehicle was DMSO. The doses were 5, 16.6, 50, 166.6 or 500 µg/plate (limit of solubility). All positive controls significantly increased the number of revertants. No significant increase in revertants was seen with AHTN at any dose with or without activation (Mersch–Sundermann et al., 1998a).

A cytogenetic assay with Chinese Hamster ovary (CHO-K_1) cells was conducted with AHTN (GLP; in acetone; purity > 98%) according to OECD Guideline 473 at concentrations of 5.9, 11.7 or 23.4 µg/ml in the non-activated study, using 4, 20 and 44 hr exposure periods. In the S-9 (from rat liver induced by Aroclor 1254) activated study, dose levels of 17.8, 20 and 25 µg/ml were tested for the 4-hr exposure period with a 20-hr harvest time and at dose levels of 3.9, 7.8 and 15.6 µg/ml for the 4-hr exposure period with a 44-hr harvest time. At the 4, 20 and 44-hr harvest times; the cells were assessed for structural chromosome aberrations, and at the 44-hr harvest time also for numerical chromosome aberrations. N-methyl-N'-nitro-N-nitrosoguanidine was used as a positive control in the non-activated study and benzo(a)pyrene in the activated study. The mitotic index was significantly lowered at the highest dose in all cases. Positive controls caused increases in structural (significant) and numerical aberrations (significant in the case of B(a)P) in all cases. No significant increases in structural or numerical chromosome aberrations were observed for AHTN without metabolic activation at any dose. With metabolic activation, AHTN induced statistically significant increases in structural aberrations at all doses at the 20-hr harvest time (not dose-related and with significant cytotoxicity at all doses), as well as a statistically significant increase in numerical aberrations at the highest dose at the 44-hr harvest time. The latter increase was, however, still

within historical control range. There was no dose response observed in this portion of the assay. Hence, the authors concluded AHTN to be negative for structural and numerical chromosome aberrations in this test (Curry, 1995; Api and San, 1999).

Steinberg et al. (1999) studied the ability of AHTN (non-GLP; purity 98%) to induce sister-chromatid exchanges (SCEs) in cultured human lymphocytes obtained from healthy non-smoking donors ranging in age from 25-30 years. Cultures were treated for 24 hr with concentrations of 2.5-40 µg/ml AHTN in DMSO with or without rat liver S9 (Aroclor 1254-induced) metabolic activation. The method used resembled OECD guideline 479. After harvest, the cells were scored for sister-chromatid exchanges. Cyclophosphamide (60 µg/ml) and ethyl methanesulfonate (120 µg/ml) were used as positive controls. Whereas the positive controls produced significant increases in sister-chromatid exchanges with (cyclophosphamide) and without (ethyl methanesulfonate) metabolic activation, AHTN up to 20 µg/ml caused no significant induction of sister-chromatid exchanges. Cytotoxicity was observed at 40 µg/ml AHTN.

The ability of AHTN (non-GLP; purity unknown) to induce SCEs was evaluated using cultured human lymphocytes obtained from healthy non-smoking donors ranging in age from 25-35 years. Cultures were treated for 24 hr with concentrations of 0.025, 0.25, 2.43, 24.25, 48.5 or 97 µM AHTN (in DMSO) with or without rat liver S9 (Aroclor 1254-induced) metabolic activation. The method used resembled OECD guideline 479. After harvest, the cells were scored for sister-chromatid exchanges. Cyclophosphamide (used as positive control) produced a significant increase in sister-chromatid exchanges. Concentrations of AHTN up to 48.5 µM caused no significant induction of sister-chromatid exchanges (97 µM was too cytotoxic to be evaluated) (Kevekordes et al., 1998).

An *in vitro* unscheduled DNA synthesis (UDS) assay in accordance with OECD guideline 482 was conducted with AHTN (GLP; purity >98%) in acetone in primary rat hepatocytes at concentrations of 0.15, 0.50, 1.5, 5.0 and 15 µg/ml (50-5000 µg/ml proved too toxic to be evaluated). The positive control (7,12-dimethylbenz(a)anthracene) induced a significant increase in the average net nuclear grain count over controls. No increase in net nuclear grain count was seen for AHTN up to and including 15 µg/ml although this dose did induce significant cytotoxicity (San and Sly, 1994; Api and San, 1999).

An *in vitro* micronucleus test was conducted with AHTN (non-GLP; AHTN; purity unknown) at concentrations of 0.05, 0.49, 4.85, 48.5, 97 or 194 µM using human peripheral lymphocyte cultures obtained from healthy non-smoking donors aged 25-35 years. After induction of mitosis, AHTN (in DMSO) was added to the cultures with and without rat liver S-9 (Aroclor 1254-induced) metabolic activation for 48 hr. After harvest, the cells were scored for micronuclei in binucleated cells. The positive controls (mitomycin –S9, cyclophosphamide +S9) significantly increased the frequency of micronuclei. No significant increase in the frequency of micronuclei was seen with AHTN at concentrations up to 97 µM (194 µM was too cytotoxic to score) (Kevekordes et al., 1997).

Another *in vitro* micronucleus test was conducted with AHTN (non-GLP; AHTN; purity unknown) at concentrations of 0.1, 0.97, 9.7, 97, 194 and 387 µM in DMSO using metabolically competent human hepatoma cells (Hep G2 line). After two hr incubation, the cells were harvested and scored for micronuclei in binucleated cells. The positive control (cyclophosphamide) significantly increased the frequency of micronuclei. No significant increase in the frequency of micronuclei was seen with AHTN at concentrations up to 194 µM (387 µM was too toxic to score) (Kevekordes et al., 1997).

An SOS chromotest was conducted by incubating *Escherichia coli* PQ37 *sfiA::lacZ* with AHTN (non-GLP; AHTN; purity unknown) in DMSO at concentrations of 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25 or 50 (limit of solubility in this assay) µg/assay with and without rat liver S-9 (Aroclor 1254-induced) metabolic activation. 4-Nitroquinoline-N-oxide (-S9) and benzo[a]pyrene (+S9) were used as positive controls. After 2 hr incubation, the enzyme activities of β-galactosidase and alkaline phosphatase were measured. Inducing factors (IF) were calculated relative to negative controls (solvent only). Both positive controls significantly increased IF but no inducing potency nor toxicity was seen with AHTN at any dose (Mersch-Sundermann et al., 1998b).

4.1.2.7.2 Studies in vivo

In a micronucleus test according to OECD guideline 474, groups of 5 male (weight 28-37 g) and 5 female (weight 24.5-31 g) ICR mice were dosed with 400, 800, or 1600 mg/kg bw AHTN (GLP; in corn oil; purity >98%) by intraperitoneal injection at a constant volume of 20 ml/kg bw. The high dose was selected to be 80% of the estimated intraperitoneal LD_{50} (see section 4.1.2.2.1). The positive control was cyclophosphamide. Bone marrow was harvested at 24, 48 and 72 hr after dosing and examined for micronucleated polychromatic erythrocytes (PCE). No mortality occurred. Clinical signs consisted of lethargy at all dose levels and diarrhoea at 1600 mg/kg bw. Moderate reductions (up to 23%) in the ratio of PCE to total erythrocytes were observed in some treated groups suggesting toxicity and bioavailability to the bone marrow target. The positive control induced a significant increase in micronucleated PCE in both male and female mice at 24 hr (the only harvest time for this group). No significant increase in micronucleated PCE in AHTN-treated groups relative to the respective vehicle control group was observed in male or female mice at 24, 48 or 72 hr after dose administration. (Gudi and Ritter, 1997; Api and San, 1999).

4.1.2.7.3 Summary of mutagenicity

AHTN has been tested in a wide array of *in vitro* tests and in an *in vivo* mouse micronucleus test. *In vitro*, AHTN was negative in gene mutation tests with bacteria, in an SOS chromotest with bacteria, in SCE and micronucleus tests with human cells and in an UDS test with primary rat hepatocytes. Equivocal results were obtained for AHTN in one *in vitro* chromosome aberration test in CHO cells. However, AHTN did not induce chromosome aberrations in the *in vivo* micronucleus test. Hence, it can be concluded that AHTN is a non-genotoxic substance.

4.1.2.8 Carcinogenicity

There are no specific tests for carcinogenicity available.

4.1.2.8.1 Studies in animals

In vivo studies

Only an oral test on liver tumour initiating and promoting activity in rats exposed to human-relevant doses of AHTN is available (see below).

Inhalation

No data available

Dermal

No data available

Oral

ATHN has been tested (non-GLP; purity >98%) for liver tumour initiating and promoting activity in rats exposed to human-relevant doses. Female and male juvenile Wistar rats (5 weeks old at start) were exposed to AHTN (300 µg/kg bw day) dissolved in isopropyl myristate alone or to single i.p. dose of diethylnitrosamine (DEN) (100 mg/kg bw day) for 90 days. Thereafter the liver architecture as well as the presence of placental glutathione S-transferase (GST-P)-positive hepatic lesions was assessed. In male animals receiving AHTN alone or in combination with DEN the number of GST-P-positive single hepatocytes was similar to that of solvent-control treated rats, while GST-P-positive mini-foci and foci were not observed. In the case of female rats the number of GST-P-positive single hepatocytes and mini-foci in AHTN-treated rats was similar to that in untreated animals, whereas in those animals receiving AHTN either alone or in combination with DEN, GST-P-positive foci could not be detected or were present in a number as similar to that in untreated rats. In conclusion it has been shown that AHTN administered over a 90-day period in concentrations similar to those taken up daily by humans does not lead to hepatotoxicity. (Steinberg et al., 2001).

In vitro studies

No data available

4.1.2.8.2 Human data

No data available.

4.1.2.8.3 Summary of carcinogenicity

There are no carcinogenicity data available. AHTN is demonstrated to be not genotoxic. There are no indications from repeated dose toxicity studies, which could be used to judge the carcinogenic potential. It has been shown that AHTN has no liver tumour initiating and promoting activity in rats exposed to human-relevant doses.

No further testing is needed.

4.1.2.9 Toxicity for reproduction

4.1.2.9.1 Effects on fertility

Studies in animals

No multi-generation study is available.

No effect on reproductive organs was found in the 13-week oral study, after administration of doses of up to 50 mg/kg bw/day (NOAEL ≥50 mg/kg bw/day) to female and male rats, see 4.1.2.6 (Lambert and Hopkins, 1996). In a peri/postnatal study no effect on reproduction performance was found (see section 4.1.2.9.2; Jones et al., 1996; Ford and Bottomley, 1997).

Human data

No studies available

4.1.2.9.2 Developmental toxicity

Studies in animals

A GLP compliant dosage-range finding study was conducted to provide information for the selection of dosages to be used in the developmental study. In this study, groups of 8 pregnant Sprague-Dawley rats were administered AHTN (purity >98%) in corn oil by gavage (5 ml/kg bw) at doses of 10, 25, 50 or 100 mg/kg bw on days 7 through 17 of pregnancy. The control group consisted of 19 animals and received corn oil only. One rat in the high dose group was sacrificed in moribund condition on day 19. All other animals were sacrificed at day 20 and Caesarean-sectioned (including the one intercurrent death) and were studied for gross pathology. Uteri were opened and foetal body weights and gross external alterations were recorded. Foetuses with anomalies were fixed in Bouin's solution. In the high dose group decreased motor activity, chromorhinorrhea, perioral substance, excessive salvation, dehydration, emaciation, perivaginal substance alopecia and urine stained fur were observed. All of the animals in the highest dose group had green livers, 3 had small spleens and 2 had green amniotic sacs for all foetuses. In the other dose-groups no treatment related necropsy findings were recorded.

The animals in the 50 mg/kg group had decreased body weight gain and in the 100 mg/kg group weight loss was recorded. Reduced weight gains were also seen at 25 mg/kg bw during days 7-10 and 12-15. Feed intake was reduced at 50 and 100 mg/kg bw, but not in the other dose groups. No litter parameters or external alterations were seen at 50 mg/kg bw or lower. Three foetuses from two litters in the high dose group had whole body oedema. Based on these findings, doses of 5, 15 and 50 mg/kg bw were chosen for the main study (Christian et al., 1997, 1999).

In a GLP compliant teratogenicity study, AHTN (purity >98%) in corn oil was administered by gavage to groups of 25 female Sprague–Dawley rats on days 7 through 17 of presumed gestation at dosages of 0, 5, 15 and 50 mg/kg bw/day. The dams were observed for signs of toxicity and body weights and feed intake were recorded. On day 20 of gestation, the dams were sacrificed and gross necropsy was performed. The number of corpora lutea in the ovaries

were recorded and the uteri were examined for pregnancy, number and distribution of implantations, live and dead foetuses and early and late resorptions. The placenta was also examined. All foetuses were weighed and examined for sex and gross external abnormalities. One half of the foetuses in each litter were examined for soft tissue alterations. The remaining foetuses were examined for skeletal alterations.

In the 50 mg/kg bw/day dose group a statistically significant number of rats had localised alopecia and colour changes in the liver (green or mottled green and dark red). The 50 mg/kg bw/day group had an initial weight loss followed by a significant reduction in maternal bodyweight gains of 23.5% accompanied by a decreased food consumption of 23%, both over the period from GD0 to GD20.

The 5 mg/kg and the 15 mg/kg dose group animals had a transient reduced body weight gain during the first days of the dosing period, but at the end of the study maternal body weights in these two groups were approximately similar to those from the control animals. Feed intake was significantly reduced at 15 mg/kg bw with 8% over GD0 to GD20. There were no significant differences seen on clinical examination or at necropsy between the two lower dose groups and controls. Because there were no other signs of adverse effects at the 15 mg/kg bw level, this level can be considered the maternal NOAEL in this study.

Average foetal body weights were about 5% lower in the low dose group, about 3.5% lower in the mid dose group and about 6.5% in the high dose groups. Statistical significance was reached in the low and high dose groups but not in the mid-dose. The decreases in bodyweight were well within historical ranges and because of no clear dose relationship they were not considered relevant. No effects were observed on numbers of implantations, dead or live foetuses, resorptions or foetal sex ratios.

There were no skeletal changes and all gross external, soft tissue or skeletal malformations in the foetuses were incidental and considered unrelated to the test article. Retardations in ossification were observed in foetuses from all four groups (control and dosed groups). Only for the numbers of caudal and sternal centres of ossification statistically significant decreases were observed. The ossification sites per fetus per litter of caudal vertebrea was decreased from 5.1 (control) to 4.6 (5 mg/kg bw/day), 4.8 (15 mg/kg bw/day) and 4.7 (50 mg/kg bw/day). The sites in the sternal centers were decreased from 3.6 (control) to 3.0, 3.4 and 3.4 respectively, for the treated groups. These can be considered to be 1) related to slightly decreased foetal body weights, 2) not specific and 3) not of biological significance. Therefore, in this study no evidence for developmental toxicity was obtained, despite maternal exposure up to toxic levels and the developmental NOAEL is 50 mg/kg bw/day, the highest dose administered (Christian et al., 1997, 1999).

In a study designed to determine the effects of AHTN on the neonate when exposed through nursing, AHTN (GLP; purity 99.3%) was administered at dosages of 0, 2, 6 or 20 mg/kg bw/day once daily by gavage in corn oil to groups of 28 time-mated rats (Crl:CD BR VAF/Plus strain) from Day 14 of pregnancy (end of organogenesis) through to weaning on Day 21 post partum. The females were allowed to litter and rear their young to weaning (litters were standardised to 8 pups on Day 4 post partum). From these litters, selected offsprings were retained (24 males and females per group) to maturity and assessed for behavioural changes and reproductive capacity. The F1 generation was only exposed to AHTN *in utero* during the perinatal phase and through transfer in the milk of the lactating dams. The exposure of the F1 foetuses through mother's milk can be estimated based on a pharmacokinetic study in pregnant/lactating rats (Hawkins et al. (1996) reported in section

4.1.2.1.1, under heading: Animal milk studies). AHTN and its metabolites can be found in the milk of the rats at levels of ca. 1 µg equivalents/ml after daily dosing of 2 mg/kg bw/day and ca. 10 µg equivalents /ml at 20 mg/kg bw/day.

After parturition, the young were counted, sexed, weighed and examined for external abnormalities. On day 4 *post partum* the pups were weighed and all litters containing more than 8 pups were culled to 8 retaining, where possible 4 males and 4 females. During the pre-weaning period, all pups were examined to determine the age of reaching certain developmental stages: by examining surface righting reflex, startle reflex, air righting reflex and pupil reflex. This F1 generation was also evaluated for behavioural effects by examining changes in motor coordination and balance, activity and avoidance. When the F1 generation reached approximately 84 days of age (having been continuously observed for signs of adverse health) they were mated one male to one female avoiding brother-sister pairings. The females were examined before and after mating to determine time of pregnancy, marked anomalies of the oestrous cycle, median pre-coital time, whether pregnancy had occurred and terminated and duration of pregnancy.

The offspring (F2 generation) were examined for abnormalities at parturition and periodically until day 21 *post partum* at which time the study was terminated.

There were no effects of treatment in any of the treated parent females during pregnancy or lactation. No effects were apparent on development of the F1 generation during the late pre-natal phase, or on postnatal growth, no changes in post weaning behavioural tests or mating performance were seen and post mortem examination of F1 males and females, reproductive capacity, litter data and macroscopic post mortem examination of F2 pups did not reveal abnormalities. There were no adverse effects to the dams or offspring up to and including the highest dose level (20 mg/kg bw/day). This study was conducted in accordance with GLP and based on the guidelines endorsed by the ICH Steering Committee on the Detection of Toxicity to Reproduction for Medicinal Products (Jones et al., 1996; Ford and Bottomley, 1997).

It should be noted that this dose cannot be considered a NOAEL for the purpose of risk characterisation for the evaluation of neonatal exposure via milk, since it is the dose received by the dams and the study was designed to detect adverse effects on the pups. However, the top dose from this study can be used to assess the risk for pup weight, pup survival and postnatal death in neonates resulting from maternal exposure. Since no adverse effects were detected, no LOAEL can be established, and the NOAEL is higher than or equal to the highest dose tested, thus ≥20 mg/kg bw/day.

Human data

No studies are available

4.1.2.9.3 Endocrine reactions

In vitro studies

In a non-GLP study, AHTN (purity; >98%) in ethanol was added to transiently human estrogen receptor (ER) α- or ERβ- transfected human embryonal kidney (HEK293) cells for 24 hr. AHTN weakly stimulated the transcriptional activities (about 6 orders of magnitude less than estradiol) with ERα- but not with ERβ-transfected cells (Seinen et al., 1999).

In a non-GLP study, AHTN (purity unknown) in ethanol (10 mmole/L) was added to estrogen receptor-positive human mammary carcinoma cells (MCF-7) and incubated for 6 days according to the method for the E-screen assay of Soto et al. (1995). It was tested at 7 different concentrations, the highest being 10 µmole/L with a solvent concentration of 0.1% at the highest. The rate of proliferation of the cells was compared to that of a hormone-free control sample as determined by photometric analysis of the total protein content of the fixed cells. The relative rate of proliferation (test substance relative to control) was then compared to that of 17β-estradiol. AHTN showed a higher and statistically significant rate of proliferation relative to the hormone-free control (ratio 1.53). This increase was 1 x 10^{-5} that of 17β-estradiol (Bitsch et al., 2002).

In a non GLP-study, AHTN was again tested in human embryonal kidney 293 (HEK293) and human osteoblastic (U2-OS) cells which were stably transfected with plasmids containing the human ER isoforms α and β and transiently transfected with an estrogen responsive reporter gene construct (ERE-luciferase). In both cell types only the highest AHTN concentration (10 µM) was able to only marginally activate the transcripton of hERα and not hERβ. AHTN in 0.01, 1 and 10 µM concentrations did repress the 17β-estradiol induced transcription in both cell types and with both receptor isoforms dose-dependently (Schreurs et al., 2002).

Human embryonal kidney 293 (HEK293) cells were transient transfected with plasmids containing the human and zebrafish estrogen receptor isoforms (hERα and hER β, and zfERα, β and γ) and an estrogen responsive reporter gene construct (ERE-luciferase). AHTN was tested up to 10 µM. AHTN alone showed a marginal transcriptional activation of hERα at the highest test concentration. The other receptors were not affected at all. The anti-estrogenic activity was tested using a submaximal dose of 0.01 nM estradiol (E2) for hERα, 0.1 nM E2 for hERβ and zfERγ and 1 nM E2 for zfERα and zfERβ, together with 0.1, 1 or 10 µM AHTN. A dose-dependent suppression by AHTN of E2 induction was shown toward hERβ and zfERγ. A weak antagonistic effect could be observed on hERα and zfERβ only at the highest test concentration of 10 µM, whereas no effect was seen at zfERα (Schreurs et al., 2004).

In a non GLP-study, AHTN was tested in human osteoblastic (U2-OS) cells, which were stably transfected with either the androgen receptor (AR) or the progesterone receptor (PR), and a sensitive reporter gene construct (3xARE-TATA-luciferase). Towards the AR, AHTN at concentrations of 1 and 10 µM could dose-dependently repress the transcriptional activity induced by 0.1 nM dihydrotestosterone to a maximum of 30% at the highest dose. Towards the PR, AHTN at concentrations of 0.01, 0.1, 1 and 10 µM could dose-dependently repress the transcriptional activity of 30 pM ORG2058, a stable PR agonist. AHTN did not show any agonistic effects towards the AR or PR in this cell line (Schreurs et al., 2005a).

In a non GLP-study, AHTN was tested in human embryonal kidney 293 (HEK293) cells stably transfected with either hERα or hERβ, and a sensitive estrogen responsive reporter gene construct (3xERE-TATA-luciferase). Furthermore, AHTN was tested in human osteoblastic (U2-OS) cells stably transfected with hAR and an androgen responsive reporter gene construct (3xARE-TATA-luciferase). As seen in previous studies of the same research group, AHTN showed dose-dependent antagonism towards hERβ only, with an IC50 value of 1.9 µM. Towards the hAR, AHTN showed dose-dependent antagonism with an IC50 value of 3.6 µM. No agonistic or antagonistic effects were observed towards the aryl hydrocarbon receptor (dioxin receptor) (Schreurs *et al.*, 2005b).

In vivo studies

In a non-GLP study, weanling (21 days old) female Balb/c mice (6 per dose group) were maintained on a diet containing 10 or 50 mg AHTN/kg (purity not specified) for 2 weeks. This resulted in mean daily intakes of about 2 or 6.5 mg/kg bw. At the end of 2 weeks, the mice were sacrificed and uterus, thymus, liver and bodyweights were recorded. Positive control mice were injected with 17β-estradiol (0.14 mg) on days 1, 5, 9 and 12 of the study. Possible uterotrophic activity of AHTN was assessed according to the protocol of Thigpen et al. (1987)(as referenced in Seinen et al., 1999), a standardised method to demonstrate estrogenic activity. The estradiol treated mice had significantly increased uterine weights and decreased thymus weights, but no effect on liver weight. AHTN had no significant effects on either organ, but it did cause an increase in absolute liver weight (Seinen et al., 1999).

This same group also investigated the possible anti-estrogenic effects in zebrafish in vivo (Schreurs et al., 2004). Transgenic zebrafish, containing a similar reporter gene construct as used in the above mentioned in vitro experiments, were exposed to AHTN with and without E2. AHTN at a concentration of 10 μM was toxic to the fish. AHTN did not show any estrogenic effect with the tested concentrations of 0.01, 0.1 and 1 μM. The concentrations of 0.1 and 1 μM resulted in a dose-dependent antagonistic effect on E2 in the juvenile zebrafish.

4.1.2.9.4 Summary of toxicity for reproduction

No multigeneration study is available.

In an oral peri/postnatal toxicity study (exposure of the F_1-generation to AHTN was only *in utero* during the perinatal phase or through any transfer in the milk of the lactating dams) no toxicity was seen at dose levels of 2, 6 or 20 mg/kg bw/day in the dams or their F1 and F2 offspring. The exposure of F1 foetuses through mother's milk can be estimated based on a pharmacokinetic study with pregnant/lactating rats given oral doses of 2 and 20 mg 14C-AHTN/kg bw/d. Levels up to 1.89 and 25 mg AHTN equivalents (i.e. AHTN + metabolites)/l of whole milk were reported, for maternal oral doses of 2 and 20 mg/kg bw/d, respectively (see **Table 4.17**).

In an oral developmental study with rats, maternal toxicity occurred at 50 mg/kg bw/day. Developmental toxicity was not seen at the highest dose administered, 50 mg/kg bw/day. Therefore, the NOAEL for maternal toxicity can be established at 15 mg/kg bw/day. There is no evidence for developmental toxicity and the developmental NOAEL is ≥50 mg/kg bw/day, the highest dose administered. From the peri/postnatal study described above, a NOAEL of ≥20 mg/kg bw/day can be established for pup weight, pup survival and postnatal death, the highest dose tested. These effects are not included in the oral teratogenicity study. Since this NOAEL is also lower than the NOAEL for teratogenic effects generated during earlier periods of foetal development (50 mg/kg bw/day; see above), this NOAEL (≥20 mg/kg bw/day) will cover also these early teratogenic events. A NOAEL for developmental toxicity of ≥20 mg/kg bw/day will be taken forward to the risk characterization.

No effects on reproductive organs of male or female rats were seen in a 13-week oral study at doses up to 50 mg/kg bw/day (NOAEL ≥50 mg/kg bw/day).

AHTN has a very weak estrogenic and anti-estrogenic potency *in vitro*, dependent on the estrogen receptor type. Marginal repressing effects were also found *in vitro* on the androgen

and progesterone receptor. However, *in vivo* no estrogenic effects were seen in the uterotrophic assay.

4.1.3 Risk characterisation

4.1.3.1 General aspects

There are no data available on the toxicokinetics of AHTN after inhalation exposure. For inhalation exposure, an assumption of 100% absorption as a worst-case will be used in the risk characterization. The latter assumption would probably overestimate exposure from dust because absorption in the lung is likely to occur only for dissolved AHTN and AHTN is poorly soluble in water (1.25 mg/L).

The available oral absorption data do not allow establishment of an exact absorption percentage. Taking into account physico-chemical properties neither no nor complete oral absorption is likely. Hence, an intermediate default percentage of 50% for oral absorption is taken forward to the risk characterisation. As a support, based on urine, cage washing and tissue levels in the study by Wu (2002), absorption of at least 50% can be concluded.

Route-to-route extrapolation introduces an additional uncertainty, not taking into account first pass metabolism.

An *in vitro* dermal absorption study with ^{14}C-ring-labelled AHTN using human epidermal membranes indicated that 4.1% of the applied dose is absorbed over 24-hr. This figure is taken forward to the risk characterisation. This is considered to be a worst-case assumption, even for damaged skin, because the *in vivo* data indicate a lower dermal absorption in humans.

Intravenous administration of AHTN to rats and the pig results in rapid distribution. Excretion in the rat is primarily via the faeces as was seen in the dermal study (~ 76% of total excretion compared to ~84% after dermal exposure) but in the pig the principle route of excretion is via urine similar to what was seen in the human study. In neither of these studies, any evidence of accumulation was seen. However, clearance from the fat was slower than from other organs. It is noteworthy that in the intravenous studies, no unmetabolised AHTN is present in the urinary radioactivity. This means that all AHTN present in urine is metabolised (for rat 21.5%, and for pig 86.2%). The faeces (the major excretion route of the rat) were not analysed for metabolites or parent.

AHTN is found in human milk in several studies, ranging from undetectable levels up till 565 µAHTN /kg milk fat. Values for risk characterization were chosen from the recent study from the Czech Republic (Hasjlova and Setkova, 2004) with 59 mothers where the highest mean (112 µg AHTN/kg fat) and maximum level (565 µg AHTN/kg milk fat) were found.

In summary, for the purpose of risk characterization, 50% absorption for oral exposure and 100% for inhalation will be used. For dermal absorption of AHTN in rats and humans, values of 20% and 4.1% respectively, are taken forward to the risk characterisation.

The data provided are considered sufficient to meet base set requirements for acute toxicity. Based on the oral LD_{50} values of 570-1377 mg/kg bw, AHTN should be classified as harmful

if swallowed (Xn R 22). The dermal LD_{50}-values are >5000 mg/kg bw, so there is no need to classify AHTN for acute dermal toxicity.

Data for acute inhalation toxicity are not available.

AHTN has been tested in two dermal irritation studies in animals. In one study, no dermal effects were observed. In the other study as a 50% solution in diethyl phthalate (DEP), slight dermal irritation was observed with the solution as well as DEP although the score for DEP was less. Based on recommended studies for hazard classification in rabbits, with the undiluted substance, AHTN does not need to be classified as a skin irritant. Dermal effects observed after topical application of AHTN in repeated dose toxicity studies may reflect (photo)-sensitisation, rather than irritation. Several sensitisation studies in humans showed no signs of dermal irritation by AHTN.

The photoirritation studies in animals indicate that AHTN is more irritating to the skin after irradiation with UV light. The results in human tests do not indicate a photoirritating effect in humans. Also, an *in vitro* phototoxicity test (in compliance with test guideline B.41 (EU/COLIPA Test)) was negative. No criteria for the classification of substances for photoirritation are available in Annex VI of Directive 67/548.

AHTN has been tested for ocular irritation in rabbits in two studies. In both studies, slight ocular irritation was observed. However, the magnitude of the effects is not high enough to require classification according to the EU guidelines.

No data on respiratory tract irritation are available.

The available data include sensitisation and photosensitisation studies in both animals and humans. The sensitisation studies with animals indicate some potential for sensitisation but the studies are only limitedly reported and were not done according to guidelines. Sensitisation studies in humans were negative.

In animal studies investigating photosensitising effects, mostly positive results were reported for AHTN, whereas negative results were reported in human studies on photosensitisation. It is to be noted that for this endpoint there is no validated test method available. The negative human data do not overrule the positive animal data, in line with 3.1.1 of Annex VI of Directive 67/548, which states that tests on man (human volunteers) should be discouraged and should not normally be used to negate positive animal data. Hence, it is concluded that AHTN is a photosensitiser. This may be due to photosensitising effects of AHTN itself, or to sensitising effects from photodegradation products arising from the interaction of AHTN and UV light. Evidence for the latter was obtained from a study where two of the four photodegradation products of AHTN reacted positive. This phenomenon may also explain the dermal effects observed in a dermal repeated dose toxicity study (see below).

In the absence of criteria for classification for photosensitisation in Annex VI of Directive 67/548, the need to communicate the photosensitising potential of AHTN to users is dealt with by way of additional safety phrases and a Note, rather than applying R43. However, in the October 2006 meeting of the TC-C&L, the Commission stated that such a note will not be developed under the current legislation. .

In a 28-day oral gavage study, no effects of AHTN were seen at doses up to and including 10 mg/kg bw/day.

In an adequate 90-day oral study, clear mild haematological effects were seen at the highest dose administered, 50 mg/kg bw/day. These effects may be associated with observations of dark discolouration of the liver and mesenteric lymph nodes seen in most high dose animals but not in animals at lower doses. Observations in animals maintained on a treatment-free regime for 28 days following the 90-day treatment period indicate that the effects are reversible. Although the differences from controls were small and generally within historical ranges seen for rats in this laboratory, the overall pattern is such that it cannot be excluded that these effects are of adverse nature. At the lower doses, some statistically significant differences from controls in blood biochemistry and haematology were found, but these differences were small and within the values for historical controls. Some of these, however, showed a dose-response relationship at 15 and 50 mg/kg bw/day. The green colouration of the lachrymal gland was clearly dose-related but not associated with any histopathology at any dose in any animal. The most likely explanation for this observation is accumulation of a pigment resulting from reaction of a photo-oxidation product of AHTN with proteins, and this finding, albeit undesirable, is not considered an adverse effect. Based on the marginal effects observed at 15 mg/kg bw/day, the NOAEL is set at 5 mg/kg bw/day, which will be used in the risk characterization.

Three subchronic dermal studies of AHTN are available. In two of these, 13-weeks at 1, 10 and 100 mg/kg bw/day and 26-weeks at 0, 9, 18 and 36 mg/kg bw/day both applied unoccluded, the purpose was to screen AHTN for neurotoxicity against AETT as positive control. While clear evidence of neurotoxicity, both clinically and pathologically, was seen with the positive control AETT no such evidence for AHTN was found in either study at any dose level. Clear evidence of haematological and hepatotoxicity were seen at 100 mg/kg bw/day for 13 weeks and at 36 mg/kg bw/day for 26 weeks, however, because of the limited nature of the report, it is not possible to judge the severity of these effects. In the third study, AHTN was included only for comparison purposes at one dose level, which proved to be so irritating (possibly resulting from (photo)sensitisation) that the results with respect to systemic effects were confounded. In none of these studies is it possible to determine the actual doses received and because of the lack of collars the real route of exposure and no NOAEL could be established. Therefore, these studies are not used in the risk characterisation.

In a sub-acute study with i.p. administration, AHTN did not show peroxisome proliferating and cytochrome P450 inducing properties.

Repeated dose toxicity studies after inhalation exposures were not available for AHTN.

AHTN has been tested in a wide array of *in vitro* tests and in an *in vivo* mouse micronucleus test. *In vitro*, AHTN was negative in gene mutation tests with bacteria, in an SOS chromotest with bacteria, in SCE and micronucleus tests with human cells and in an UDS test with primary rat hepatocytes. Equivocal results were obtained for AHTN in one *in vitro* chromosome aberration test in CHO cells. However, AHTN did not induce chromosome aberrations in the *in vivo* micronucleus test. Hence, it can be concluded that AHTN is a non-genotoxic substance.

There are no carcinogenicity data available. AHTN is demonstrated to be not genotoxic. There are no indications from repeated dose toxicity studies, which could be used to judge the carcinogenic potential. It has been shown that AHTN has no liver tumour initiating and promoting activity in rats exposed to human-relevant doses. No further testing is needed.

No multigeneration study is available.

In an oral peri/postnatal toxicity study (exposure of the F_1-generation to AHTN was only *in utero* during the perinatal phase or through transfer in the milk of the lactating dams) no toxicity was seen at dose levels of 2, 6 or 20 mg/kg bw/day in the dams or their F1 and F2 offspring. The exposure of F1 foetuses through mother's milk can be estimated based on a pharmacokinetic study with pregnant/lactating rats given oral doses of 2 and 20 mg 14C-AHTN/kg bw/day. Levels up to 1.89 and 25 mg AHTN equivalents (i.e. AHTN + metabolites)/l of whole milk were reported, for maternal oral doses of 2 and 20 mg/kg bw/d, respectively (see **Table 4.17**).

In an oral developmental study with rats, maternal toxicity occurred at 50 mg/kg bw/day. Developmental toxicity was not seen at the highest dose administered, 50 mg/kg bw/day. Therefore, the NOAEL for maternal toxicity can be established at 15 mg/kg bw/day. There is no evidence for developmental toxicity and the developmental NOAEL is ≥50 mg/kg bw/day, the highest dose administered. From the peri/postnatal study described above, a NOAEL of ≥20 mg/kg bw/day can be established for pup weight, pup survival and postnatal death, the highest dose tested. These effects are not included in the oral developmental study. Since this NOAEL is also lower than the NOAEL for teratogenic effects generated during earlier periods of foetal development (50 mg/kg bw/day; see above), this NOAEL (≥20 mg/kg bw/day) will cover also these early teratogenic events. A NOAEL for developmental toxicity of ≥20 mg/kg bw/day will be taken forward to the risk characterization.

No effects on reproductive organs of male or female rats were seen in a 13-week oral study at doses up to 50 mg/kg bw/day (NOAEL ≥50 mg/kg bw/day).

AHTN has a very weak estrogenic potency *in vitro* but no such effects were seen *in vivo.*

4.1.3.2 Workers

4.1.3.2.1 Introduction

Assuming that oral exposure is prevented by personal hygienic measures, the risk characterisation for workers is limited to the dermal and inhalation routes of exposure.

4.1.3.2.2 Comparison of exposure and effects

Acute toxicity

Dermal exposure

Given the dermal LD_{50} values of >5000 mg/kg bw and the highest anticipated exposure level of 39 mg/d (or 39 mg / 70 kg = 0.55 mg/kg bw) of a molten form, it is concluded that AHTN is of no concern for workers with regard to acute dermal effects **(conclusion ii)**.

Inhalation exposure

There are no data on acute inhalation toxicity. Therefore, short term inhalation exposure has to be compared to available acute toxicity data for other routes. For clarity, potential risks are assessed for vapour as well as dust. Given the oral LD_{50} values of 570-1377 mg/kg bw and the dermal LD_{50} values of >5000 mg/kg bw and the highest anticipated exposure of 0.1 mg/m^3 to

vapour in scenario 2 (Small size plants) during 0.15 hour per day, which is (0.25 h * $10m^3$ / 8h * 0.1 mg/m^3) / 70 kg = 0.0005 mg/kg bw, it is concluded that there are no indications for concern with respect to acute toxicity by inhalation exposure of vapour **(conclusion ii)**.

Given the highest exposure for dust of 0.6 mg/m^3 during 2 h/d, which is (2 h * $10m^3$ / 8h * 0.6 mg/m^3) / 70 kg = 0.021 mg/kg bw, in scenario 1 (for the incidental maintenance of the cooler) it is concluded that there are no indications for concern with respect to acute toxicity by inhalation exposure of dust **(conclusion ii).**

Irritation including photoirritation, and corrosivity

Acute dermal irritation

AHTN is not a skin irritant **(conclusion ii)**.

Corrosivity

AHTN is not corrosive to the skin **(conclusion ii).**

Photoirritation

AHTN is not a photoirritant **(conclusion ii).**

Dermal irritation after repeated exposure

AHTN is not a skin irritant after repeated exposure **(conclusion ii)**.

Eye irritation

AHTN is not an eye irritant **(conclusion ii).**

Respiratory irritation

No data are available on effects on the local respiratory tract after acute exposure. Given the lack of skin and eye irritation potential, no significant respiratory irritation potential is expected **(conclusion ii).**

Sensitisation including photosensitisation

Sensitisation

AHTN is not a sensitiser (**conclusion ii**).

Photosensitisation

In animal studies a photosensitising potential was observed. In the four exposure scenarios workers may be exposed dermally to the following percentages of AHTN (see **Table 4.2**):

- Scenario 1: 100% AHTN (crystallisation);
- Scenario 2: 2% AHTN (compounding – all size plants – liquid); 100% (compounding – all size plants – dust); 100% (compounding – molten form);
- Scenario 3: 2% AHTN (handling); 0.002% AHTN (clearing and maintenance);
- Scenario 4: 0.02% AHTN (handling).

Based on the available animal data (photosensitisation was observed from concentrations of 1% AHTN (lowest concentration tested)) and on the % AHTN in the exposure scenarios, it cannot be excluded that photosensitising effects may occur in scenarios 1, 2 and 3 (handling) (**conclusion iii**).

The concern for photosensitisation for workers in scenario 3 (cleaning and maintenance) and scenario 4 is considered low, because the exposure concentration of AHTN in these scenarios (<1%) is below the general concentration limit normally applied for classification of preparations for sensitisation. Besides, negative results have been reported in human studies on photosensitisation with 1%, 5% and 10% AHTN preparations (**conclusion ii**).

In the absence of a risk phrase for photosensitisation, a specific Note can be used to warn workers for the photosensitising potential of AHTN. However, at the October 2006 meeting of the TC-C&L the Commission stated that such a Note will not be developed under the current legislation. If a specific Note to warn workers for the photosensitising potential of AHTN will be available, conclusion ii may be applicable for all scenarios.

Repeated dose toxicity

With regard to conclusions regarding the risk characterisation for local effects after repeated exposure to AHTN it is referred to the sections 'Irritation including photoirritation, corrosivity' and 'Sensitisation including photosensitisation'.

Unlike local effects, systemic effects depend on the internal body burden. Doses or exposures at different routes of application may be converted to an internal body burden (IBB) by taking into account the different absorption factors. NOAELs are not available for dermal repeated dose toxicity or for toxicity upon repeated doses via inhalation. The starting point for the risk assessment is the oral NOAEL of 5 mg/kg bw/day from the oral 90-day repeated dose study with rats. Assuming an oral absorption value of 50% for rats, this NOAEL corresponds to an internal no-effect dose of 2.5 mg/kg bw/day. For exposure after inhalation no data are available, the absorption is assumed to be 100 %. Although it is recognized that quite different dermal exposure conditions exist between the different scenarios, e.g. in terms of exposure times and area doses, a value of 4.1% is taken for dermal absorption in all worker scenarios.

Dermal exposure

The (highest) dermal exposure estimates from **Table 4.2** are taken forward in the risk characterisation.

The values of the MOS between the dermal exposure levels and the internal no-effect dose for repeated dose toxicity are mentioned in **Table 4.35.**

Table 4.35 Occupational risk assessment of dermal exposure to AHTN for repeated dose toxicity

Workers scenario	**Liquid/Dust** **Highest exposure of both**	**Internal body burden** [A] **(mg/kg bw/d)**	**MOS** [B]	**Conclusion** [C]
Scenario 1				
- crystallisation	5.5 mg (420 cm^2)	0.0032	781	ii
- maintenance of cooler	Negligible / No data		-	ii

Workers scenario	Liquid/Dust Highest exposure of both	Internal body burden [A] (mg/kg bw/d)	MOS [B]	Conclusion [C]
Scenario 2				
- delivery	Negligible		-	ii
- compounding All size plants	6.5 mg (420 cm^2) (sum of liquid and dust)	0.0038	658	ii
- compounding (molten form)	39 mg/day (100 cm^2)	0.023	109	ii
- analysis	Negligible		-	ii
- odour control	Negligible		-	ii
Scenario 3				
- handling	0.85 mg/day (420 cm^2)	5×10^{-4}	5,000	ii
- cleaning & maintenance	0.04 mg/day (1300 cm^2)	$2,3 \times 10^{-5}$	109,000	ii
Scenario 4				
- handling	0.16 mg/day (840 cm^2)	9.4×10^{-5}	26,500	ii

A Taking into account a dermal absorption of 4.1% and a body weight of 70 kg

B Based on comparison of internal body burden to an internal no-effect dose of 2.5 mg/kg bw/d

C Conclusion is reached considering the magnitude of the MOS in comparison with a minimal MOS of 100 (which is based upon the default values for assessment factors as specified in the draft version of the TGD (2005): an interspecies factor of 10 (4 for metabolic size differences * 2.5 for sensitivity differences), an intraspecies factor of 5 and a factor of 2 for semichronic to chronic exposure extrapolation).

Table 4.35 shows that for all scenarios the MOS is above the minimal MOS of 100, so **conclusion ii** applies.

Inhalation exposure

Scenario 1

The inhalation exposure estimates from **Table 4.2** are taken forward in the risk characterisation.

Inhalation exposures at production and cleaning are considered negligible (not depicted in the table).

At the crystallisation exposure for 4 hours with inhalation of dust that contains 0.1 mg AHTN/m^3 gives an inhaled amount of 0.5 mg/day (0.1 mg/m^3 x 1.25 m^3/h x 4 h). The internal body burden is 0.007 mg/kg bw/day.

During maintenance of the cooling belt and crystallisation system the inhalation of dust with 0.600 mg AHTN/m^3 for two hours gives a internal dose of 0.02 mg/kg bw. This exposure is incidental.

Scenario 2

The daily absorption due to inhalation of vapour and dust in scenario 2 can be based on the 8 hours inhalation volume of 10 m^3 at 0.013 mg/m^3 and 100 % absorption. This gives a daily dose of 0.13 mg or 0.0019 mg/kg bw/day for large and medium size plants.

For small size plants the ventilation may be reduced and the conservative estimate gives 0.0093 mg/kg bw/day.

The short-term inhalation of air with vapour and dust containing 0.023 mg AHTN/m^3 (large & medium size plants) or 0.1 mg AHTN/m^3 (small size plants) during 15 minutes/day in scenario 2 (compounding) gives an inhalation of 0.0072 and 0.031 mg/day, respectively. The daily internal body burden is 0.0001 and 0.00045 mg/kg bw/day, respectively. However, these exposures are incidental.

The values of the MOS between the inhalation exposure levels and the internal no-effect dose for repeated dose toxicity are mentioned in **Table 4.36.**

Table 4.36 Occupational risk assessment of inhalation exposure to AHTN for repeated dose toxicity.

Workers scenario	**Internal body burden (IBB) [A] (mg/kg bw/d)**			**MOS [B]**	**Conclusion [C]**
	Vapour	**Dust**	**Combined**		
Scenario 1					
- crystallisation	Negligible	0.007	0.007	357	ii
- maintenance of cooler	Negligible	only incidental	only incidental	-	-
Scenario 2					
- delivery	Negligible	Negligible	Negligible	-	-
- compounding					
large & medium size plants	0.0019		0.0019	1316	ii
small size plants	0.0093		0.0093	269	ii
compounding (molten form)					
- analysis	Negligible	Not applicable	Negligible	-	-
- odour control	Negligible	Not applicable	Negligible	-	-
Scenario 3					
- handling	Negligible	Not applicable	Negligible	-	-
-cleaning & maintenance	Negligible	Not applicable	Negligible	-	-
Scenario 4					
- handling	Negligible	Not applicable	Negligible	-	-

A Taking into account an inhalation absorption of 100%, a body weight of 70 kg and a respiratory volume of 1.25 m^3/h

B Based on comparison of internal body burden to an internal no-effect dose of 2.5 mg/kg bw/d

C Conclusion is reached considering the magnitude of the MOS in comparison with a minimal MOS of 100 (which is based upon the default values for assessment factors as specified in the draft version of the TGD (2005): an interspecies factor of 10 (4 for metabolic size differences * 2.5 for sensitivity differences), an intraspecies factor of 5 and a factor of 2 for semichronic to chronic exposure extrapolation.

From **Table 4.36** it can be concluded that the MOS values are higher than the minimal MOS (**conclusion ii**).

Combined exposure

The total body burden is determined by uptake after dermal as well as exposure by inhalation of AHTN. This combined exposure should not be applied if a simultaneous exposure can be excluded. As shown in **Table 4.2** in the exposure assessment chapter, combination of various exposure routes is only relevant for the crystallisation in scenario 1 (total IBB of 0.0102 mg/kg bw/d) and the compounding in scenario 2 (total IBB of 0.0057 and 0.0131 mg/kg bw/d for large & medium size plants and small size plants, respectively). The resulting MOS values are 245, 438, and 190 for crystallisation and compounding in large & medium size plants and small size plants, respectively. Comparing these MOS values with the minimal MOS value (100), **conclusion ii** is proposed for all three scenarios where combined (dermal and inhalation) exposure is relevant.

Mutagenicity

AHTN is a non-genotoxic substance **(conclusion ii).**

Carcinogenicity

ATHN lacks liver tumour initiating and promoting activity in rats when exposed to human-relevant doses. There are no other carcinogenicity data available. The mutagenicity data on AHTN do not indicate a concern with regard to carcinogenicity nor does AHTN possess any structural features that would raise a concern (**conclusion ii).**

Toxicity for reproduction

No multigeneration study is available. There are no indications for effects on fertility in the oral 90-day study with rats although in this study investigation was limited to histological examination of the reproductive organs and no adverse effects were reported up to the highest dose tested (the NOAEL was =50 mg/kg bw/day).

Reproduction toxicity studies by inhalation or dermal exposure are lacking.

In an oral developmental study with rats, maternal toxicity occurred at 50 mg/kg bw/day. Developmental toxicity was not seen at the highest dose administered, 50 mg/kg bw/day. The peri/postnatal study, including endpoints as pup weight, pup survival and postnatal death, resulted in a NOAEL (highest dose level) of =20 mg/kg bw/day. Therefore, the NOAEL for maternal toxicity can be established at 15 mg/kg bw/day assuming 50% oral absorption (internal no-effect dose 7.5 mg/kg bw/d). There is no evidence for developmental toxicity and the developmental NOAEL is =20 mg/kg bw/day (internal no-effect dose =10 mg/kg bw/d), the highest dose administered. AHTN has a very weak estrogenic potency *in vitro* but such effects were not seen *in vivo*.

Given the lowest internal no-effect dose (7.5 mg/kg bw/d) and the highest internal body burdens of 0.0093 mg/kg bw/d (scenario 2) for inhalation exposure and 0.023 mg/kg bw/d (scenario 2) for dermal exposure, the resulting MOS values are 806 and 326, respectively. For combined exposure (dermal and inhalation), the highest combined internal body burden of 0.0131 mg/kg bw/d results in a MOS of 572. A minimal MOS of 50 is considered appropriate for this effect. The latter is established by taking into account an interspecies factor of 10 (4 for metabolic size differences * 2.5 for remaining differences) and an intraspecies factor of 5.

Comparison of the calculated MOS values with the minimal MOS value leads to **conclusion ii** for all scenarios.

4.1.3.2.3 Occupational limit values

At the moment no occupational limit values for AHTN have been established. The health risk assessment of inhalation exposure does not give reasons to establish occupational exposure limit values.

4.1.3.2.4 Summary of risk characterisation for workers

Conclusion iii is reached with regard to photosensitising effects because it cannot be excluded that these effects may occur in scenarios 1, 2 and 3 (handling).
For all other relevant endpoints **conclusion ii** is reached.

4.1.3.3 Consumers

4.1.3.3.1 Introduction

Consumer exposure occurs from consumer products to which AHTN is added intentionally as a component of the fragrance that enhances the product. It is used as an ingredient in commercial preparations (fragrance oils) intended to be used to fragrance a wide variety of consumer products such as perfumes, creams, toiletries, soaps and shampoos. It is also used in household and laundry cleaning products and air fresheners. The exposure is of a repeated nature and the main exposure route is dermal, with some inhalation exposure. There is no oral exposure from consumer products.

The starting point for the risk characterisation is the external dermal exposure level of 0.34 mg/kg bw/day together with the inhalatory exposure level of 0.0046 mg/kg bw/day. Because the absorption of AHTN through human skin is 4.1% (worst-case assumption), the external dermal exposure level results in an internal exposure level of 0.014 mg/kg bw/day. For inhalation, 100% absorption is assumed, so the internal exposure level is 0.0046 mg/kg bw/day. The total internal exposure amounts 0.019 mg/kg bw/day.

4.1.3.3.2 Comparison of exposure and effects

Irritation

The available data on AHTN do not indicate a skin irritating or photoirritating potential. Hence, there is no concern for consumers for skin (photo-)irritation (**conclusion ii)**.

There is no concern for consumers for eye irritation, because AHTN is not an eye irritant (**conclusion ii)**.

No data are available on local effects in the respiratory tract. However, given the lack of skin and eye irritation potential, no significant respiratory irritation potential is expected. (**conclusion ii**).

Sensitisation

Whereas the available data do not indicate a skin sensitising potential of AHTN (**conclusion ii)**, a photosensitising potential was identified in animal studies. The concern for consumers for photosensitisation, however, is low, because the concentration of AHTN in consumer products (<1%) is below the general concentration limit normally applied for classification of preparations for sensitisation. Besides, negative results have been reported in human studies on photosensitisation with 1%, 5% and 10% AHTN preparations (**conclusion ii**).

Repeated dose toxicity

The starting point for the risk assessment is the oral NOAEL of 5 mg/kg bw/day from the 90-day study with rats. Assuming an oral absorption value of 50% for rats, this NOAEL corresponds to an internal no-effect dose of 2.5 mg/kg bw/day.

Comparing this internal no-effect dose with the calculated human systemic exposure level of 0.019 mg/kg bw/day, a margin of safety (MOS) of 132 can be calculated. Using assessment factors of 10 for intra- and interspecies (2.5 x 4) differences, a factor of 2 for duration extrapolation and a factor of 1 for dose-response, the minimal MOS would be 200. However, it should be taken into account that the NOAEL is set rather conservatively, given the marginal effects observed at 15 mg/kg bw/day. Taking also into account the worst-case character of the exposure estimate, the MOS of 132 indicates no concern for consumers following repeated dermal exposure (**conclusion ii).**

Mutagenicity

AHTN is a non-genotoxic substance (**conclusion ii).**

Carcinogenicity

ATHN lacks liver tumour initiating and promoting activity in rats when exposed to human-relevant doses. There are no other carcinogenicity data available. The mutagenicity data on AHTN do not indicate a concern with regard to carcinogenicity nor does AHTN possess any structural features that would raise a concern (**conclusion ii).**

Reproductive toxicity

There are no indications for effects on fertility in the oral 90-day study with rats although in this study investigation was limited to histological examination of the reproductive organs, and no adverse effects were reported up to the highest dose tested (the NOAEL was ≥50 mg/kg bw/day).

Dermal developmental studies are lacking.

In an oral developmental toxicity study with rats, developmental toxicity did not occur at maternal toxic dose levels ($NOAEL_{developmental\ toxicity}$ ≥50 mg/kg bw/day, $NOAEL_{maternal\ toxicity}$ 15 mg/kg bw/day). A peri/postnatal study with rats, including endpoints such as pup weight, pup survival and postnatal death, resulted in a NOAEL for developmental toxicity of ≥20 mg/kg bw/day (the highest dose tested). Assuming an oral absorption value of 50% for rats, this $NOAEL_{developmental\ toxicity}$ corresponds to an internal no-effect dose of ≥10 mg/kg bw/day.

Comparing this internal no-effect dose with the calculated human systemic exposure level of 0.019 mg/kg bw/day, a MOS of 526 can be calculated. This MOS indicates no concern for consumers for developmental toxicity (**conclusion ii)**, based on comparison with a minimal

MOS of 100, taking into account intra- (factor of 10) and interspecies differences (factor of 10 (2.5 x 4)) and the lack of effect at the highest dose tested (factor of 1 for dose-response).

4.1.3.3.3 Summary of risk characterisation for consumers

For consumers, **conclusion ii** is reached for all endpoints.

4.1.3.4 Humans exposed via the environment

4.1.3.4.1 Introduction

For man exposed via the environment the inhalation and oral route are applicable. The contribution of the inhalation of AHTN via air is negligible compared to other uptake routes, hence only the main oral exposure route via fish and root crops is taken into account. Because of the occurrence of AHTN in mother's milk, a separate risk characterization is necessary for breast-fed babies.

4.1.3.4.2 Exposure via food and water

Using EUSES, the total daily intake is estimated at 1.8 µg/kg bw/day for the local scenario (large/medium compounding scenario) and 0.012 µg/kg bw/day for the regional scenario.

Repeated dose toxicity

The starting point for the risk assessment is the oral NOAEL of 5 mg/kg bw/day from the oral 90-day repeated dose study with rats. Assuming an oral absorption value of 50% for rats, this NOAEL corresponds to an internal no-effect dose of 2.5 mg/kg bw/day. Using assessment factors of 10 for intra- and interspecies (2.5 x 4) differences, a factor of 2 for duration extrapolation and a factor of 1 for dose-response, the minimal MOS would be 200. For both local and regional scenario's, the margin of safety is higher than the minimal MOS (1389 and 2.1E+05, respectively), which results in a (**conclusion ii).**

Mutagenicity

AHTN is a non-genotoxic substance (**conclusion ii).**

Carcinogenicity

ATHN lacks liver tumour initiating and promoting activity in rats when exposed to human-relevant doses. There are no other carcinogenicity data available. The mutagenicity data on AHTN do not indicate a concern with regard to carcinogenicity nor does AHTN possess any structural features that would raise a concern (**conclusion ii).**

Reproductive toxicity

There are no indications for effects on fertility in the oral 90-day study with rats although in this study investigation for reproductive toxicity was limited to histological examination of the reproductive organs, and no adverse effects were reported up to the highest dose tested (the NOAEL was ≥50 mg/kg bw/day).

In an oral developmental toxicity study with rats, developmental toxicity did not occur at maternal toxic dose levels ($NOAEL_{developmental\ toxicity}$ ≥50 mg/kg bw/day, $NOAEL_{maternal\ toxicity}$ 15 mg/kg bw/day). A peri/postnatal study with rats, including endpoints such as pup weight, pup survival and postnatal death, resulted in a NOAEL for developmental toxicity of ≥20 mg/kg bw/day (the highest dose tested). Assuming an oral absorption value of 50% for rats, this $NOAEL_{developmental\ toxicity}$ corresponds to an internal no-effect dose of ≥10 mg/kg bw/day. Comparing this internal no-effect dose with the local and regional values, MOSses of 5555 and 8.3E+5 respectively can be calculated. These MOSses indicate no concern for humans exposed indirectly via the environment for developmental toxicity (**conclusion ii)**, based on comparison with a minimal MOS of 100, taking into account intra- (factor of 10) and interspecies differences (factor of 10 (2.5 x 4)) and the lack of effect at the highest dose tested (factor of 1 for dose-response).

4.1.3.4.3 Exposure via mother's milk

AHTN has been determined in human milk samples. The source of AHTN in these samples is not entirely clear. Maternal exposure to consumer products, intake via food, water or air and occasionally also occupational exposure may contribute to the AHTN level in milk. However, from the point of view of the child, AHTN in milk is an indirect environmental exposure. Therefore this exposure is dealt with in this section, rather than the sections on consumer or combined exposure.

An analysis of the milk from 59 nursing mothers revealed the presence of AHTN with a mean value of 112 µg/kg milk fat. The minimum and maximum values found were undetectable and 565 µg/kg milk fat, respectively. A fat content ranging from 1.5 to 4.2% was also reported. Based on the highest fat content (worst case), human milk contains 4.7 µg/kg whole milk (mean) or 23.7 µg/kg whole milk (maximum). In an oral peri/post natal study in which female rats were exposed orally to AHTN from day 14 of gestation through weaning, there were no effects on the dams at maternal doses of up to 20 mg/kg bw/day nor on the pups which were exposed via the milk during nursing. Measurements of levels of AHTN (9.4 and 2.1 µg/ml at 4 or 8 hr post dosing, respectively; parent AHTN only) in the milk of the dams dosed at 20 mg/kg bw/day compared to the levels found in human milk samples indicate that the pups in the high dose group were exposed to levels approximately 460 to 2000 times the mean level. This corresponds to approximately 90 to 400 times the maximum level found in human milk samples (4.7 and 23.7 µg AHTN/kg whole milk, respectively).

Even for the highest concentration in human milk samples, compared to the highest concentration in rat milk, a sufficiently high MOS can be calculated (~100). Taking into account that at the top maternal dose no effects were observed at all (i.e. the real NOAEL is at least equal but probably above this top dose), a **conclusion ii** is reached.

Additional to the assessment above, which is only based on concentrations in human *versus* rat milk, an assessment is carried out which also takes into account, the amount of milk that is consumed by infants and rat pups, in a way similar to the assessment applied in the Risk Assessment Report on MCCP (ECB, 2004). For the mentioned references see this RAR.

It is assumed that an infant breast feeds for 1 year, and this year of life is subdivided into two periods – 0 to 3 months and 3 to 12 months – reflecting the changing feeding demands of the infant. It is assumed that over the first 3 months the infant has an average weight of 6 kg (data taken from the UK growth charts, published by the Child Growth Foundation, 1995; Freeman

et al, 1995 and Cole, 1994), that the infant ingests 0.8 kg of milk per day, that 50% of the ingested AHTN is absorbed and that the breast milk has an average fat content of 4.2% (see above). From 3 to 12 months, it is assumed that the infant has an average weight of 10 kg (data taken from same source as above), that the infant ingests 0.5 kg of milk per day, that 50% of the ingested AHTN is absorbed and that the breast milk has a fat content of 4.27% (see above). It is also assumed that the content of AHTN remains constant during the breast-feeding period.

Using the following equation and the assumptions detailed above, the average daily uptake of the breast-feeding infant (ADU_{infant}) is estimated for both the 0-3 month and 3-12 month periods of infant life. The resultant uptakes are then summed to generate an average uptake for the infant in $mg.kg^{-1}.day^{-1}$.

$$ADU_{\text{inf }ant} = \frac{C_{milk-fat} xf\,3 xf\,4 xIR_{milk}}{BW_{\text{inf }ant}}$$

where:

$C_{milk\text{-}fat}$	is the concentration of AHTN in $mg.kg^{-1}$ fat in breast milk
f3	is the fraction of fat in breast milk (0.042 kg fat/kg milk)
f4	is the absorbed fraction of ingested AHTN (0.5)
IR_{milk}	is the ingestion rate of milk ($kg.day^{-1}$)
BW_{infant}	is the average infant body weight over the exposure period (kg)

AHTN uptake during 0-3 months, assuming a concentration of AHTN in human breast milk of 565 µg/kg (maximum measured value):

$$ADU_{\text{inf }ant} = \frac{565x0.042x0.5x0.8}{6} = 1.6x10^{-3} mg/kg/day$$

AHTN uptake during 3-12 months, assuming a concentration of AHTN in human breast milk of 565 µg/kg:

$$ADU_{\text{inf }ant} = \frac{565x0.042x0.5x0.5}{10} = 0.59x10^{-3} mg/kg/day$$

Based on these estimates, the time-weighted year-average uptake of AHTN for the first 12 months of life is 0.84 x 10^{-3} mg/kg/day.

A similar calculation can be performed for the rat. Pup body weight at birth is around 6 g and at weaning is about 40-50 g; an average weight of about 20 g will be assumed for the purposes of this calculation. Milk production in the lactating rat varies over the lactation period. Sampson and Jansen (1984) derived a model to estimate daily milk yield in the lactating rat. Based on this model, on day 10 of lactation, milk yield was estimated to be 29.5 ml for a dam nursing 8 pups. This equates to about 3.7 ml (or 3.7 g) milk per pup, and will be used as the average daily milk consumption for this calculation.

Based on these assumptions, and using the value of 9.4 mg/l milk for AHTN content in rat milk, estimated daily pup uptake is about 0.9 mg/kg/day (i.e. level of AHTN per kg whole milk x daily milk consumption (l) x absorption fraction / pup body weight (kg) = 9.4 mg/l x 3.7 x 10^{-3} x 0.5 / 20 x 10^{-3} mg/kg/day).

Comparing these two estimates of uptake, there is a difference of approximately 1000 between the levels of AHTN exposure in the rat study (in which no adverse effects were found) and human infant exposure. This large Margin of Safety (MOS) leads to little cause for concern and thus a ***conclusion (ii)***.

4.1.3.4.4 Summary of risk characterisation for exposure via the environment

A conclusion **ii** was reached for man exposed indirectly via the environment at the local scale as well as at the regional scale, and also for breast-fed babies.

4.1.3.5 Combined exposure

A worst case estimate for the combined (internal) exposure to AHTN would be the sum of the worst case estimates for the three individual populations, i.e. 0.023 mg/kg bw/day (dermal, scenario 2 compounding "molten, for workers) + 0.019 mg/kg bw/day (dermal and inhalation, consumers) + 0.0018 mg/kg bw/day (oral and inhalation, locally via the environment). This results in a total internal (worst case) combined exposure estimate of 0.044 mg/kg bw/day. The contribution of the exposure via the environment attributes only about 4%. The contribution to the total exposure as worker or as consumer is about equal. This value is compared to the two relevant chronic endpoints, namely repeated dose toxicity and reproductive toxicity.

Comparing this value to an internal no-effect dose of 2.5 mg/kg bw/day from the repeated dose toxicity study, a MOS of 57 can be derived. Based on a comparison with a minimal MOS of 100 (established by taking into account an interspecies factor of 10 (4 for metabolic size differences * 2.5 for remaining differences), an intraspecies factor of 5 for workers and a factor of 2 for semichronic to chronic exposure extrapolation, is considered a borderline case. However, given the worst case approaches taken in both exposure (worker and consumer) assessments, this MOS is also considered acceptable (**conclusion ii**).

Comparing this value to an internal no-effect dose of ≥10 mg/kg bw per day for maternal toxicity, a MOS of ≥ 294 can be derived. A minimal MOS of 50 is considered appropriate for this effect. The latter is established by taking into account an interspecies factor of 10 (4 for metabolic size differences * 2.5 for remaining differences) and an intraspecies factor of 5 for worker. Comparison of the calculated MOS values with the minimal MOS value leads to **conclusion ii** for workers after total combined exposure (no concern).

4.2 HUMAN HEALTH (PHYSICO-CHEMICAL PROPERTIES)

4.2.1 Exposure assessment

As AHTN is not explosive, flammable and has no oxidising potential, this exposure assessment is not filled in.

4.2.1.1 Workers

4.2.1.2 Consumers

4.2.1.3 Humans exposed via the environment

4.2.2 Effects assessment: Hazard identification

4.2.2.1 Explosivity

AHTN is not explosive (expert judgement).

4.2.2.2 Flammability

AHTN is not flammable.

4.2.2.3 Oxidizing potential

AHTN is not oxidizing

4.2.3 Risk characterisation

Based on the absence of physico-chemical hazards, there is no reason for concern. **Conclusion (ii)** applies.

4.2.3.1 Workers

4.2.3.2 Consumers

4.2.3.3 Humans exposed via the environment

5 RESULTS [15]

5.1 ENVIRONMENT

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

Conclusion (ii) applies to all compartments and all scenarios.

5.2 HUMAN HEALTH

5.2.1 Human health (toxicity)

5.2.1.1 Workers

Conclusion (iii) There is a need for limiting the risks; risk reduction measures which are already being applied shall be taken into account.

Conclusion (iii) is reached because it cannot be excluded that photosensitising effects may occur in scenarios 1, 2 and 3 (handling).

In the absence of a risk phrase for photosensitisation, a specific Note can be used to warn workers for the photosensitising potential of AHTN. However, at the October 2006 meeting of the TC-C&L the Commission stated that such a Note will not be developed under the current legislation. If a specific Note to warn workers for the photosensitising potential of AHTN will be available, conclusion ii may be applicable.

5.2.1.2 Consumers

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

5.2.1.3 Humans exposed via the environment

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

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[15] Conclusion (i) There is a need for further information and/or testing.
Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.
Conclusion (iii) There is a need for limiting the risks; risk reduction measures which are already being applied shall be taken into account.

5.2.1.4 Combined exposure

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

5.2.2 Human health (risks from physico-chemical properties)

Conclusion (ii) There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already.

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ABBREVIATIONS

ADI	Acceptable Daily Intake
AF	Assessment Factor
AS	Activated Sludge
ASTM	American Society for Testing and Materials
ATP	Adaptation to Technical Progress
AUC	Area Under The Curve
B	Bioaccumulation
BBA	Biologische Bundesanstalt für Land- und Forstwirtschaft
BCF	Bioconcentration Factor
BMC	Benchmark Concentration
BMD	Benchmark Dose
BMF	Biomagnification Factor
bw	body weight / *Bw*, *b.w.*
C	Corrosive (Symbols and indications of danger for dangerous substances and preparations according to Annex III of Directive 67/548/EEC)
CA	Chromosome Aberration
CA	Competent Authority
CAS	Chemical Abstract Services
CEC	Commission of the European Communities
CEN	European Standards Organisation / European Committee for Normalisation
CMR	Carcinogenic, Mutagenic and toxic to Reproduction
CNS	Central Nervous System
COD	Chemical Oxygen Demand
CSTEE	Scientific Committee for Toxicity, Ecotoxicity and the Environment (DG SANCO)
CT_{50}	Clearance Time, elimination or depuration expressed as half-life
d.wt	dry weight / dw
dfi	daily food intake
DG	Directorate General
DIN	Deutsche Industrie Norm (German norm)
DNA	DeoxyriboNucleic Acid
DOC	Dissolved Organic Carbon
DT50	Degradation half-life or period required for 50 percent dissipation / degradation
DT90	Period required for 50 percent dissipation / degradation
E	Explosive (Symbols and indications of danger for dangerous substances and preparations according to Annex III of Directive 67/548/EEC)
EASE	Estimation and Assessment of Substance Exposure Physico-chemical properties [Model]

EbC50	Effect Concentration measured as 50% reduction in biomass growth in algae tests
EC	European Communities
EC10	Effect Concentration measured as 10% effect
EC50	median Effect Concentration
ECB	European Chemicals Bureau
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ECVAM	European Centre for the Validation of Alternative Methods
EDC	Endocrine Disrupting Chemical
EEC	European Economic Communities
EINECS	European Inventory of Existing Commercial Chemical Substances
ELINCS	European List of New Chemical Substances
EN	European Norm
EPA	Environmental Protection Agency (USA)
ErC50	Effect Concentration measured as 50% reduction in growth rate in algae tests
ESD	Emission Scenario Document
EU	European Union
EU-15	European Union, 15 member states
EU-15+2	EU-15 plus Norway and Switzerland
EUSES	European Union System for the Evaluation of Substances [software tool in support of the Technical Guidance Document on risk assessment]
F(+)	(Highly) flammable (Symbols and indications of danger for dangerous substances and preparations according to Annex III of Directive 67/548/EEC)
FAO	Food and Agriculture Organisation of the United Nations
FELS	Fish Early Life Stage
GLP	Good Laboratory Practice
HEDSET	EC/OECD Harmonised Electronic Data Set (for data collection of existing substances)
HELCOM	Helsinki Commission -Baltic Marine Environment Protection Commission
HPLC	High Pressure Liquid Chromatography
HPVC	High Production Volume Chemical (> 1000 t/a)
IARC	International Agency for Research on Cancer
IC	Industrial Category
IC50	median Immobilisation Concentration or median Inhibitory Concentration
i.e.	Inhabitant equivalents
ILO	International Labour Organisation
IPCS	International Programme on Chemical Safety
ISO	International Organisation for Standardisation
IUCLID	International Uniform Chemical Information Database (existing substances)
IUPAC	International Union for Pure and Applied Chemistry

JEFCA	Joint FAO/WHO Expert Committee on Food Additives
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
Koc	organic carbon normalised distribution coefficient
Kow	octanol/water partition coefficient
Kp	solids-water partition coefficient
L(E)C50	median Lethal (Effect) Concentration
LAEL	Lowest Adverse Effect Level
LC50	median Lethal Concentration
LD50	median Lethal Dose
LEV	Local Exhaust Ventilation
LLNA	Local Lymph Node Assay
LOAEL	Lowest Observed Adverse Effect Level
LOEC	Lowest Observed Effect Concentration
LOED	Lowest Observed Effect Dose
LOEL	Lowest Observed Effect Level
MAC	Maximum Allowable Concentration
MATC	Maximum Acceptable Toxic Concentration
MC	Main Category
MITI	Ministry of International Trade and Industry, Japan
MOE	Margin of Exposure
MOS	Margin of Safety
MW	Molecular Weight
N	Dangerous for the environment (Symbols and indications of danger for dangerous substances and preparations according to Annex III of Directive 67/548/EEC
NAEL	No Adverse Effect Level
NOAEL	No Observed Adverse Effect Level
NOEL	No Observed Effect Level
NOEC	No Observed Effect Concentration
NTP	National Toxicology Program (USA)
O	Oxidizing (Symbols and indications of danger for dangerous substances and preparations according to Annex III of Directive 67/548/EEC)
OECD	Organisation for Economic Cooperation and Development
OEL	Occupational Exposure Limit
OJ	Official Journal
OSPAR	Oslo and Paris Convention for the protection of the marine environment of the Northeast Atlantic
P	Persistent
PBT	Persistent, Bioaccumulative and Toxic

PBPK	Physiologically Based PharmacoKinetic modelling
PBTK	Physiologically Based ToxicoKinetic modelling
PEC	Predicted Environmental Concentration
pH	logarithm (to the base 10) (of the hydrogen ion concentration $\{H^+\}$
pKa	logarithm (to the base 10) of the acid dissociation constant
pKb	logarithm (to the base 10) of the base dissociation constant
PNEC	Predicted No Effect Concentration
POP	Persistent Organic Pollutant
PPE	Personal Protective Equipment
QSAR	(Quantitative) Structure-Activity Relationship
R phrases	Risk phrases according to Annex III of Directive 67/548/EEC
RAR	Risk Assessment Report
RC	Risk Characterisation
RfC	Reference Concentration
RfD	Reference Dose
RNA	RiboNucleic Acid
RPE	Respiratory Protective Equipment
RWC	Reasonable Worst Case
S phrases	Safety phrases according to Annex III of Directive 67/548/EEC
SAR	Structure-Activity Relationships
SBR	Standardised birth ratio
SCE	Sister Chromatic Exchange
SDS	Safety Data Sheet
SETAC	Society of Environmental Toxicology And Chemistry
SNIF	Summary Notification Interchange Format (new substances)
SS	Suspended Sludge
SSD	Species Sensitivity Distribution
STP	Sewage Treatment Plant
T(+)	(Very) Toxic (Symbols and indications of danger for dangerous substances and preparations according to Annex III of Directive 67/548/EEC)
TDI	Tolerable Daily Intake
TG	Test Guideline
TGD	Technical Guidance Document
TNsG	Technical Notes for Guidance (for Biocides)
TNO	The Netherlands Organisation for Applied Scientific Research
UC	Use Category
UDS	Unscheduled DNA Synthesis
UN	United Nations

UNEP	United Nations Environment Programme
US EPA	Environmental Protection Agency, USA
UV	Ultraviolet Region of Spectrum
UVCB	Unknown or Variable composition, Complex reaction products of Biological material
vB	very Bioaccumulative
vP	very Persistent
vPvB	very Persistent and very Bioaccumulative
v/v	volume per volume ratio
w/w	weight per weight ratio
WHO	World Health Organization
WWTP	Waste Water Treatment Plant
Xn	Harmful (Symbols and indications of danger for dangerous substances and preparations according to Annex III of Directive 67/548/EEC)
Xi	Irritant (Symbols and indications of danger for dangerous substances and preparations according to Annex III of Directive 67/548/EEC)

Appendix 1

Evaluation of the aquatic toxicity test with *Nitocra spinipes*

Nitocra spinipes test (Breitholtz et al. 2003)

The test was carried out in 10 ml of test medium to which salmon food was added at a level of 75 mg per liter. Every other day 70% of the overlying water was replaced with fresh medium and new food was added (38 mg/L). The organic residues are not removed, so an increasing amount of organic matter was available for sorption.

Breitholtz et al. (2003) characterize the test organism *Nitocra spinipes*: 'it lives mainly on sandy bottoms, feeding on bacteria or particles.' In their discussion it is stated that 'it is likely that a significant fraction of the synthetic musks were adsorbed to particulate organic material and to the glass…. . Ingestion of synthetic musks adsorbed to particles could therefore be an equally or more significant route of exposure than uptake through the water for the bottom dwelling *N. spinipes* which feeds on bacteria and particles'.

Potential routes of disappearance of the test substance include sorption to organic matter, to the vessel glass wall, degradation and evaporation. Breitholtz et al. (2003) refer to the presence of various sinks for hydrophobic test substances, as was shown by Breitholtz and Wollenberger (2003)[16] on brominated flame retardants (their figure 2). In this study the walls of the test vials and especially particulate matter were clearly the most important targets of the test substances. At day 8 about 35% - 45% of the compounds was sorbed to the glass wall and 50 – 60% to the particulate matter. After another week the percentage found on particulate matter had increased to 60 – 80%, illustrating indeed that this test design led to increased accumulation of the material on the particulate matter in time. Although sorption to organic material can explain the decrease in concentrations of the two studied polybrominated diphenyl ether (PBDE) congeners (PBDE 47 and PBDE 99), this may be only partly valid for the polycyclic musk compounds AHTN and HHCB, which are less hydrophobic than the PBDE congeners. The log K_{ow} of AHTN and HHCB is about 1 to 2 log units lower than of the two PBDEs, on basis of accurate slow-stirring values[17]. The estimated log K_{oc} values of PBDEs are therefore higher as well.

With the lower log Koc values for AHTN and HHCB, sorption to organic matter may not be a sufficient explanation of the loss of test material. Methodological studies on the further development of a suitable test system for *Acartia tonsa* showed that up to 50% of AHTN and HHCB are lost from open test vessels after 1 day (Egeler et al 2007)[18]. The results of Kroon once more illustrate the relevance of sorption of polycyclic musks to algae cells (see below).

For substances with high log K_{ow} (>5) oral exposure is considered to be a significant route of exposure. In the EU-TGD (EC 2003) this exposure is taken into account by lowering PEC/PNECsediment by a factor of 10 when PNEC is based on equilibrium partitioning. In true sediment toxicity tests, oral exposure is inherent in the test system. In their discussion on

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[16] Breitholtz M, L Wollenberger (2003) Effects of three PBDEs on development, reproduction and population growth rate of the harpactticoid copepod *Nitocra spinipes*. Aquat. Toxicol. **64**, 85-96.

[17] Braekevelt E, SA Titlemier, GT Tomy (2003) Direct measurement of octanol–water partition coefficients of some environmentally relevant brominated diphenyl ether congeners. Chemosphere **51**, 563-567.

[18] Egeler P, JP Ferreira, D. Gilberg (2007). AHTN and HHCB: Preliminary studies on the toxicity to the larval development of the marine calanoid copepod *Acartia tonsa.* Report to PFW Aroma Chemicals and to IFF, ECT Oekotoxikologie GmbH, April 2007.

the toxicity of PBDEs, Breitholtz and Wollenberger (2003) draw the attention to the importance of oral ingestion of the sorbed fraction to the toxicity:

'The test organism used in the present study, *Nitocra*, feeds on bacteria and small particles.' and 'We are convinced that the main route of exposure in Nitocra in our test system is via ingestion of particle-adsorbed PBDEs.

As cited already above, Breitholtz et al. (2003) recognize the importance of the sorbed fraction for both polycyclic musk compounds AHTN and HHCB as well.

Sensitivity of Nitocra as as sediment organism

For comparison to the results of the sediment toxicity tests, the estimated concentration sorbed to the particulate matter (expressed per kg organic carbon) can be related to a concentration in sediment. The concentration on organic carbon (f_{oc}) was normalised to a sediment containing 5% organic carbon:

$$\textit{Estimated conc. in normalised sediment} = \textit{Conc in on particulate matter}_{[OC]} * \textit{foc}$$

The results of the calculation for HHCB and AHTN are shown in table A2-1 and figure A2-1 and A2-2.

Table A1-1: Calculated HHCB and AHTN 'sediment concentrations' based on log K_{oc} values.

	Calculated concentration in sediment with 5% OC in mg/kg dwt
HHCB	
Lumbriculus sediment test	NOEC (foc 5%) 38.6 (-13% biomass)
Hyalella sediment toxicity test	NOEC (foc 5%) 19.7 (-3% length, 0% biomass)
Nitocra 'aquatic toxicity test'	0.31 (-3% LDR) 1.1 (-20% LDR) 5.6 (-26% LDR) 20 (-21% LDR) 54 (-51% LDR)
AHTN	
Hyalella sediment toxicity test	NOEC (foc 5%) 42 (-3.5% length, -15% biomass)
Nitocra 'aquatic toxicity test'	0.77 (+ 10% LDR) 38 (-6% LDR) NOEC ≥38
Lumbriculus sediment toxicity test	NOEC (foc 5%) 17.2 (-13% biomass)

From this table it is clear that with the present measured concentrations in overlying water and log K_{oc}, the concentrations in particulate matter are similar to those in the sediment toxicity test with the sensitive benthic crustacean, *Hyallela azteca*. For *Nitocra spinipes* the variation in the parameter LDR seems relatively large. The LOEC for HHCB lies at 21-26%, while a similar positive effect ('stimulation') was found at equal concentration of AHTN. However, given the uncertainty in the calculation of the corresponding sediment concentrations, it can not be excluded that *Nitocra* is indeed the most sensitive of the tested benthic species.

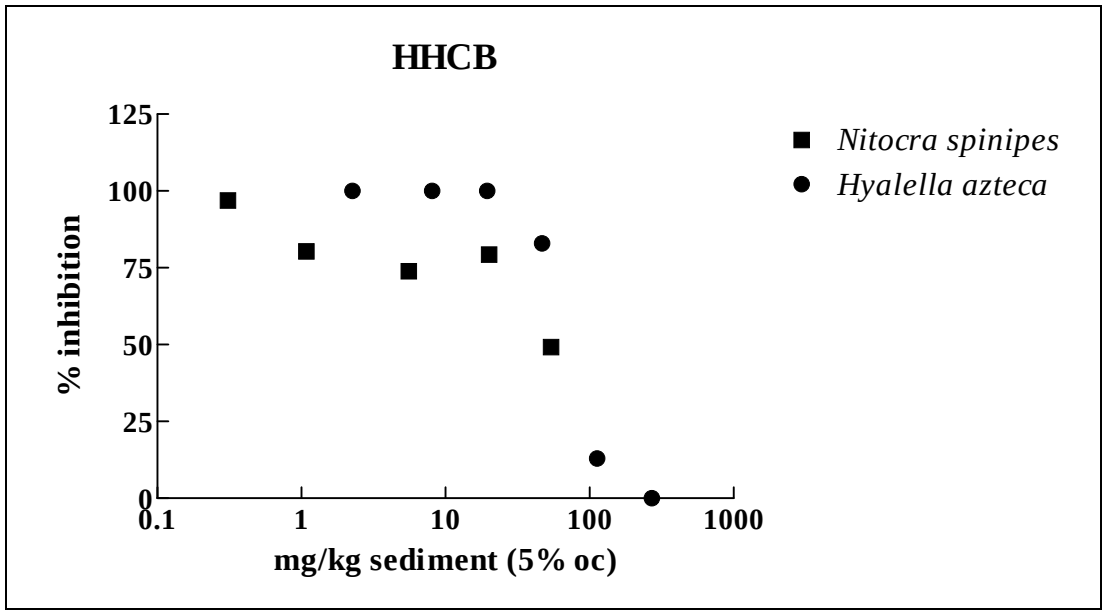


Figure A2-1. Comparison of the partial effect concentrations for Hyalella azteca (Egeler 2004), Nitocra spinipes (Breitholz et al. 2003) and Acartia tonsa (Wollenberger et al. (2003) for HHCB

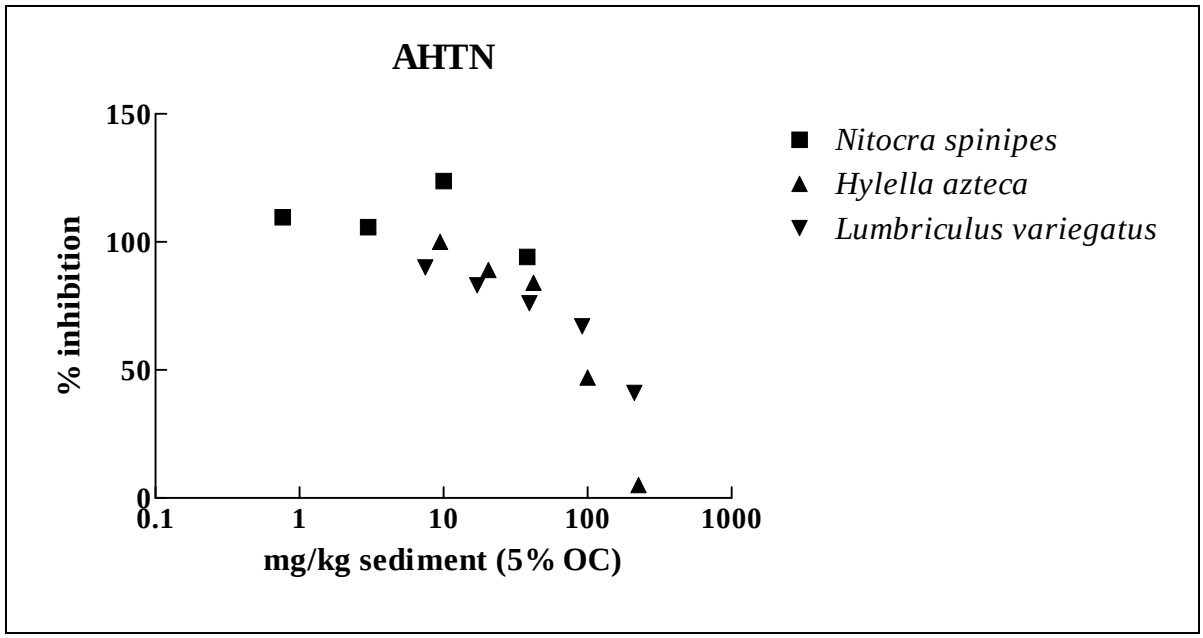


Figure A2-2. Comparison of the partial effect concentrations for Hyalella azteca (Egeler 2004), Lumbriculus variegatus (Egeler and Gilberg 2004b), Nitocra spinipes (Breitholz et al. 2003) for AHTN

Therefore, a comparison of the acute toxicity studies, in which the organimsms are only exposed via the aqueous phase, is necessary. From the comparison with other acute toxicity data for benthic organisms tested in a water-only system it appears that *Nitocra spinipes* is not particularly sensitive for the polycyclic musk compounds.

For AHTN the 5-d EC50 for *Lumbriculus variegatus* was 0.397 mg/l (measured concentration). No acute toxic effects were observed for *Chironomus riparius* up to 0.460 mg/l (measured concentration). The 96-h LC50 for *Nitocra spinipes* was 0.61 mg/l (nominal concentration). For HHCB the 5-d EC50 for *Lumbriculus variegatus* was 0.394 mg/l

(measured concentration). For *Chironomus riparius* the 96-h LC50 was 0.288 mg/l (measured concentration). The 96-h LC50 for *Nitocra spinipes* was 1.9 mg/l (nominal concentration).

In conclusion both for AHTN and HHCB at least the acute toxicity to *Lumbriculus variegatus* resulted in lower EC50s than the LC50 for *Nitocra spinipes*. For HHCB the LC50 for *Chironomus variegatus* was lower too, while for AHTN this could not be established, because the highest tested concentration was lower than the LC50 for *Nitocra spinipes*.

Conclusion

1. The decrease of the test substance concentration is probably caused by a combination of volatilisation and sorption to organic residues. This has caused a lower exposure through the water phase as well as an additional exposure by oral uptake. Therefore the actual exposure durng the test cannot be established.

2. Based on the nominal concentrations for the *Nitocra* test, it is concluded that *Nitocra*, *Lumbriculus* and *Hyallella* have 'sediment' effective concentrations in the same range, but *Nitocra* seems to be slightly more sensitive in the case of HHCB. In principle, *Nitocra spinipes* is a benthic organism, and therefore repeating the aquatic toxicity test is not very useful for the risk assessment of the water compartment. Moreover, the same problems of decreasing aquatic concentrations and oral exposure to substance sorbed to particulate matter will occur. The conclusion is that the results of this test with the benthic species *Nitocra* can not be used as an aquatic toxicity test in which exposure should take place only via the water phase. If a toxicity test with this benthic species should be performed this should be a sediment test in which exposure takes place directly via the sediment. However, considering the other tests with benthic organisms and the absence of a suitable test protocol, this has not much additional value compared with the already available tests with benthic organisms. Therefore, a new test with *Nitocra spinipes* is not considered necessary.

Adsorption and volatilisation of a polycyclic musk in an algae test

The study of Kroon with *Scendesmus subspicatus* (see figure 3) showed that:

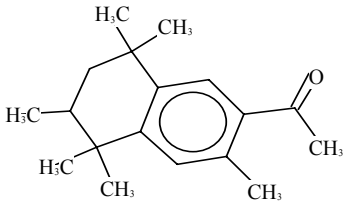
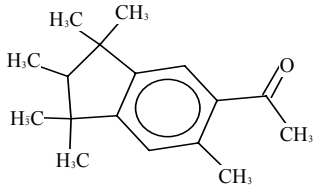
1. 20 – 25% of the test material is missing at the start of the test (other 'sinks' e.g., sorption to glass)
2. a considerable part (40% (= 1- (44/76)) of AHMI present in the algal suspension (10,000 cells/ml) was sorbed to the algal cells. Also when the total concentration in the vessel decreased in time, the fraction on the cells remained stable
3. the disappearance is linear in time. This could be explained by evaporation

If sorption is a partitioning process between the water phase and a lipid or organic matrix which is governed by log K_{ow}, the sorption of AHTN will probably be even higher than that of AHMI due to the higher log K_{ow} of AHTN. However, the results shown above would lead to bioconcentration factors by the algae, that would be much higher than would be predicted based on log K_{ow}.

The sorption in the test containing the larger alga *R. salina* at 50,000 cells/ml will be higher than the sorption in the test containing the smaller alga *S. subspicatus* at 10,000 cells/ml by an order of magnitude if sorption is based on log K_{ow} (cell volume ratio estimated to be about 20). However, if sorption is governed by the surface area available for sorption, the sorption

to the cell area will still be higher with *R. salina* compared to *S. subspicatus* by a factor of about 10.

Table A1-2. Structure of AHTN and AHMI

AHTN	**AHMI**
ClogP estimated log K_{ow} = 6.25	ClogP estimated log K_{ow} = 5.69
SRC estimated log K_{ow} = 6.35 measured log K_{ow} = 5.4	SRC estimated log K_{ow} = 5.85 with experimental value adjusted based on AHTN: estimated log K_{ow} = 4.91
	

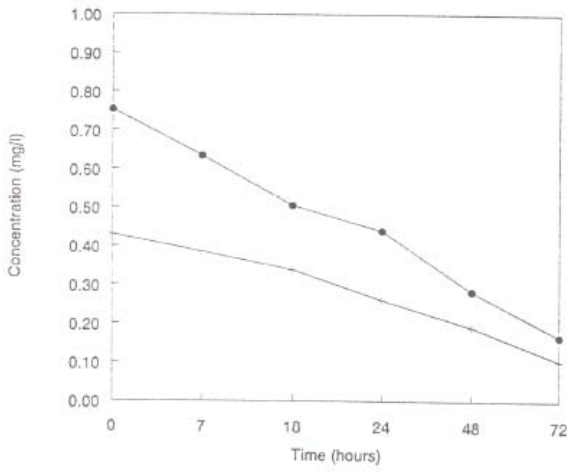


Fig. 2 Measured concentration of Phantolid® in aqueous solution incubated in the light with (+) or without (●) the addition of algae at the start of the test (1x10^4 cells/ml).

Figure A1-3. Adsorption of AHMI to algal cells (from Kroon 1998)

The report provides the comprehensive risk assessment of the substance 1-(5,6,7,8-TETRAHYDRO-3,5,5,6,8,8-HEXAMETHYL-2-NAPTHYL)ETHAN-1-ONE (AHTN). It has been prepared by The Netherlands in the frame of Council Regulation (EEC) No. 793/93 on the evaluation and control of the risks of existing substances, following the principles for assessment of the risks to man and the environment, laid down in Commission Regulation (EC) No. 1488/94.

The evaluation considers the emissions and the resulting exposure to the environment and the human populations in all life cycle steps. Following the exposure assessment, the environmental risk characterisation for each protection goal in the aquatic, terrestrial and atmospheric compartment has been determined. The environmental risk assessment concludes that there is no concern for any of the environmental compartments.

For human health the scenarios for occupational exposure, consumer exposure and humans exposed via the environment have been examined and the possible risks have been identified. The human health risk assessment concludes that there is concern for workers with regard to photosensitising effects. For consumers, for humans exposed via the environment and for human health (physico-chemical properties) there is no concern.