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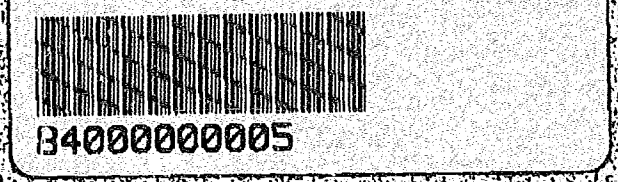
401 M Street, S.W. Room G-099
Washington, DC 20460



Re: Product Stewardship Information - Phantolid®

On behalf of PFW Aroma Chemicals BV, a foreign manufacturer and importer located at Nijverheidsweg 60, 3770 AK Barneveld, The Netherlands, please find enclosed the Draft Background Document Concerning the Elaboration of the Programmes and Measures on Musk Xylene and other Musks. This document was prepared for the Working Group on Diffuse Sources meeting of 18-22 October 1999 of the OSPAR Convention for the Protection of the Marine Environment of the North-East Atlantic in Bern, Switzerland. The lead country and author of this background document on the health and environmental assessment of nitromusks and polycyclic musks is Switzerland as represented by the Federal Office for Environment, Forests and Landscape (Bundesamt fur Umwelt, Wald and Landschaft - BUWAL).

Also enclosed for the Agency are three unpublished reports describing the results of acute toxicity testing with freshwater fish (*Brachydanio rerio*), invertebrates (*Daphnia magna*) and the green algae, *Selenastrum capricornutum* with the commercial substance Phantolid® (CAS 15323-35-0) and known by the common chemical name 6-Acetyl-1,1,2,3,3,5-hexamethylindan (AHMI) and CA Index Name -Ethanone, 1-(2,3-dihydro-1,1,2,3,3,6-hexamethyl-1H-inden-5-yl). The results reported in these studies are also summarized in the draft document on human and environmental risks of the musk compounds.

This information is being voluntarily provided by the sole importer of the substance into the USA, PFW Aroma Chemicals BV, as part of their on-going product stewardship program and to ensure that the TSCA program has the best available and current data for the scientific evaluation and technical assessment of existing chemical substances. The substance Phantolid® is one of several polycyclic musk compounds used in fragrances for detergents, fabric softeners and conditioners and cosmetic products such as soaps, shampoos and perfumes and is not known to have been previously tested for effects on aquatic organisms. The substance was on the original TSCA inventory and is also included on the DSL (Canada), EINECS (Europe), ENCS (Japan), AICS (Australia) and ECL (Korea) chemical inventories.

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Acute (EC50/LC50) values reported are similar to other commercial polycyclic musks such as Hexahydrohexamethylcyclopentabenzopyran-HHCB (CAS 1222-05-05) and Acetyl hexamethyltetralin-AHTN (CAS 1506-02-1) as presented in the OSPAR evaluation. The main difference would be usage levels as the Phantolide® or AHMI represents a small portion (1% or less) of fragrance additives used worldwide. Interestingly, the OSPAR report also includes the results of valuable environmental monitoring data (surface water, sewage sludge, suspended matter and sediments) which allows the environmental safety of synthetic nitro musks and polycyclic musks to be established for the European Community based on PEC/PNEC ratios and adequate margins of safety.

Should you have any questions or comments on the enclosures please do not hesitate to correspond or contact the undersigned as a technical representative of PFW Aroma Chemicals here in the USA.

T: 508-830-1025
F: 508-830-0918

Respectfully Submitted

Strategic Marketing
& Management Associates

Robert B. Foster

Robert B. Foster
Managing Director

Cc: H. D. Gaisser - PFW Barneveld

Enclosures

1. Draft Background Document Concerning the Elaboration of Programmes and Measures on Musk Xylene and other Musks. OSPAR Convention for the Protection of the Marine Environment of the North-East Atlantic. Working Group on Diffuse Sources (DIFF). 18-22 October 1999. Presented by Switzerland.
2. Evaluation of the toxic effects of Phantolid® to *Brachydanio rerio*. Aquasense Report 98.1202-17 Issued 14/01/99. Sponsor PFW Aroma Chemicals BV, Barneveld, The Netherlands.
3. Evaluation of the toxic effects of Phantolid® to *Daphnia magna*. Aquasense Report 98.1202-16 Issued 14/01/99. Sponsor PFW Aroma Chemicals BV, Barneveld, The Netherlands.
4. Evaluation of the toxic effects of Phantolid® to on the growth of the freshwater alga *Selenastrum capricornutum*. Aquasense Report 98.1202-15. Issued 15/01/99. Sponsor PFW Aroma Chemicals BV, Barneveld, The Netherlands.

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OSPAR CONVENTION FOR THE PROTECTION OF THE MARINE ENVIRONMENT OF THE NORTH-EAST ATLANTIC

WORKING GROUP ON DIFFUSE SOURCES (DIFF)

BERNE, 18 – 22 OCTOBER 1999

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Draft Background Document Concerning the Elaboration of Programmes and Measures on Musk Xylene and other Musks

Presented by Switzerland

Background

1. OSPAR 1997 agreed that musk xylene was to be included as a candidate into the list of substances which were annexed to the Draft Strategy to Implement OSPAR's Objective with regard to Hazardous Substances. At DIFF 1997 Germany presented a background paper about synthetic nitro musk compounds to justify their inclusion in the work programme of DIFF. DIFF 1997 agreed that Switzerland should act as a lead country and prepare a background document about the whole family of musks for presentation at DIFF1998.

2. OSPAR 1998 adopted:

   a. the OSPAR Strategy with regard to Hazardous Substances (reference number: 1998-16, cf. DIFF 98/3/1) in which musk xylene appears on the OSPAR List of Chemicals for Priority Action (Annex 2);

   b. the OSPAR Action Plan 1998 - 2003 (cf. DIFF 98/2/3) in which musk compounds appear on, *inter alia*, the List of Substances and Groups of Substances Originating from Diffuse Sources and Identified for the Purpose of Programmes and Measures (Annex 4).

3. The OSPAR Action Plan 1998 – 2003 stipulates that musk compounds belong to the category of diffuse sources and groups of substances to be considered for action by DIFF.

4. Switzerland presented a draft background document concerning the elaboration of programmes and measures on nitro musk and polycyclic musk at DIFF 1998. DIFF 1998 agreed that Switzerland should present a revised draft background document for presentation and examination at DIFF 1999.

5. OSPAR 1999 agreed (OSPAR 99/15/1) that lead countries should be invited to follow an interim guidance on how to address hazardous substances for priority action (Annex 7).

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6. Switzerland presented a draft background document on musk xylene and other musks as at Appendix 1

Action requested

7. DIFF is invited

a. to examine and, as it deems appropriate, elaborate the draft background document further;

b. to discuss the conclusions and recommendations of the draft background document and, if possible, endorse these conclusions and recommendations;

c. consequently, to assess whether there is sufficient support to forward this draft background document to PRAM 2000 with a view to its publication;

d. to discuss the need for further actions, e.g. the development of programmes and measures with regard to diffuse sources of musk xylene and other musks;

e. to provide the lead country with DIFFs view on the choice and the appropriateness of such actions.

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Appendix

Draft Background Document Concerning the Elaboration of Programmes and Measures on Musk Xylene and other Musks

Contents

1	**Introduction**
2	**Identification of the Sources of Musks and their Pathway to the Marine Environment**
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Annex 2:	**Ecotoxicity Data**
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Annex 4:	**Physical Chemical Properties**
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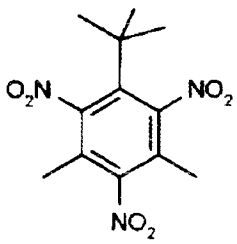
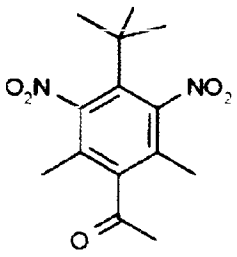
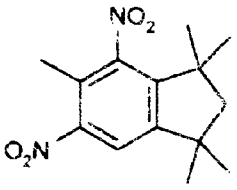
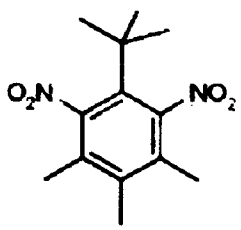
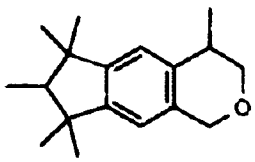
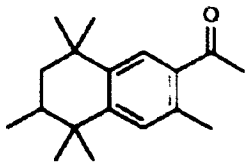
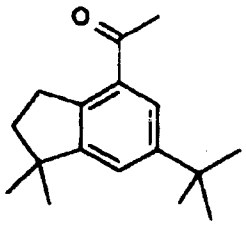
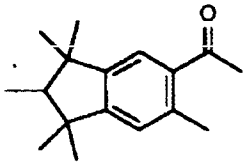
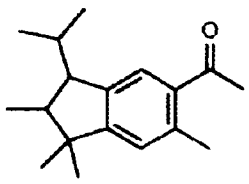
1 Introduction

Musk ingredients are substances with a typical musky scent, which determines largely the odour of a product. They are important for the fragrance industry and are widely used in cosmetics, detergents, fabric softeners, cleaning products and other household products

Two groups of substances with similar substantive properties but otherwise completely different chemical structure are used: nitro musks and polycyclic musks. Musk xylene - which appears on the list of substances for priority action in the OSPAR Strategy with regard to Hazardous Substances - is one of the nitro musks. Other three nitro musks of commercial interest - Musk ketone, Moskene and Musk tibetene - and the 5 polycyclic musks HHCB, ADBI, AHTN, AITI and AHMI listed in table 1 are also included in this review because they have to be considered as possible substitutes for Musk xylene. Musk xylene, Musk ketone, AHTN and HHCB represent about 95% of the market. Musk ambrette, an additional nitro musk, and the polycyclic musk AETT (Versalide®) have not been included in this review because they are no longer used.

Table 1: Musks included in this review

Common Name	CAS-No	Chemical Formula	Chemical Name
Musk xylene	81-15-2		1-tert-Butyl-3,5-dimethyl-2,4,6-trinitrobenzene
Musk ketone	81-14-1		4-tert-Butyl-2,6-dimethyl-3,5-dinitroacetophenone
Moskene	116-66-5		1,1,3,3,5-Pentamethyl-4,6-dinitroindane

Musk tibetene	145-39-1		1-tert-Butyl-3,4,5-trimethyl-2,6-dinitrobenzene
HHCB **Galaxolide®** **Abbalide®**	1222-05-5		1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta(g)-2-benzopyran
AHTN **Tonalide®** **Fixolide®** **Astralide®**	1506-02-1		6-Acetyl-1,1,2,4,4,7-hexamethyltetralin
ADBI **Celestolide®** **Crysolide®**	13171-00-1		4-Acetyl-6-tert-butyl-1,1-dimethylindane
AHMI **Phantolide®**	15323-35-0		6-Acetyl-1,1,2,3,3,5-hexamethylindane
AITI **Traseolide®**	68140-48-7		5-Acetyl-3-isopropyl-1,1,2,6-tetramethylindane

Due to their dispersive use and the relatively low rate of degradation many musk ingredients have been detected in environmental samples and human milk and tissue. These findings have prompted activities in different countries and national and international fora, and the data on musk ingredients have already been compiled and assessed in several review articles and reports. At the request of the Ministry of Environment, Space Planning and Housing (VROM) the Dutch National Institute of Public Health and the Environment (RIVM) carried out an

(RIVM, March 1996) and of the two most used polycyclic musks AHTN and HHCB (RIVM, December 1997). A refined version of the RIVM assessment of Musk xylene and Musk ketone was later published (Tas et al. 1997). A refined environmental risk assessment of the two polycyclic musks AHTN and HHCB is in press (Balk and Ford I and II).

The papers mentioned above, including the background document prepared by Germany and presented at DIFF 1997 (DIFF 97/13/1-E) and the report presented by the Netherlands on the marine occurrence and toxicity of nitro musks at SIME 1999 (SIME/99/3/20), were the basis for the present work. Existing data compilations and assessments have been supplemented by results of the environmental and safety testing programmes of the fragrance industry, made available by the International Fragrance Association (IFRA), and by other easily accessible data. Additional - partially unpublished monitoring data - have also been included. Results from existing assessments based on internationally accepted rules have not been re-evaluated and were included as such in the present report unless new information and findings differed considerably from the data already assessed.

2 Identification of the Sources of Musks and their Pathway to the Marine Environment

Nitro musks and polycyclic musks are low-cost fragrance ingredients with a musky odour. They are used in most fragrances for detergents, fabric softeners, fabric conditioners, cleaning agents, air fresheners and cosmetic products such as soaps, shampoos and perfumes. Musk xylene is primarily used in detergents and soaps. The principal use of Musk ketone is in cosmetics.

The materials are discharged after use in domestic waste water to the sewer and find their way to a sewage treatment plant. There some part will be removed by adsorption to sludge and biodegradation. The remaining fraction in the effluent will be discharged in the freshwater environment. In the waterways leading to sea the concentrations will be lowered by dilution, adsorption to organic matter and biodegradation.

Other uses are not known. Releases into the environment due to other uses can therefore be excluded.

Nitro musks are not produced in Europe. Formulation is a possible point source, but probably does not contribute significantly to the overall release of these substances to the marine environment.

Musks are important ingredients for the fragrance industry not only because of their typical and unique smell which determines the odour of a product to a great extent. In addition, some of them have a positive effect on the quality of a fragrance. They make it more balanced and longer lasting because of their ability to bind fragrances to fabrics and to the skin. These substantive properties relate to their poor water solubility, the relatively low volatility and to their high solubility in organic solvents and tissues.

3 Monitoring Data, Quantification of Sources and Assessment of the Extent of Problems

3.1 Quantification of the Sources

All musk ingredients are used in fragrances for products such as cosmetics, detergents, fabric softeners, household cleaning products etc. A large amount used for these purposes will therefore be released into domestic waste water. According to IFRA no geographical differences should be expected for the release pattern within Europe, unless a country has taken specific measures to phase out a certain substance. As a worst case for the aquatic environment it can therefore be assumed that 100% of the musks used in Europe is released into the waste water and that no substance remains on the fabric, skin or surfaces or has evaporated.

Data on consumption in Europe have been estimated by the Research Institute for Fragrance Materials (RIFM) for the years 1992 and 1995 based on surveys carried out in 1993 and 1996 and made available for the Swiss authorities by IFRA (letter of 30 April 1998). An other survey was carried out by IFRA for estimating the consumption in 1998 (letter of 8 July 1999). The results are presented in table 2 and table 3.

Table 2: Use of Musk xylene and other nitro musks in Europe (tonnes)

Year	Musk xylene	Musk ketone	Moskene	Musk tibetene
1992	174	124		
1995	110	61	5	0.8
1998	86	40		

Table 3: Use of polycyclic musks in Europe (tonnes)

Year	HHCB	AHTN	ADBI	AHMI	AITI
1992	2400	885			
1995	1482	585	34	50	40
1998	1473	385	18	19	2

Currently, Musk ketone, Musk xylene, HHCB and AHTN represent about 95% of the market in Europe for all nitro musks and polycyclic musks. No reliable data seem to be available for the time before 1992. Data that have been published vary considerably and are often difficult to interpret. Facts on trends for a long-term increase or a decrease in consumption in Europe are therefore not available. After 1992 the use of Musk xylene and Musk ketone and AHTN

3.2 Monitoring Data

A compilation of monitoring data is given in Annex 2

3.2.1 Water

Only a few monitoring data are available for the North Sea. In the German Bight Musk ketone was found in concentrations up to 0.08 ng/l (90th-percentile: 0.05 ng/l) and Musk xylene up to 0.17 ng/l (90th-percentile: 0.12 ng/l). In the same area the concentrations of HHCB were 0.26 ng/l (median value) and those of AHTN 0.2 ng/l (median value). No other musk ingredients have been detected (Gattermann et al. 1995).

More data are available for freshwater samples from the catchment area. Concentrations are highly variable. The ranges and 90th percentile concentrations reported are < 0.2 - 240 ng/l (30 ng/l) for Musk ketone and < 0.2 - 180 ng/l (30 ng/l) for Musk xylene. Musk xylene concentrations in samples from large rivers that have been taken recently are often close to the detection limit. Concentrations in rivers of similar size in different countries are comparable. The highest concentrations are found in small rivers receiving effluent discharges. For tributaries of the River Ruhr concentrations up to 40 ng/l were reported for Musk xylene (Eschke et al. 1994a).

Concentrations of HHCB and AHTN are in general higher than those of the major nitro musks Musk xylene and Musk ketone. This is consistent with the lower consumption volume of nitro musks. The ranges and 90th percentile concentrations reported are < 25 - 12470 ng/l (400 ng/l) for HHCB and < 25 - 6780 ng/l (300 ng/l) for AHTN. One set of data sampled in Berlin is out of the range compared to the others. In this study (Heberer et al. 1999) concentrations were found in surface waters that are normally reported for waste water effluents. According to the authors, these unusually high concentrations might be explained by the high percentage of sewage in the surface waters and the high population density in the Berlin area.

Measured waste water and surface water concentrations of the major nitro musks (Musk xylene and Musk ketone), the major polyclinic musks (HHCB and AHTN), and some musks of less commercial interest are summarised in tables 4, 5, and 6 of Annex 1).

Metabolites of Musk xylene and Musk ketone have been detected in recent studies. Samples of effluent from the Hamburg sewage plant contained 34 ng/L of 4-amino Musk xylene, 10 ng/L of 2-amino Musk xylene and 250 ng/L of 2-amino Musk ketone. These transformation products were not detectable in the influent samples. The decrease in the concentrations of the parent compounds and the increase of the amino derivatives suggest that, besides adsorption to sludge, the transformation of the nitro musks is an important process in the treatment plant. In the river Elbe concentrations of 4-amino Musk xylene attained 3 - 10 fold higher values than the parent compound (Gatermann et al. 1998).

3.2.2 Sediments, suspended matter, sewage sludge

Musk xylene, Musk ketone and some of the polycyclic musk ingredients have also been found in sewage sludge, suspended matter and sediments (See Annex 1, tables 7-9). Due to their lipophilic character, the concentrations in these samples are considerably higher than in the water samples. Sediment was measured only in fresh water. For musk xylene concentrations

higher than those of the two major nitro musks. This is consistent with the higher consumption volume and the higher octanol/water partition coefficient.

In a recent study, degradation products of nitro musks were analysed in digested sewage sludge from Swiss treatment plants (Berset & Herren 1999). 4-Amino Musk xylene was detected in 6 of 12 samples and 2-Amino Musk ketone in 1 of 12 samples. Degradation products of Musk xylene have also been detected in sediments of river Elbe (Rimkus et al. 1999).

3.2.3 Biota

For Musk xylene many data are available for both marine and freshwater fish, mussels and crustaceans. They have been compiled and presented by the Netherlands for SIME 1999 (SIME 99/3/20): Wet weight concentration for marine organisms were reported to be < 0.1 - 5.3 µg/kg for molluscs and 0.63 µg/kg for fish. Freshwater concentrations were reported to be up to 95 µg/kg.

Measured concentrations of certain musk ingredients in freshwater biota samples (mainly fish) have also been compiled by Switzerland for DIFF 1998. They are summarised in tables 10 - 14 in Annex 1. To be comparable some values, given as µg/kg lipid by the authors, have been recalculated to values based on fresh weight (indicated in the table). In the tables a distinction is made between eel and other fish species because of the high fat content of eel. Mean fresh weight contents of Musk xylene in eels from the river Ruhr have been reported to be 66 µg/kg. Mean concentrations for non-eel fish species in rivers and lakes are well below these values, normally lower than10 µg/kg.
For an effluent pond, a mean value as high as 639 µg/kg for samples taken before 1994 was reported. In recent studies (samples from 1997) concentrations of Musk xylene in eels in effluent ponds were significantly lower (mean: 37 µg/kg). Samples from effluent ponds, however, reflect more the conditions in a specific effluent than those under normal environmental conditions.

As in freshwater and sediment samples, amino metabolites of Musk xylene and Musk ketone have also been detected in biota samples (table 12). In fish samples and in 1 mussel sample the main metabolite 4-amino Musk xylene was detected in concentrations of 5-161 µg/kg fat. The concentration of this metabolite exceeded those of the parent compound in some samples. In many samples 2-amino Musk xylene (<1-18 µg/kg fat) and 2-amino Musk ketone (<1-34 µg/kg fat) could also be detected.

3.2.4 Musks in Human Samples

The nitro musks Musk xylene and Musk ketone and the polycyclic musks have been detected in human milk and fat since the early nineties. Concentrations found in human milk samples range from 0.01 - 0.29 mg/kg fat for Musk xylene, from 0.01 - 1.220 mg/kg fat for Musk ketone (Eschke et al. 1995b, Geyer et al. 1994, Kypke-Hutter 1993, Kypke-Hutter 1995, Liebl & Ehrenstorfer 1993, Rimkus & Brunn 1996, Rimkus & Wolf 1993, Waizenegger et al. 1998, Waizenegger & Scherbaum 1995) and 0.1 - 0.36 mg/kg for polycyclic musks (Eschke et al. 1995b, Kypke-Hutter 1995). No essential differences were observed between synthetic musk concentrations in human milk and human fat (Eschke et al. 1995b, Geyer et al. 1994, Heibich 1995, Kokot-Helbling et al. 1995, Müller et al. 1996, Rimkus & Brunn 1996, Rimkus & Wolf 1993). Musk xylene represented the first identified and formerly also the most highly concentrated synthetic musk found in human milk. According to more recent European data a

decline in concentrations of both nitro musks, Musk xylene and Musk ketone seems to be likely (Waizenegger et al. 1998), presumably as a consequence of restrictions in the use of these compounds in laundry detergents and cosmetics and a decline in production. Similar levels as for nitro musks were also found for AHTN and HHCB in human milk and adipose tissue.

3.3 Risk Assessment

3.3.1 Risks for the Aquatic Environment

A wide-ranging risk assessment was carried out for Musk xylene and Musk ketone by RIVM according to the EU Technical Guidance Document for the Risk Assessment of New and Existing Substances (RIVM, March 1996, Tas et al. 1997). The Predicted Environmental Concentrations (PECs) were determined using the 1992 use data recalculated for the Netherlands based on number of inhabitants and the release assumptions reported in chapter 3.1. Further assumptions: degradation and volatilisation in the sewage treatment plant are not considered. Sorption to activated sludge is the only removal process. Measured concentrations (see chapter 3.2) were significantly lower than predicted. The calculated PECs and the measured concentrations in the environment have been compared with the Predicted No Effect Concentrations (PNECs) using the toxicity data for aquatic species and the assessment factors given in table 16 below. $PNEC_{soil}$ was calculated from $PNEC_{aqua}$ using the equilibrium partitioning theory. A compilation of the most reliable ecotoxicity data for Musk xylene and other nitro musks is given in annex 2.

Table 16: NOEC's and Assessment Factors used for the Risk Assessment

Substance	Aquatic toxicity data	Assessment Factor
Musk xylene	21 d NOEC Daphnia rep. = 0.056 mg/l	50
Musk ketone	21 d NOEC Fish = 0.063 mg/l	10

The PEC/PNEC ratios for aquatic organisms and sediment organisms have been found to be <1 for both Musk xylene and Musk ketone. For Musk xylene, the PEC/PNEC ratio for the soil compartment was just above one. However this value was associated with a rather high uncertainty. High assessment factors have been used to determine the PNEC. In addition, the PEC has been calculated assuming ten yearly applications of sludge to agricultural land and nitro musk contents of about 20 mg/kg. Recent data on concentrations in digested sludge indicate levels below this value and sometimes below the detection limits, indicating that the estimated concentrations in sludge and also in soil were overestimated by a factor in the range of 20 to 1'000. This implies that the risk quotient for soil organisms for both Musk xylene and Musk ketone is well below 1.

Musk ketone		Exposure		Effect	Ratio	
		PEC	Median measured	PNEC	Exposure predicted	Exposure measured
Aquatic organisms [mg/l]	Local	0.00051	0.00003	0.0063	0.08	0.005
	Regional	0.000029	<0.00001		0.005	<0.002
Sediment organisms [mg/kg dw]	Local	0.10	-	0.99	0.01	-
	Regional	0.0052	<0.025		0.005	<0.03
Soil organisms [mg/kg dw]	Local	0.34	-	0.64	0.53	-
Fish-eating predators [mg/kg ww]	Local and regional	0.37	0.007	25	0.01	0.0003
	Regional	0.039	0.02		0.002	0.0008
Musk xylene		**Exposure**		**Effect**	**Ratio**	
Aquatic organisms [mg/l]	Local	0.00011	0.00001	0.0011	0.10	0.009
	Regional	0.0000048	<0.00001		0.004	<0.009
Sediment organisms [mg/kg dw]	Local	0.08	-	0.69	0.12	-
	Regional	0.0034	<0.025		0.005	<0.04
Soil organisms [mg/kg dw]	Local	0.29	-	0.23	1.3	-
Fish-eating predators [mg/kg ww]	Local and regional	0.089	0.006	8	0.01	0.0008
	Regional	0.0077	0.03		0.001	0.004

This preliminary assessment needs to be refined by taking into account the presence of degradation products in the environment. This is, however, not possible for the time being

The acute toxicity of 4-Amino Musk xylene (1-tert-butyl-3,5-dimethyl-4-amino-2,6-dinitrobenzene) and 2-Amino Musk xylene (1-tert-butyl-3,5-dimethyl-2-amino-2,6-dinitrobenzene) on *Daphnia magna* has recently been determined (Behechti et al. 1998). The toxicity of 4-Amino-musk-xylene was reported to be about three orders of magnitude higher than the parent compound. Sponsored by RIFM the acute toxicity test with Daphnia magna was repeated (Putt 1999) under four different test conditions (standard water and natural water, with light and under complete darkness). As can be seen from the table below, the results of Behechti et al. could not be reproduced. The new study indicates that the toxicity of 4-AminoMusk xylene is in the same order as reported before for the parent compound. A comprehensive evaluation and comparison of the two Daphnia tests and eventually additional toxicity tests with Daphnia magna and other aquatic species are needed to draw reliable conclusions on the aquatic toxicity of the metabolites.

	4-Amino Musk xylene	2-Amino Musk xylene
Daphnia magna (Behechti et al., 1998)	EC_{50} = 0.00025 mg/l	EC_{50} = 1.07 mg/l
Daphnia magna (Putt 1999) Laboratory water, standard lighting Laboratory water, total darkness Natural water, standard lighting Natural water, total darkness	 EC_{50} = 0.490 mg/l EC_{50} = 0.510 mg/l EC_{50} = 0.370 mg/l EC_{50} = 0.440 mg/l	

In February 1999, a report "Marine occurrence and toxicity of nitro musks" prepared by the Netherlands, has been presented to the OSPAR Working Group SIME (SIME 1999). In this report the following provisional Ecotoxicological Assessment Criteria (EACs) have been derived for Musk xylene and Musk ketone, obviously based on the same test data as presented in this report:

EACs for Musk xylene:	for water	0.01-0.1 µg/l
	for sediment	5-50 mg/kg
	for mussel	0.5-5 mg/kg
	for fish	0.5-5 mg/kg
EACs for Musk ketone:	for water	0.05-0.5 µg/l
	for sediment	1-10 mg/kg
	for mussel	0.1-1 mg/kg
	for fish	0.1-1 mg/kg

The concentrations of musk xylene in the maritime area are lower than EACs. At some sites of the catchment area freshwater concentrations are above EACs, while at others concentrations are well below EACs. The authors concluded that the concentrations of Musk xylene and Musk ketone in the maritime environment appear to be at a safe level (lower than EACs). For the assessment of environmental concentrations however, a better understanding of the risk an extremely toxic metabolite of Musk xylene may pose to marine organisms is necessary. For Musk moskene, Musk tibetene and Musk ambrette it was not possible to assess the concentrations by means of EACs due to the lack of sufficient toxicity data.

The risks associated with the use of Moskene and Musk tibetene are considered to be negligible due to the similar properties but the considerably smaller use volume of these compounds.

A similar risk assessment has been performed for HHCB and AHTN (RIVM 1997). The PEC/PNEC ratios for aquatic organisms and for sediment organisms were clearly less than 1. Again the compartment of concern was the soil compartment where the PEC/PNEC ratio has been calculated to be greater than 1 for HHCB and close to 1 for AHTN. However, these calculations assume absence of any biodegradation in soil during 120 years, whereas soil degradation data for HHCB show 60% disappearance within one year. Recent soil studies show half-lives of 3 months or less. A refined assessment based on more realistic but still conservative assumptions is in press (Balk and Ford I and II). The results are given in the table below. Taking a very conservative half-life of 6 months in soil, risk quotients are in the order of 0.1 for the soil compartment.

AHTN	Exposure concentrations	PNEC	Ratio
Aquatic organisms [µg/l]	0.30 (m)	3.5	0.086
Sediment organisms [mg/kg dw]	0.48 (m) *10 [1)]	11	0.44
Soil organisms [mg/kg dw]	0.029 (m+p)	0.32	0.091
Fish-eating predators [mg/kg]	0.12 (m)	10	0.012
Worm-eating predators [mg/kg]	0.065 (m+p)	10	0.007
HHCB			
Aquatic organisms [µg/l]	0.5 (m)	6.8	0.074
Sediment organisms [mg/kg dw]	0.16 (m) *10 [1)]	25	0.064
Soil organisms [mg/kg dw]	0.032 (m+p)	0.32	0.1
Fish-eating predators [mg/kg]	0.125 (m)	100	0.0013
Worm-eating predators [mg/kg]	0.099 (p)	100	0.001

(m): measured concentration

(p): predicted concentration

(m+p): predicted from measured concentration

[1)]: measured concentration multiplied by 10 as log Pow of AHTN and HHCB is above 5 (EU Technical Guidance Document)

Since the preparation of the initial assessment reports for Musk xylene, Musk ketone, AHTN and HHCB some additional monitoring data have become available. They confirm the overall conclusion that PEC/PNEC ratios are below 1.

A preliminary risk assessment was also performed for the 'minor' polycyclic [illegible] DBI, AHMI and AITI by HASKONING on behalf of RIFM (Research Institute [illegible] Fragrance Materials). A draft assessment report was made available in June 1998 but has not yet been officially published (Balk 1998). As expected the outcome of the assessment demonstrates a low risk for aquatic organisms and sediment organisms (similar properties to other polycyclic musks, smaller use volume).

3.3.2 Bioaccumulation and Secondary Poisoning

3.3.2.1 Risks for Predators

Musk xylene and other musk ingredients are lipophilic, poorly degradable and have tendency to bioaccumulate. In a recent review article the author even concluded that these substances should be considered as Persistent Organic Pollutants POPs (Rimkus 1998).

Indeed, several biodegradation tests performed with the four nitro musks demonstrate that these chemicals do not mineralise under standard test conditions. The test results are tabled in Annex 3 together with degradation data for polycyclic musks. The UNEP Expert Group for Persistent Organic Pollutants recently proposed half-lives in soil or sediments longer than six months for identifying substances as POP candidates. Opinions of experts were divided with regard to half-lives in water. Two and six months, respectively, were proposed (UNEP/POPs/INC/CEG/2/3). Degradation half-lives in water are not known for Musk xylene and the other musk ingredients. For some polycyclic musks, however, the ability to degrade has been shown for example in soil studies, in microcosm studies and in a biotransformation study in activated sludge. Recent soil studies show half-lives of 3 months or less. In addition, degradation has been shown for AHTN, HHCB and Musk ketone in fish bioaccumulation studies. Finally, evidence that nitro musks can be degraded has been given from monitoring data. Metabolites of Musk xylene and Musk ketone were detected in influents, sewage sludge, effluents and downstream of sewage treatment plants.

The bioconcentration of Musk xylene and Musk ketone was determined in well-conducted studies using radiolabelled material (Tas et al. 1997). The results are presented in table 19. A BCF of 1600 was reported for Musk xylene based on radiolabelled residue in fish. BCF based on parent material will be lower. In another bioconcentration study performed at concentrations well above the solubility limit of Musk xylene, BCF values between 1440 and 6740 were found.

In the test with Musk ketone the uptake and depuration was carried out in edible and non-edible parts of fish. Careful analysis during the accumulation period indicated that three polar metabolites were formed. From the mass balance for the system fish plus water it is concluded that between 70 and 100% of the excreted radioactivity may consist of polar metabolites of Musk ketone. The elimination rate constant has been found to be 0.27-0.47 d^{-1}, which gives a recalculated elimination half-life of 2.2-2.6 days. Hence it seems that the uptake of Musk ketone was highly reversible. For Musk xylene no studies with investigation of metabolites and/or determination of elimination rates are available.

Table 19: Bioconcentration factors for Musk ketone and Musk xylene

		Bioconcentration factor (whole fish, wet weight)1)	
Musk ketone	Rainbow trout	1380	Tas et al. 1997
Musk xylene	Bluegill sunfish	1600	Tas et al. 1997

1) BCF based on radiolabelled residue in fish. The BCF based on parent material will be lower.

The bioconcentration factors of AHTN and HHCB have recently been measured in bluegill sunfish in a flow-through system (table 20). No datas are available for the other polycyclic musks.

The bioconcentration studies for both AHTN and HHCB were performed with radiolabelled material following the formation of metabolites. The studies showed that both compounds are metabolised to one or more polar metabolites in a relatively short time, with estimated elimination half-lives of 1-2 days for AHTN and 2-3 days for HHCB (RIVM 1997).

Table 20: Bioconcentration factors for AHTN and HHCB

	Bioconcentration factor (whole fish, wet weight)	
AHTN	1320* 597**	RIVM, December 1997
HHCB	1624* 1584**	RIVM, December 1997

* BCF based on total radioactivity

** BCF based on actual concentration of the parent compound

The test design of the studies for Musk ketone, AHTN and HHCB permitted it to be established that these substances are metabolised under the bioconcentration test conditions in a relatively short time and that these polar metabolites will be eliminated by the fish at a higher rate than the parent compounds. It can therefore be concluded that the real bioconcentration of these compounds is much smaller than could be expected based on the rather high octanol/water partition coefficients of Musk ketone, AHTN and HHCB. The test design for Musk xylene did not permit a comparable analysis.

Both the PEC/PNEC approach and the method for derivation of Ecotoxicological Assessment Criteria (EACs) reported in chapter 3.3.1 do not indicate a risk for secondary poisoning. Using calculated PECs, the PEC/PNEC ratio for Musk xylene is 0.01. Using monitoring data, this figure drops to 0.0008, and the provisional EAC_{biota} is also below the concentrations found in marine and freshwater species. However, all these figures are very uncertain. Toxicity studies using predators present in the environment such as fish-eating birds are not available. As a surrogate toxicity test, data for rats and mice were used for the assessment. It has also to be kept in mind that metabolites of Musk xylene and Musk ketone have recently been detected in samples of water and biota. It is uncertain whether the toxicity of these metabolites and the risk they pose to predators is adequately reflected in these assessments.

3.3.2.2 Human Risks

Musk xylene and other musks have been detected in human milk, fatty tissue and blood (see chapter 3.2 and annex 1). This raises the question of whether food chain accumulation takes place and secondary poisoning can occur. Measurements on predatory animals or birds would be useful for indicating if the substances can persist in the food chain. Unfortunately, such data are not available.

It seems, however, that direct exposure through the use of cosmetics is the main route of

be negligible compared to direct exposure through the use of cosmetic products (Kokot-Helbling et al. 1995). Reviews of previously published experiments and estimations confirmed dermal uptake from cosmetics and fabrics to be the main exposure route for human beings (Rimkus 1997, Kevekordes et al. 1999).

Estimates of dermal absorption factors calculated from human, pig and rat skin penetrations were used to calculate systemic exposure of rats and humans to nitro musks from cosmetic products. The average human exposure corrected for dermal adsorption is calculated as 0.014 mg/kg/d for Musk xylene and 0.025 mg/kg/d for Musk ketone. These exposure values were compared to the systemic exposure at NOAEL calculated for rats from dermal applications (For toxicological data of musk ingredients see Annex 5). By comparing the two exposure concentrations, safety factors(s) were estimated to range between 300 to 3000 for the different compounds (Ford & Api 1990, table 21).

Table 21. Human risk assessment; comparison of human dermal absorption to rat dermal absorption at the rat NOAEL level (Ford 1998)

Musk xylene	**Musk ketone**	**Moskene**	**Musk tibetene**
NOAEL, rat (dermal): Males: 75 mg/kg/day Females: 24 mg/kg/day *Rat dermal abs.: 20%* NOAEL rat Systemic exposure (based on female values): **4.8 mg/kg/d**	NOAEL, rat (dermal): 75 mg/kg/d *rat. Dermal abs.: 28%* NOAEL rat systemic exposure: **21 mg/kg/d**	NOAEL, rat (dermal): males: 24 mg/kg/d females 75 mg/kg/d *rat dermal abs.: 34%* NOAEL rat systemic exposure (based on male values): **8.2 mg/kg/d**	NOAEL, rat (dermal): 75 mg/kg/d *rat dermal abs.: 28%* NOAEL rat systemic exposure: **21 mg/kg/d**
Estimated total exposure (dermal) : 0.18mg/kg/d *Human, rat: 7.5%* **Human systemic exp.** From total dermal exp.: 7.5% of 0.18mg = **0.014mg/kg/d;** *s = 342*	**Estimated total exposure (dermal): 0.18mg/kg/d** *Human; rat: 14%* **Human systemic exp.** From total dermal exp.: 14% of 0.18mg = **0.025 mg/kg/d;** *s = 840*	**Estimated total exposure (dermal): 0.15mg/kg/d** *pig: 6.2%* **human systemic expos.** From total dermal exp.: 6.2% of 0.15 mg = **0.009 mg/kg/d** *s = 911*	**Estimated total exposure (dermal): 0.15mg/kf/d** *pig, rat: 4.6%* **human systemic expos.** from total dermal exp.: 4.6% of 0.15 mg = **0.007 mg/kg/d** *s = 3000*

If the estimated human systemic exposure is compared to the NOAEL of 0.7 - 0.8 mg/kg/day (Suter-Eichenberger et al. 1998b) determined on the basis of a developmental pre- and post-natal exposure study with rats (see Annex 3) a safety factor of 50 to 57 results. The dose at the lowest observed effect level differs from the calculated human exposure by cosmetics by a factor of 178. Fat levels in the offspring at the lowest effective dose (LOAEL) were a factor of 50 to 150 higher than human fat levels and pup fat levels at the NOAEL are 10 to 50 times higher than concentrations in human fat tissues.

4 Identification of Possible Measures

4.1 Review of Agreed National and International Measures

So far, no action to limit or phase out the use of Musk xylene and other musks has been taken by OSPAR. Other national and international bodies, however, have already taken such action.

In June 1981 the International Fragrance Association (IFRA) in Geneva, Switzerland, recommended in its Code of Practice to limit the use of Musk ambrette to 4% in new fragrance compounds. According to the last amendment in July 1994, the Committee recommends that Musk ambrette should no longer be used as a fragrance ingredient. The same recommendation was made for 1,1,4,4-tetramethyl-7-acetyl-1,2,3,4-tetrahydronaphthalene (AETT) in February 1980.

In July 1995 the use of Musk ambrette was banned in the EU by including it in the list of banned components in cosmetics (95/34/EEC; 18th amendment of the Cosmetic Directive 76/768/EEC). The same action was taken in 1998 for Musk tibetene and Moskene (98/62/EC of 3 September 1998). The use of Musk ambrette in cosmetics was also banned in Switzerland (Ordinance on Cosmetic Products, SR 817.042.1; Amendment of 30. January 1998). For other nitro musks limit concentrations for several cosmetic products were introduced (50 mg/kg in deodorants, products for skin care etc.; 200 mg/kg in aftershaves, skin tonics etc; 500 mg/kg in Eaux de Cologne and other toilet waters). There are no limitations for perfumes and extraits. A ban for Musk tibetene and Moskene is in preparation.

In Germany (1993) and in Switzerland (1994), the industry associations responsible for detergents recommended to their member companies not to use Musk xylene any more in washing and cleaning agents. So far no contracting party of the OSPAR Convention has taken legally binding measures to restrict the use of Musk xylene or other musks in such products. Musk ketone, Musk xylene, HHCB and AHTN met the criteria to be high production volume chemicals in the sense of Art. 3 of EEC Council Regulation No 793/93 on the evaluation and control of the risks of existing substances. Musk ketone and Musk xylene have been included in the third EU priority list of existing substances (Regulation 143/93) and the Netherlands was appointed as rapporteur. Draft risk assessment reports are not yet available and follow-up actions of the EU risk assessment cannot be expected in the near future. HHCB and AHTN are substances for which industry has filed full HEDSETs (Harmonised Electronic Data Set) under the Existing Substances Regulation 793/93/EC because they are produced in the EU in excess of 1000 tonnes per year. For the other musks the produced or imported quantity into the customs territory of the European Union is below 1000 tonnes per year and regulation 793/93 does not apply at all or only a limited data reporting is required according to the provisions of article 4.

Due to the actions already taken, and the general concern about the environmental fate and toxicology of nitro musks, their importance as fragrances is decreasing. As can be seen from the data presented in chapter 3 the use of Musk xylene has decreased by about 50% (from 174 tonnes in 1992 to 86 tonnes in 1998) in the last six years. For Musk ketone the decrease is even more important during the same period. In Germany and Switzerland voluntary actions to phase out Musk xylene in detergents and cleaning agents have been successful. This is demonstrated by recent monitoring data. Musk xylene could be detected only in one of 30 surface water samples taken in 1996 at different sites in the Berlin area (Heberer et al. 1999), and in sewage sludge, Musk xylene was detected only in one of 12 treatment plants sampled at different locations in Switzerland (Berset and Herren 1999).

The importance of polycyclic musks as fragrances, on the other hand, was expected to increase because they can be used as substitutes for nitro musks. The use volumes reported in chapter 2, however, do not confirm this concern.

4.2 Desired Reduction and Need for Programmes and Measures

4.2.1 Nitro musks

Musk xylene appears on the list of chemicals for priority action in the OSPAR Strategy with regard to Hazardous Substances. The strategy stipulates that the Commission will carry forward the drawing up of programmes and measures in relation to this list. The Commission will continue to work towards the reduction, by the year 2000, of discharges, emissions and losses of hazardous substances which could reach the marine environment, to levels that are not harmful to humans or nature. The ultimate target is the cessation of discharges, emissions and losses of hazardous substances by the year 2020.

The risk assessment based on PEC/PNEC considerations according to internationally agreed methods for fresh water does not indicate a concern for the aquatic environment based on the data actually available. Only a few monitoring data are available for the North Sea. Based on the highest reported concentration in the North Sea a PEC/PNEC ratio of 0.0002 results for Musk xylene, which corresponds to a safety factor of about 6500. For the catchment area the PEC/PNEC ratios for the aquatic environment, including sediment and fish eating predators are higher, but still at or below 0.1 if calculated exposure data are used. Even lower ratios result when monitoring data are used instead of calculated PECs. Therefore, the risk of Musk ketone and Musk xylene for organisms in the aquatic environment, including sediment organisms, is low, as well as the risk of secondary poisoning of fish-eating birds and mammals.

The method for derivation of Ecological Assessment Criteria (EAC) gives similar results. The concentrations of musk xylene in the maritime area are lower than EACs. At some sites of the catchment area freshwater concentrations are above EACs, while at others they are well below. It can be concluded that the concentrations of Musk xylene in the maritime environment appear to be at a safe level.

These assessments, however, have some shortcomings:

- The PEC/PNEC approach is regarded as an inappropriate tool for the protection of the marine environment by many experts. The OSPAR ad hoc Working Group of a Dynamic Selection and Prioritisation Mechanism for Hazardous Substances (DYNAMEC) examines the development of risk assessment techniques for the marine environment in co-operation with experts of the European Union
- The risk of secondary poisoning was not assessed directly based on the risk Musk xylene may pose for marine predators due to the lack of toxicity data for such species.
- Metabolites have not been included in the assessments. Degradation products of Musk Xylene have repeatedly been detected in freshwater samples, sewage effluents, sewage sludge and biota. So far little is known about their toxicity. Tests with Daphnia magna demonstrate that primary degradation of Musk xylene does not result in detoxification. It could, however, not be confirmed that the 4-amino metabolite of Musk xylene is by three orders of magnitude more toxic than the parent compound

As a consequence the levels of Musk xylene in the North Sea and the OSPAR catchment area cannot be considered as safe. More data are needed to refine the assessment (toxicity of metabolites for additional species; toxicity for marine predators; levels of Musk xylene in predatory mammals). As a precautionary measure discharges, losses and emissions of Musk xylene should be reduced further.

4.2.2 Polycyclic musks

Polycyclic musks - possible substitutes for musk xylene - have a similar hazard profile to nitro musks. HHCB and AHTN have been detected in many samples of wastewater, surface water, sewage sludge, suspended matter, sediments and biota at levels exceeding those of Musk xylene. The ubiquitous presence of these substances in the aquatic environment may be explained by the continuous input of these chemicals through sewage treatment plant effluent discharges into the aquatic environment and the low degradation rates. The concentrations detected so far seem to be at a safe level and the formation of toxic metabolites has not been reported. The risk of causing adverse effects, therefore, is lower for polycyclic musks than for nitro musks and immediate action is not necessary.

4.3 Choice of action/measures

Due to their use in consumer products (laundry detergents, cleaning agents, fabric softeners, cosmetics etc.) musk ingredients are discharged into the domestic wastewater. Technical measures for emission control are not practicable. The quantities released into the environment are directly correlated with the use figures. Possible measures and recommended actions to reduce the emissions into the environment are:

- As a precautionary measure nitro musks should not be added to consumer products which are discharged with waste water and which are consumed in large quantities such as detergents and household cleaning agents.
- The presence of nitro musks should be indicated on the label of consumer products.
- The content of all nitro musks and polycyclic musks in consumer products that are discharged with waste water should be reduced to the lowest level needed for technical reasons.
- The quantities of nitro musks and polycyclic musks used in Europe should be monitored and reported regularly.
- The concentrations of nitro musks and polycyclic musks should be measured by analysis in selected samples of the aquatic compartment and reported regularly.
- An additional review for actions or measures should be considered if quantities used or concentrations found are increasing during consecutive years.
- In the long term, alternatives with a more favourable hazard profile, e.g. substances for which evidence for inherent primary degradation in standardised tests for inherent biodegradability (> 20 % elimination) is given, should be developed as substitutes for nitro musks and polycyclic musks.

Voluntary agreements with industry associations have been proven to be an appropriate tool for the phasing out of Musk xylene in washing and cleaning agents. As a possible alternative tool, most elements of the recommended actions above could also be integrated into a PARCOM Recommendation or a BEP. If such measures are not successful, the introduction

legislation on detergents and cleaning agents must be envisaged to achieve the long-term goal. Other bodies (EC, OECD) would have to be approached with a view to establishing such a measure.

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Annex 1: Monitoring Data

Table 4: Concentrations of Musk ketone, Musk xylene and metabolites in waste water and surface water

Sample/Location	n	year	Musk ketone [µg/l]	Musk xylene [µg/l]	reference
Influent 9 STPs Switzerland	9	1993	mean: 0.22 0.09-0.44	mean: 0.27 0.09-0.70	SAEFL 1995
Influent 9 STPs	9	1994	mean: 0.16 0.07-0.44	mean: 0.16 0.08-0.30	
Effluent 9 STPs	9	1993	mean: 0.18 0.06-0.44	mean: 0.06 <0.02-0.10	
Effluent 9 STPs	9	1994	mean: 0.09 <0.02-0.32	mean: 0.03 <0.02-0.09	
River water Switzerland (n=3)	3	1995	med.: 0.025 0.005-0.03	med.: 0.0013 <0.0002-0.007	Schmid 1997
Lake water Switzerland (n=3)	4	93/95	med.: 0.0025 <0.0002-0.0034	med.: 0.0006 <0.0002-0.001	
Influent 25 STPs Ruhr basin (Germany)	19	1994	med.: 1.5 0.57-2.4	med.: 0.68 0.09-1.7	Eschke et al. 1994a
Effluent 25 STPs	36		med.: 0.63 0.22-1.3	med.: 0.12 0.03-0.31	
Tributaries Ruhr	3	1994	0.18-0.24	0.03-0.04	
Ruhr	31		mean: 0.03	mean: 0.01	
Influent STP (Hamburg)	1	1996	MK: 0.55 2-AMK*: nq	MX: 0.15 2-AMX/4-AMX*: nq	Gatermann et al. 1998
Effluent STP (Hamburg)	1		MK: 0.006 2-AMK: 0.25	MX: 0.01 2-AMX: 0.01 4-AMX: 0.034	
Elbe (Hamburg, downstream effluent of the STP)	3	1995	MK: nq-0.004 2-AMK: 0.007 (n = 1)	MX: nq-0.002 2-AMX: nq-0.001 4-AMX: 0.001-0.009	
Effluent STP (Schleswig-Holstein, Germany)	2	1997	MK: 0.07-0.09 2-AMK: 0.015-0.017	MX: 0.002-0.003 2-AMX: <0.001 4-AMX: 0.012-0.015	Rimkus et al. 1999
Effluent STP (Schleswig-Holstein, Germany)**	1	1997	MK: 0.013 2-AMK: 0.020	MX: <0.003 2-AMX: <0.003 4-AMX: 0.007	
Elbe (Brunsbüttel)	1	1993	0.0046	0.0008	Gatermann et al. 1995
Elbe (Brunsbüttel)	1	1995	MK: nq (<0.001)	MX: nq (<0.001) 2-AMX: nq (<0.0005) 4-AMX: 0.002	Gatermann et al. 1998

* Transformation products of MK and MX; 2-AMK = 2-amino musk ketone; 2-AMX/4-AMX = 2-amino musk xylene/4-amino musk xylene

** 24h bulked composite water sample

nq: not quantifiable

Table 4: Concentrations of Musk ketone, Musk xylene and metabolites in waste water and surface water

Sample/Location	n	year	Musk ketone [µg/l]	Musk xylene [µg/l]	reference
Elbe (Magdeburg)	31	96/97	mean: 0.006 med.: 0.006 <0.002-0.01 90%: 0.009	all: nq (<0.002)	Winkler et al. 1998
Elbe (Magdeburg) water samples after centrifugation	31	96/97	mean: 0.005 med.: 0.005 <0.002-0.011 90%: 0.008	all: nq (<0.002)	
Surface water Berlin (canals, rivers, lakes upstream and downstream of STP outlets)	30	1996	mean: 0.08 med.: 0.035 nq-0.39 90%: 0.23	med.: nq 0.18 (1 of 30 samples)	Heberer'99
Rhine & Meuse (The Netherlands)	31 34	1996	nq (<0.01)	nq (<0.01)	Breukel'96 in Tas et al 1997
Seawater North Sea (German Bight)	30	1993	med.: nq (<2e-5) nq-0.00008 90%: 0.00005	med.: 0.000035 nq-0.00017 90%: 0.00012	Gatermann et al. 1995

* Transformation products of MK and MX; 2-AMK = 2-amino musk ketone; 2-AMX/4-AMX = 2-amino musk xylene/4-amino musk xylene

** 24h bulked composite water sample

nq: not quantifiable

Table 5: Concentrations of HHCB and AHTN in waste water and surface water

Sample/Location	n	year	HHCB [µg/l]	AHTN [µg/l]	reference
Effluent 17 STPs	17	1998	med.: 2.3	med.: 1.4	SAEFL 1998
River Aare basin (Switzerland)			1.1-5.6 90%: 4.7	0.5-2.4 90%: 2.0	
Tributaries River Aare (Switzerland)	11	1998	med.: 0.06 <0.025-0.244 90%: 0.095	med.: 0.029 <0.025-0.095 90%: 0.047	
River Aare	9		med.: 0.06 <0.025-0.118	med.: nq (<0.025) <0.025-0.04	
River Glatt	1	...	0.136	0.075	Müller et al. 1996
River water Switzerland (n=3)	3	1995	med.: 0.125 0.044-0.435	med.: 0.053 0.026-0.128	Schmid 1997
Lake water Switzerland (n=3)	4	93/95	med.: 0.062 0.026-0.206	med.: 0.024 0.015-0.043	

Table 5: Concentrations of HHCB and AHTN in waste water and surface water

Sample/Location	n	year	HHCB [µg/l]	AHTN [µg/l]	reference
Effluent 3 STPs Ruhr basin (Germany)	21	1994	med.: 1.2 90%: 2.3	med.: 1.6 90%: 3.0	Eschke et al. 1995a
River Ruhr	23		mean: 0.37 <0.03-0.50	mean: 0.20 <0.03-0.30	Eschke et al. 1994b
River Elbe (Stade)	1	1995	0.095	0.067	Bester 1998
Elbe (Magdeburg)	31	96/97	mean: 0.114 med.: 0.118 0.036-0.152 90%: 0.146	mean: 0.066 med.: 0.073 0.024-0.088 90%: 0.083	Winkler et al. 1998
Elbe (Magdeburg) water samples after centrifugation	31	96/97	mean: 0.089 med.: 0.094 0.036-0.126 90%: 0.117	mean: 0.050 med.: 0.054 0.024-0.071 90%: 0.067	
River Saale (Germany)	6	96/97	mean: 0.4 0.2-0.61	mean: 0.24 0.14-0.33	ARGE'96 in Rimkus et al. 1998a
Surface water Berlin (canals, rivers, lakes upstream and downstream of STP outlets)	30	1996	mean: 2.5 med.: 0.85 0.02-12.5 90%: 8.5	mean: 1.34 med.: 0.50 0.03-6.78 90%: 4.81	Heberer'99
Influent 3 STPs The Netherlands	9	...	med.: 6.4 Max.: 14.5	med.: 4.0 max.: 8.7	Rijs 1998 in Balk 1998
Effluent 3 STPs	8		med.: 1.4 Max.: 1.6	med.: < detect. limit max: 0.77	
Surface water The Netherlands	24	...	med.: 0.05 90%: 0.40	med.: 0.15 90%: 0.96	
River Rhine (NL) filtered water samples	32	94-96	med.: 0.06 0.01-0.22	med.: 0.05 0.01-0.13	Breukel '96 in RIVM, Dec. 1997
River Meuse (NL) filtered water samples	32	94-96	med.: 0.08 0.01-0.26 90%: 0.19	med.: 0.07 0.01-0.4 90%: 0.11	
Seawater North Sea (German Bight)	12	90/95	1995 (n=6): med.: 0.00026 0.00015-0.0048	med.: 0.0002 0.0001-0.0026 90%: 0.0009	Bester 1998

Table 6. Concentrations of other nitro musks and polycyclic musks in waste water and surface water (ng/l)

Sample/Location	n	year	Moskene	musk tibetene	ADBI	other musks	reference
Effluent 9 STPs (Switzerland)	...	1994	nq (<10)	nq (<10)			SAEFL 1995
Effluent 17 STPs River Aare Basin (Switzerland)	17	1998			med.: nq (<100) <100-160 90%: 124		SAEFL 1998
Tributaries River Aare	11	1998			nq (<10)		
River Glatt (downstream STP)	1	...	0.08	0.04	3.2		Müller et al. 1996
River water Switzerland (n=3)	3	1995	nq (<0.2)	med.: <0.2 <0.2-0.6	med.: 2.2 0.8-10.9		Schmid 1997
Lake water Switzerland (n=3)	4	93/95	med.: nq (<0.2) <0.2-0.7	med.: 0.45 <0.2-1.3	med.: 2.65 1.3-3.4		
Effluent 3 STPs catchment area Ruhr (Germany)	15	1994			med.: 80 90%: ≈ 100		Eschke et al. 1995a
River Ruhr	23				nq (<30)		Escke et al. 1994b
River Elbe (Magdeburg)	31	96/97			mean: 4 <2-8 90%: 7		Winkler et al. 1998
River Elbe (Magdeburg) water samples after centrifugation	31	96/97			mean: 3 <2-5 90%: 5		
Surface water Berlin (canals, rivers, lakes upstream and downstream of STP outlets	30	1996	29 of 30 samples: nq (<10) max: 170	all: nq (<10)	mean: 104 med.: 50 10-520 90%: 311	musk ambrette: all: nq (<10)	Heberer'99

Table 6: Concentrations of other nitro musks and polycyclic musks in waste water and surface water (ng/l)

Sample/Location	n	year	Moskene	musk tibetene	ADBI	other musks	reference
Influent 3 STPs (The Netherlands)	9	...			med.: nq max: 290	AHMI: med.: nq; max.: 700 AITI: med.: nq; max.: 570	Rijs 1998 in Balk 1998
Effluent 3 STPs	8				all: nq	AHMI, AITI: nq	
Surface water (The Netherlands)	24	...			all: nq	AHMI, AITI: all: nq	
River Rhine & Meuse (conc. truly dissolved)	32	94-96				AHMI: occasionally detected AITI: regul. 20	Breukel'96 in RIVM, Dec. 1997

Table 7: Concentrations of nitro musks in sewage sludge, suspended matter and sediments

Sample/Location	n	year	Musk ketone	musk xylene	other NM	reference
Sewage sludge [µg/kg d.s.]						
12 STPs Switzerland	12	1998	mean: 5.2 (7 of 11) 90%: 6.9 2-AMK: nq in 11 of 12 samples max: 13.1	nq in 11 of 12 samples max: 32.5 4-AMX: mean: 24.6 (6 of 12) med.: 5.5 90%: 30.8	Moskene: all: nq Amosk: mean: 10.7 (5 of 12) med.: nq 90%: 7.8	Berset & Herren 1999
2 STPs Germany	2	...	mean: 30 <10-60	mean: <5 <5		Sauer et al. 1997
Suspended matter [µg/kg]						
River Elbe (Magdeburg)	31	96/97	mean: 9 med.: 7 4-22 90%: 13	all: nq		Winkler et al. 1998
River Schwarzbach & Rodau (G'many)	13	1996	med.: 106 24-408	med.: 8 nq-46		Fooken'97 in Rimkus et al. 1998a
River Rhine (NL)	14	94-96	nq (<500)	nq (<500)		Breukel'96 in Tas et al. 1997
River Meuse	14	94-96	nq (<500)	med.: nq (<500) max.: 1000		
Sediments of rivers [µg/kg]						
5 Rivers Niedersachsen (Germany)	16	95/96	med: 0.45 0.2-3.8 90%: 1.0	med.: 0.6 <0.1-2.2 90%: 0.95		Lach & Steffen 1997
River Elbe (Hamburg-Dresden)	9	1997	mean: 1.1 <0.5-4.4	mean: 2 <0.5-7.7		ARGE'97 in Rimkus et al. 1998a
River Elbe (Hamburg-Teufelsbrück)*	3	1996	0.14-0.19	0.19-0.3 2-AMX: 0.003-0.054 4-AMX: 0.08-0.13		Rimkus et al. 1999

Transformation products: 2-AMK = 2-amino musk ketone, 4-AMX = 4-amino musk xylene; Amusk = Amino muskene

* Concentrations on a wet weight basis. nq: not quantifiable

Table 8: Concentrations of HHCB and AHTN in sewage sludge, suspended matter and sediments

Sample/Location	n	year	HHCB	AHTN	Reference
Sewage sludge [µg/kg d.s.]					
12 STPs (Switzerland)	12	1998	mean: 4853 med.: 3896 2293-12'157 90%: 7853	mean: 1537 med.: 1321 741-4161 90%: 1964	Berset & Herren 1999
2 STPs (Germany)	2	...	mean: 8870 4300-13'400	mean: 8300 4000-12'600	Sauer et al. 1997
STPs (Hessen, Germany)	9	1996	mean: 16'930 11'886-21'626	mean: 15'450 11'992-20'107	Fooken'97 in Rimkus et al. 1998a
STPs (NL)*	13	...	med.: 23'000 9000-31'000	med.: 16'000 4900-22'000	Blok 1998
4 STPs (NL) digested sludge	8	1997	mean: 23'000 11'000-31'000 s.d.: 7200	mean: 14'800 6200-21'000 s.d.: 5000	Blok 1998
Suspended matter [µg/kg]					
River Elbe (Magdeburg)	31	96/97	mean: 440 med.: 442 148-736 90%: 613	mean: 468 med.: 458 194-770 90%: 613	Winkler et al. 1998
River Schwarzbach & Rodau (G'many)	13	1996	med.: 2641 897-13'722	med.: 2948 544-12'666	Fooken'97 in Rimkus et al. 1998a
	14		med.: 60 <50-160	med.: 240 100-540	Breukel'96 in RIVM, Dec. 1997
River Meuse (NL)	14	94-96	med.: 200 50-580	med.: 840 60-1200	
Rhine & Meuse	28	94-96	90%: 310	90%: 960	
Sediments of rivers [µg/kg]					
River Elbe (Hamburg-Dresden)	9	1997	mean: 101 16-180	mean: 47 7.2-104	ARGE'97 in Rimkus et al. 1998a
5 Rivers Niedersachsen (Germany)	8	1996	med.: 7.15 <0.5-54	med.: nq (<0.5) <0.5-3.9	Lach & Steffen 1997

*Concentrations in digested, thickened and composted sludge
s.d.: standard deviation

Table 9: Concentrations of other polycyclic musks in sewage sludge, suspended matter and sediments

Sample/Location	n	Year	ADBI	Other PM	Reference
Sewage sludge [µg/kg d.s.]					
12 STPs Switzerland	12	1998	mean: 130 med.: 113 41-330 90%: 234	AHMI: mean: 303 med.: 203 65-842 90%: 781 DPMI: mean: 93 med.: 68 38-332 90%: 142	Berset & Herren 1999
2 STPs Germany	2	...	mean: 200 120-290		Sauer et al. 1997
4 STPs (NL) digested sludge	8	1997	med.: 1100 <1000-1800	AHMI: med.: 1250 <1000-2200 AITI: mean: 2200 s.d.: 855 med.: 1950 1200-3600	Blok 1998
Suspended matter [µg/kg]					
River Elbe (Magdeburg)	31	96/97	mean: 15 med.: 16 <4-43 90%: 23		Winkler et al. 1998
Sediments of rivers [µg/kg]					
5 Rivers Niedersachsen (Germany)	8	1996	med.: nq (<0.5) <0.5-0.7	AITI: med.: nq (<0.5) <0.5-1.8 DPMI, AHMI: all: < 0.5	Lach & Steffen 1997

s.d.: standard deviation

Table 10: Concentrations of nitro musks in biota samples (muscle samples) from various sampling sites (concentrations in µg/kg fresh weight)

Sample/Location	n	Year	Musk ketone	Musk xylene	Reference
Freshwater fish (Switzerland)	41	1992		nq-70 (nq in 20 of 41 samples)	Kant. Lab. BE'92
Freshwater fish (Alosa fallax lacustris) Lake Verbano	270	1995	med.: 0.5 0.3-0.7	med.: 0.8 0.5-1.1	Ceschi et al. 1996
Freshwater fish (Southern Bavaria)	102	1992		mean: 12 nq-240	In Geyer et al. 1994
Trout from fish farm (Southern Bavaria)	50	1992		mean: 3 nq-31 (nq in 23 of 50 samples)	
Eel (The Netherlands)	13		med.: 20 <4-60	med.: 30 <4-80	Wiertz'95 in Tas et al. 1997
Fish (non-eel) from River Ruhr	7	...	mean: 6.7 3-13	mean: 5.7 <1-9	Eschke et al. 1994a
Eel from River Ruhr	2	...	mean: 52 37-66	mean: 66 37-95	
Fish (non-eel) from River Elbe*	5	1997	med.: 0.14 0.07-1.27	med.: 0.27 0.07-0.50	Rimkus et al. 1998b
Fish (non-eel) from effluent pond	8	...	mean: 119 74-204	mean: 37 21-58	Eschke et al. 1994a
Eel from effluent pond	5	...	mean: 1278 1024-1605	mean: 639 559-753	
Fish (non-eel) from effluent pond*	13	1997	med.: 17 2-67 90%: 30	med.: 4.8 0.1-17 90%: 8.8	Rimkus et al. 1998b
Eel from effluent pond*	1**	1997	63	37	

nq = not quantifiable

* Values based on fresh weight for data from Rimkus et al. (1998b) were calculated from data based on fat content and fat contents of fish given by the authors

** pooled sample of 12 eels

Table 11: Concentrations of polycyclic musks in biota samples (muscle samples) from various sampling sites (concentrations in µg/kg fresh weight)

Sample/Location	n	Year	HHCB	AHTN	Reference
Fish (non-eel) from River Ruhr	7	...	med.: 17 10-45	med.: 29 18-34	Eschke et al. 1994b
Eel from River Ruhr	2	...	mean: 112 97-126	mean: 134 124-143	
Fish (non-eel) from effluent pond*	7	...	med.: 144 23-345	med.: 291 69-647	Eschke et al 1995a
Eel from effluent pond	3	...	med.: 13'200 1530-19'200	med.: 13'200 2560-17'500	

* Values based on fresh weight for data from Eschke et al. (1995) were calculated from data based on fat content and fat contents of fish given by the authors

Table 12: Concentrations of nitro musks and their amino metabolites in biota samples (muscle samples) from various sampling sites 1997. Concentrations on a lipid basis, in µg/kg fat (Rimkus et al. 1998b)

Sample/Location	Lipid %	MX	4-NH_2-MX	2-NH_2-MX	MK	2-NH_2-MK
Fish (pike/perch) from river Elbe; n=5	0.3-0.7	med.: 39 23-99	med.: 14 8-161	med.: 6 <3-14	med.: 27 23-182	med.: 13 <3-17
Fish (bream) from tributary of Elbe, a.) upstream	1.2	78	122	7	235	16
b.) downstream	1.7	10	41	2	85	10
Rainbow trout from various aquacultures, n=7	2.3-4.2	med.: 56 11-332	med.: 30 20-64	med.: <2 <1-3	med.: 50 22-117	med.: 2.5 <1-23 (n=6)
Fish from effluent pond; n=13	0.7-4.3	med.: 280 18-395 90%: 359	med.: 72 28-151 90%: 117	med.: 8 <2-18 90%: 15	med.: 1170 300-1560 90%: 1440	med.: 24 7-34 90%: 33
Eel from effluent pond; n=1**	17.9	205	5	2	350	6
Mussels (Dreissena polymorpha) from effluent pond	1.4	121	152	13	1230	not analysed

* Rudd (Scardinius erythrophthalmus) tench (Tinca tinca), crucian carp (Carassius carassius)

** Pooled sample of 12 eels

Table 13: Concentrations of polycyclic musks in biota samples (muscle) from various sampling sites (concentrations on a lipid basis, µg/kg fat)

Sample/Location	n	year	HHCB	AHTN	ADBI	AHMI	AITI	reference
Fish from River Ruhr (Germany)	9	...	med.: 2500 400-3800	med.: 3200 500-7100	all: nq			Eschke et al. 1995a
Fish (eel) from River Elbe	5	...	med.: 40 30-90	med.: 50 40-120	all: <10	all: <10	med.: <10 <10-20	Rimkus & Wolf 1998 in Rimkus 1997
Fish (pike/perch) from River Elbe	4	...	med.: 2395 600-3840	med.: 505 320-990	all: <100	all: <100	med.: <10 <10-110	
Rainbow trout from aqua-culture (Denmark)	4	...	med.: 340 110-650	med.: 320 200-590	all: < 30	all: < 30	med.: <30 <30-40	
Fish from effluent pond (Germany)	11	...	med.: 7600 1000-63'600	med.: 15'300 3000-57'900	med.: nq nq-1000			Eschke et al. 1995a
Mussels (Dreissena poly-morpha) from effluent pond (Germany)	!		120	45				Rimkus & Wolf 1998 in Rimkus et al. 1998a
Fish (brook trout) from Tributary of Elbe, down-stream STP outfall	2	...	mean: 17'000 13'700-20'300	mean: 12'000 10'600-13'400	mean: 925 730-1120	mean: 205 200-210	mean: 720 600-840	Rimkus & Wolf 1998 in Rimkus 1997
Herring East sea	1	...	750	530	<10	10	30	
Herring (DK, Lofoten)	1	...	<10	<10	<10	<10	<10	
Mussels (Miesmuscheln) North sea	3	...	<30-110	<30-60				Rimkus & Wolf 1998 in Rimkus et al. 1998a
Craps North sea	4	...	<40-370	<40-60				

Table 14 Concentrations (Conc.) and enantiomeric ratios (ER) of polycyclic musks in biota caught in the pond of a municipal STP in 1997. Concentrations on a lipid basis, in µg/kg fat (Hühnerfuss et al. 1999)

Species	n		HHCB	Trans-HHCB	cis-HHCB	AHTN		trans-ATII		AHDI	
			Conc.	ER	ER	Conc.	ER	Conc.	ER	Conc	ER
Rudd	3	mean	6.93	0.59	1.14	5.60	0.88	0.50	0.98	0.30	0.99
		v	8	8	7	8	7		1		10
Tench	4	mean	155	1.0	0.98	36	1.8	2.27	0.45	2.17	1.35
		v	3	9	2	12	8	11	12	12	6
Crucian carp	7	mean	66	0.13	0.47	32	1.16	1.57	0.09	2.54	0.74
		v	26	22	9	12	5	15	56	16	14
Eel	2	mean	4.7	0.89	1.21	2.65	0.87	0.3	1.73	0.2	0.61
Mussel	1		120	0.91	1.26	45	0.90	3.4	0.67	2.3	1.02

v = s.d./mean [%], s.d. = standard deviation

Table 15: Concentrations of nitro musks and polycyclic musks in human samples (Concentrations on a lipid basis, in µg/kg lipid except blood samples in µg/l plasma)

Human Sample/Location	n	year	musk xylene	musk ketone	Other nitro musks	HHCB	AHTN	other poly-cyclic musks	reference
Breast milk (Southern Bavaria, Germany)	391	91/92	med.: 70 10-1220 90%: 210	med.: 30 <10-240 90%: 80	musk ambrette: med.: 30 <10-290 90%: 70				Liebl & Ehrens-torfer 1993
Breast milk (Schleswig-Holstein, Germany)	23	92/93	med.: 60 20-190	med.: 20 10-90	musk tibetene: all: <10 (d.l.)				Rimkus et al. 1994
Breast milk (Schleswig-Holstein, Germany)	5	93/95	med.: 30 10-30	med.: 10 5-15		med.: 37 16-108	med.: 22 11-58	ADBI: med.: 6 1-18 AHMI, AITI, AETT: < d.l (all)	Rimkus & Wolf 1996
Breast milk (Essen, Germany)	2	...	29-56	7-21		310-360	250-290	ADBI: 20-24	Eschke et al. 1995b in Rimkus 1997
Breast milk (Middle Hessen, Germany)	55	1995	mean: 57 11-252 75%: ≈65	mean: 20 <1-102 75%: ≈25					Ott et. al. 1999 in Rimkus 1998
Adipose tissue (Essen, Germany)	2	...	26-80	4-5		145-149	56-72	ADBI: 3-9	
Adipose tissue (Switzerland)	4	83/84	med.: 26.5 13-59	med.: 4.5 4.2-40	musk ambrette: med.: 12.5 <1-67 others[1]: < 1	med.: 15.5 12-93	med.: 8.6 1.9-12	ADBI: med.: 0.95 0.22-3.4	Müller et al. 1996

[1] musk moskene, musk tibetene

Table 15: Concentrations of nitro musks and polycyclic musks in human samples (Concentrations on a lipid basis, in µg/kg lipid except blood samples in µg/l plasma)

Human Sample/Location	n	year	musk xylene	musk ketone	Other nitro musks	HHCB	AHTN	other poly-cyclic musks	reference
Adipose tissue (Switzerland)	11	1994	med.: 36 6.7-288	med.: 5.8 <1-173	musk ambrette: 18 (1/11)[2] musk moskene: <1-42 (4/11)[2] musk tibetene: all: < 1 (d.l)	med.: 69 12-171	med : 5.1 1.0-23	ADBI: med.: 0.57 0.12-3.5	
Adipose tissue samples from females (Germany)	13	1992	med.: 60 20-220	med : 20 10-220	musk tibetene: all: <10 (d.l.)				Rimkus et al. 1994
Adipose tissue samples from males (Germany)	19	1993	med.: 50 20-90	med : 20 10-30	musk tibetene: all: <10 (d.l.)				
Adipose tissue (Schleswig-Holstein, Germany)	14	93/95	med.: 20 5-50	med : 7.5 5-30		med.: 78.5 28-189	med.: 18.5 8-33	ADBI: med.: 2 (<1-3) AHMI: 1-5 (8/14)[2] ATTI: 8-10 (4/14)[2]	Rimkus & Wolf 1996
Blood (Germany)	72	...	mean: 0.31 0.05-1.12						Käfferlein et al. 1997
Blood (fish consumer, once per week)	24	...	mean: 0.33 0.05-1.12						
Blood samples from males (Germany)	85	1998	mean: 0.077 95%: 0.158	mean 0.067 95%: 0.181	musk ambrette: all: <0.02 (d.l.)	mean 0.764 95%: 1.51	mean 0.340 95%: 0.69		Bauer & Frössl 1999
Blood samples from females (Germany)	328	1998	mean: 0.091 95%: 0.243	mean: 0.072 95%: 0.186		mean: 0.713 95%: 1.74	mean: 0.257 95%: 0.63		
Blood (Switzerland)	11	...	0.07-0.27						Helbling et al.'94

[2] Frequency of detection; d.l = detection limit

Annex 2: Ecotoxicity Data

Several studies concerning the aquatic toxicity of the nitro musk ingredients are available. The most reliable studies are summarised in table 17. The lowest reported NOEC values, which have been used for the risk assessment, are: 0.063 mg/l (fish) for Musk ketone and 0.056 mg/l (Daphnia) for Musk xylene.

Table 17: Aquatic toxicity of nitro musks

	Bacteria	Algae	crustacean	Fish
Moskene	Vibrio fischeri EC50 30min > 0.037 mg/l Schramm et al. 1996	Scenedesmus subspicatus EC50 72h > 0.046 mg/l (no effect at water solubility) Schramm et al. 1996	Daphnia magna EC50 48h > 0.046 mg/l (no effect at water solubility) Schramm et al. 1996	
Musk tibetene	Vibrio fischeri EC50 30min > 0.042 mg/l Schramm et al. 1996	Scenedesmus subspicatus EC50 72h > 0.052 mg/l (no effect at water solubility) Schramm et al. 1996	Daphnia magna EC50 48h > 0.052 mg/l (no effect at water solubility) Schramm et al. 1996	
Musk ketone	Vibrio fischeri EC50 30min > 0.34 mg/l Schramm et al. 1996	Selenastrum capricornutum ErC50 72h = 0.244 mg/l Tas et al. 1997	Daphnia magna EC50 48h > 0.46 mg/l (no effect at water solubility) Schramm et al. 1996	Oncorhynchus mykiss NOEC 21d = 0.063 mg/l Tas et al. 1997
		Scenedesmus subspicatus EC50 72h > 0.46 mg/l (no effect at water solubility) Schramm et al. 1996	Daphnia magna EC50 21d (reproduction) = 0.169-0.338 mg/l Tas et al. 1997	
Musk xylene	Vibrio fischeri EC50 30min > 0.12 mg/l Schramm et al. 1996	Selenastrum capricornutum NOEC 5d > 5.6 mg/l Tas et al. 1997	Daphnia magna NOEC 48h = 0.32 mg/l Tas et al. 1997	Bluegill sunfish LC50 96h = 1.2 mg/l Tas et al. 1997
		Scenedesmus subspicatus EC50 72h > 0.15 mg/l (no effect at water solubility) Schramm et al. 1996	Daphnia magna NOEC 21d (reproduction) = 0.056 mg/l Tas et al. 1997	Brachydanio rerio LC50 14d = 0.4 mg/l Tas et al. 1997

Only a few tests have been performed with terrestrial organisms. The toxicity of Musk xylene to earthworm (Eisenia foetida) was determined in a standard OECD 207 test (Tas et al. 1997). The 14d-NOEC for survival, growth and behaviour was > 50 mg/kg soil. In a similar test with the same organism using musk ketone an 8 week-NOEC for reproduction of 32 mg/kg soil was determined (Tas et al. 1997). The reproduction toxicity of Musk ketone to springtail (Folsomia candida) was measured in an artificial soil test according to a draft ISO guideline (Tas et al. 1997). The 28d-NOEC was found to be 100 mg/kg soil and the LC50 28d greater than 1000 mg/kg soil. It is concluded that both nitro musks are moderately toxic to terrestrial organisms. No test results are available for Moskene and Musk tibetene.

The aquatic toxicity for the polycyclic musks is summarised in table 20. The lowest reported NOEC values which have been used for the risk assessment are: 0.068 mg/l (fish) for HHCB and 0.035 mg/l (fish) for AHTN.

Table 18: Aquatic toxicity of polycyclic musks

	Algae	Crustacean	Fish
AITI		Daphnia magna EC50 48h = 0.42 mg/l Balk 1998	
AHMI	Selenastrum capricornutum EbC50 72h = 0.081 mg/l ErC50 72h = 0.2 mg/l NOEC (growth rate) = 0.044 mg/l AquaSense 1998a	Daphnia magna EC50 48h = 0.33 mg/l AquaSense 1998b	Brachydanio rerio NOEC 96h = 0.9 mg/l AquaSense 1998c
HHCB	Pseudokirchneriella subcapitata EbC50 72h = 0.723 mg/l RIVM, December 1997	Daphnia magna NOEC 21d = 0.111 mg/l RIVM, December 1997	Lepomis macrochirus NOEC 21d = 0.182 mg/l RIVM, December 1997 Pimephales promelas NOEC 36d = 0.068 mg/l RIVM, December 1997
AHTN	Pseudokirchneriella subcapitata EbC50 72h = 0.468 mg/l RIVM, December 1997	Daphnia magna NOEC 21d = 0.196 mg/l RIVM, December 1997	Lepomis macrochirus LC50 21d = 0.314 mg/l RIVM, December 1997 Pimephales promelas NOEC 36d = 0.035 mg/l RIVM, December 1997

No toxicity data are available for ADBI.

The toxicity to terrestrial organisms has been determined for AHTN and HHCB (RIVM, December 1997).

	Earthworm (Eisenia foetida)	springtail (Folsomia candida)
AHTN	NOEC 8 weeks = 105 mg/kg soil	NOEC 4 weeks = 45 mg/kg soil
HHCB	NOEC 8 weeks = 45 mg/kg soil	NOEC 4 weeks = 45 mg/kg soil

Annex 3: Data on Degradation

Several biodegradation tests have been performed with the 4 nitro musks. All of them have shown that these chemicals do not mineralise under standard test conditions.

Musk ketone	Not inherently biodegradable (MITI II)	Tas et al. 1997
Musk xylene	Not readily biodegradable (MITI I)	Tas et al. 1997
Musk moskene	Not inherently biodegradable (MITI II)	Givaudan Roure 1993a
Musk tibetene	Not inherently biodegradable (MITI II)	Givaudan Roure 1993b
HHCB	Not readily biodegradable (mod. Sturm test)	RIVM, December 1997
AHTN	Not inherently biodegradable (MITI II) (<1% biodegradation after 28 days) Low mineralisation (ISO 10708)	RIVM, December 1997 Balk 1998
ADBI	Not inherently biodegradable (MITI II) (<1% biodegradation after 28 days)	Givaudan Roure 1995a
AITI	No data available	
AHMI	Low mineralisation (ISO 10708)	Balk 1998

AHTN, HHCB and AHMI were studied in biodegradation tests where the mineralisation by the production of CO_2 or the uptake of O_2 was determined (Balk 1998). Under these conditions, AHTN and HHCB did not mineralise. A low level of mineralisation (12-21% of ThOD) was found with AHTN and AHMI in a two-phase closed bottle test. All these tests showed that the mineralisation of these compounds is very slow. However AHTN, HHCB and AHMI degrade in soil samples and in cultures inoculated with soil-micro-organisms to various more polar metabolites within a few days to a week. HHCB was degraded by an air-born fungus to a mixture of metabolites. After incubation with a soil slurry, both HHCB and the metabolites were gradually transformed to more polar structures and some CO_2. In a microcosm study with various soil types, HHCB disappeared with a half-life of approx. 3 months. After one year several polar metabolites could be extracted whereas formation of CO_2 was negligible. There are also indications of anaerobic digestion of AHTN and HHCB during sludge treatment (Blok 1998). In conclusion, the mineralisation of polycyclic musks in standard test systems is negligible. Primary biodegradation does however occur in soil.

Recently, the biotransformation of HHCB was examined under realistic conditions in activated sludge (Itrich et al. 1998). A novel test system was established that permitted the detection of metabolites and the measurement of volatiles (parent compound and CO_2 evolved). ^{14}C-HHCB was dosed at a low level (50 µg/l) to freshly collected activated sludge, and disappearance of parent and formation of metabolites were monitored over time. It was found that HHCB is biotransformed during wastewater treatment with an estimated half-life of approx. 35 hours to 2 polar metabolites that have substantially lower Pow values than the parent (measured values: 5.9 for HHCB, 2.0-3.0 for metabolite 1, ca. 0 for metabolite 2). No significant volatilisation of HHCB was observed.

The photodegradation of the 4 nitro musks was examined by irradiating an aqueous solution at room temperature with artificial UV light. The measured half-lives are in the range of 2.0-8.2 minutes (Butte et al. 1996). The half-life of AHTN under similar conditions was determined to be 1.25 minutes (Butte et al. 1997).

Annex 4: Physical Chemical Properties

Nitro musks are poorly soluble in water and have relatively high octanol/water partition coefficients. Measured values for the water solubility reported in the literature (Schramm et al. 1996) vary from 0.046 for Moskene to 0.46 for Musk ketone. For Moskene and Musk tibetene a somewhat higher value of 2 mg/l has also been reported by a manufacturer (Roche 1985). For Musk ketone and Musk xylene the water solubility was also calculated (Isnard & Lambert 1983). The results are similar to the values determined experimentally. The most reliable measured values for log Pow vary from 4.3 (Musk ketone) to 5.3 (Moskene). Lower log Pow values have also been reported before: 4.4 for Moskene and 4.3 for Musk tibetene (Roche 1985), 3.2 for Musk ketone (RIFM data) and 3.4 for Musk xylene (Johnson et al. 1984). Calculated values for Musk ketone and Musk xylene are somewhat lower than the experimentally determined but still of the same order.

Table 21 Water solubility (s) and partition coefficient octanol/water (log Pow) for nitro musks and polycyclic musks

Common Name	S (measured) in mg/l	S (calculated) in mg/l	log Pow (measured)	log Pow (calculated)
Moskene	0.046 (Schramm et al. 1996)		5.3 (Givaudan Roure 1996a)	
Musk ketone	0.46 (Schramm et al. 1996)	1.9 (Isnard & Lambert 1983)	4.3 (Tas et al. 1997)	3.8; 4.3 (Tas et al. 1997)
Musk tibetene	0.052 (Schramm et al. 1996)		5.0 (Givaudan Roure 1996b)	
Musk xylene	0.15 (Schramm et al. 1996)	0.49 (Isnard & Lambert 1983)	4.9 (Tas et al. 1997)	3.7, 4.45 (Tas et al. 1997)
HHCB	1.65 (RIVM, December 1997)	0.19 (Balk 1998)	5.9	6.3 (Balk 1998)
AHTN	1.22 (RIVM, December 1997)	0.36 (Balk 1998)	5.7	6.4 (Balk 1998)
ADBI		0.22 (Balk 1998)	5.4	5.9 (Balk 1998)
AHMI		0.25 (Balk 1998)		5.85 (Balk 1998)
AITI		0.09 (Balk 1998)		6.3 (Balk 1998)

The polycyclic musks HHCB and AHTN have slightly higher water solubilities and octanol/water partition coefficients than the nitro musks. For ADBI the Pow was measured. Only a calculated value is available for water solubility. For AHMI and AITI no experimental data are available at all

Annex 5: Toxicity Data

The fragrance industry has filed dossiers on the nitro musks and two polycyclic musks AHTN and HHCB and submitted these to the EU Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP). A summary of the data available is given below.

Nitro musks

Table 17 summarises the most relevant parameters for the safety assessment.

Table 17: Toxicological data of Musk xylene and Musk ketone (Ford 1998a)

Exposure	Musk xylene	Musk ketone
Dermal toxicity 90-day study	75 mg/kg/day male 24 mg/kg/day female (1990)	75 mg/kg/day (1990)
Genotoxicity	Not genotoxic	Not genotoxic
Developmental toxicity	20 mg/kg/day maternal tox. Developmental NOAEL 200 mg/kg/day	15 mg/kg/day maternal tox. Developmental NOAEL 45 mg/kg/day
Pre- and post-natal	NOAEL 7.5 mg/kg/day	NOAEL 7.5 mg/kg/day
Skin effects: irritation, sensitisation, photoirritation, photosensitisation	No effects at 5% on human skin	No effects at 5% on human skin
NOAEL selected	4.8 mg/kg/day (24 mg/kg/day derm. absorp.)	7.5 mg/kg/day (pre- and post-natal dev. study)
Exposure dermal (as agreed with the SCCNFP)	(181 µg/kg/day) 14 µg/kg/day corr. dermal absorption (7.5% human)	(176 µg/kg/day) 25 µg/kg/day corr. dermal absorption (14% human)

In a developmental rat model (Suter-Eichenberger et al. 1998a) with exposure to Musk xylene of the parent generation, pregnant dams and their offspring, and using enzyme induction as a parameter, a lowest observed effect level of 2.3 - 2.7 mg/kg/day and a NOAEL of 0.7 - 0.8 mg/kg/day (Suter-Eichenberger et al. 1998b) were found.

Polycyclic musks

Table 18 summarizes the most relevant parameters for the safety assessment.

Table 18: Toxicological data of AHTN and HHCB (Ford 1998b)

Exposure	AHTN	HHCB
Oral toxicity 90-day study	NOAEL 15 mg/kg/day (1996)	NOAEL 150 mg/kg/day (1996)
Genotoxicity	Not genotoxic	Not genotoxic
Developmental toxicity	Oral gavage study: 50 mg/kg/day maternal toxicity; no findings	Oral gavage study: 500 mg/kg/day maternal toxicity; at highest dose reduced fetal weight and increased skeletal malformations
Pre- and postnatal milk study	No effects found at the dose of 20 mg/kg/day	No effects found at the dose of 20 mg/kg/day

Skin effects: irritation, sensitisation, photoirritation, photosensitisation	No effects at 10% on human skin	No effects at 10% on human skin
NOAEL selected	15 mg/kg/day	150 mg/kg/day
Exposure dermal as agreed with the SCCNFP	(306 µg/kg/day) 43 µg/kg/day corr. dermal absorption (14% rat)	(764 µg/kg/day) 107 µg/kg/day corr. dermal absorption (14% rat)
Margin of safety	340 (cosmetic products)	1402 (cosmetic products)

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Final Report

CONFIDENTIAL

Study started	11-May-98
Study completed	15-May-98
Study number	1202B.GLP.VIS04
Report number	98.1202-17 (re-issue of 98.1202-10)
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CONFIDENTIALITY STATEMENT

This document contains information and unpublished results of research which may be confidential, and therefore it should not be photocopied or microfilmed. It should also not be released in any form to an outside party, nor should information contained herein be used by any authority without the written permission of the Sponsor.

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with the Principles of Good Laboratory Practice as described in the Revised OECD Principles of Good Laboratory Practice (1996). All data on the test substance such as , characterization, verification and properties were submitted by the Sponsor who accepted full responsibility for the validity thereof.

The study is valid for the purpose for which it was conducted and this report is a true reflection of the raw data generated.

All persons involved in the performance of the study were experienced and qualified, able to conduct the experimental tasks assigned to them.

Drs.Ing. A.G.M Kroon,
Study Director

14/01/99

Date,

I have reviewed this report and concur with its contents.

Dr. J.T. Meulemans,
Manager Aquasense Lab

15 - 1 - 99
Date,

QUALITY ASSURANCE STATEMENT

The testing facilities utilized in this study have been inspected regularly in accordance with the Principles of Good Laboratory Practice.

This study was inspected and audited and the findings were reported to the Study Director and the Management on the dates as shown below. Inspections were performed according to the Standard Operating Procedures (SOP) of the testing facility. The final report was audited in detail against the approved Study Protocol and all pertinent raw data.

It is considered to be an accurate presentation of the methods and procedures applied in the course of the study and an accurate reproduction of the data recorded.

Inspection date	Inspection/Audit	Date of QA report
07-05-98	Study protocol inspection	08-05-98
12-05-98	Study-based inspection	13-05-98
05-06-98	Report inspection	05-06-98
18-08-98	Re-issue inspection	1[illegible]-08-98
04-11-98	Report Amendment inspection	05-11-98
14-12-98	Re-issue inspection	14-12-98

Ir. G.M. van Eijnatten,
Quality Assurance manager

14/01/99
Date,

STUDY PERSONNEL AQUASENSE LAB

Study Director	Drs.Ing. A.G.M. Kroon
Technician(s)	Ing. A. van Mullem, Ing. S. de Valk S. Rooijakkers
Management	J.T. Meulemans

ARCHIVING OF RECORDS AND SAMPLES

The study file including the Final Report, Study Protocol, ammendments, QA records, and all raw data pertaining to the study is retained in the archives of Aquasense Lab for a period of ten years. The test material is stored deepfrozen under the sample number (302900) for ten years, or as long as the quality of the test substance permits evaluation. All other records connected with the study are also registered in the Aquasense Lab archives for a period of at least ten years.

Summary

In order to predict effects of chemicals in an aquatic environment, the toxicity of Phantolid® to *Brachydanio rerio* was assessed. The toxicity was determined in an acute toxicity test which is described in the OECD 203 (1992) and EEC 67/548 C1 (1992) test guidelines, and was performed in accordance with the principles of Good Laboratory Practice (GLP). The toxicity was determined over an exposure period of 96 hours under defined conditions. During the test the pH varied no more than 0.3 units, and the oxygen concentrations were all above 60% of the saturation values. The temperature varied between 22.3 and 24.7 °C throughout the test. The test is valid as shown by the absence of mortality in the control. The LC_{50} value could not be calculated due to the low solubility and the low toxicity at the tested concentrations. The No Observed Effect Concentration (NOEC) derived from nominal concentrations is 0.902 mg/l. The chemical analysis showed a slight decrease in the concentration at the start of the test and decreased rapidly to below the detection limit. A NOEC based on measured values (0.075 mg/l) is questionable since a small toxic effect at the highest dose was observed and all concentrations, except the first measurement, are below the detection limit.

1. INTRODUCTION

The objective of this test was to assess the acute toxicity of Phantolid® to the freshwater fish *Brachydanio rerio* during 96 hours of continuous exposure in a static test. In the test the fish were exposed to five concentrations of the test substance in a geometric series under defined conditions. The test solutions were not renewed during the test duration, and mortalities were recorded every 24 hours. Where possible, the acute toxicity is expressed as the median lethal concentration (LC_{50}), that is the concentration which kills 50% of the fish within the period of testing. Furthermore, the highest concentration causing no effect (NOEC) and the lowest concentration causing 100% mortality were also determined.

The methods employed were based on the OECD test guideline 203 [4.1] and the EEC directive 67/548 C1 [4.2], and in compliance with the OECD principles of Good Laboratory Practice [4.3].

2. MATERIALS AND METHODS

2.1 Test substance

The sample of Phantolid® was received at Aquasense Lab on 15-jan-98 and assigned the sample number 302900. The following test substance data were submitted by the Sponsor. The Sponsor accepted full responsibility for the validity of these data.

• name/code	Phantolid®
• chemical name	5-acetyl-1,1,2,3,3,6-hexamethylindan
• batch/lot No.	10237
• CAS nr.	15323-35-0
• purity	96.7 %
• appearance	off-white powder
• water solubility	estimated at 0.1 - 1 mg/l
• vapour pressure	not known
• storage conditions	at room temperature in the dark
• stability	stable for at least 12 months under storage conditions
• study sample number	302900

The nominal concentrations as referred in this report are the concentrations based on the 96.7% purity of the active ingredient. The Certificate of Analysis is presented in Annex I.

2.2 Test organism

The test fish (Zebra-fish, *Brachydanio rerio)* were obtained from an established commercial hatchery (Bio International, Horn, The Netherlands), and registered in the in-house purchase register as batch : 020298. Quarantine and transportation procedures were in accordance with the Standard Operating Procedure A-324 [4.6]. All fish were held in reconstituted water (DSW) of an approximately 16-hour daylight photoperiod and observed for at least 12 days prior to testing. During the holding period the fish were fed daily with a standard commercial fish food, and this was stopped approximately 24 hours before testing. The batch had a mortality of 3 % and was accepted because less than 5% mortality was observed within 7 days of holding. The dissolved oxygen concentration was more than 80% of the air-saturation value throughout the holding period. Records were held on the quarantine procedures. The wet weight of the fish used in the test, based on the weight of ten fish of the same batch, was 0.77 gram. The mean length of the fish was 2.4 cm. The biomass loading did not exceed the maximum loading of 1 gram wet weight /l.

2.3 *Chemicals*

The chemicals for preparation of the test medium were all of analytical grade and obtained from an established commercial supplier. (Merck, Darmstadt, Germany, main supplier)

2.4 *Glassware*

The test was performed in test vessels containing 1000 ml of DSW. The test vessels were closed with plastic stoppers. All other equipment was made of glass. All glassware used was cleaned prior to testing according to Standard Operating Procedure A-340 [4.9].

2.5 *Dilution medium*

The dilution water used for testing and dilutions was Dutch Standard Water (DSW), having a pH of 7.8 ± 0.5. The DSW was prepared using deionized water which has a conductivity of less than 0.5 µS/mm, according to Standard Operating Procedure A-301 [4.7]. The dilution water was aerated before being used in the test. The air was purified by active coal, cotton filter and water. The deionized water was prepared using a water purification system (Milli-Q, Millipore, Breda, The Netherlands). The water contained the following minerals per litre: 100 mg $NaHCO_3$, 20 mg $KHCO_3$, 200 mg $CaCl_2 \cdot 2H_2O$ and 180 mg $MgSO_4 \cdot 7H_2O$.

2.6 *Apparatus and test conditions*

The test was carried out in a temperature controlled area with a temperature of 23 ± 2°C. The photoperiod was 16 hours of daylight supplied by fluorescent tubes (8 ± 2 µmol/m²/s). The test vessels were placed in a random manner. Aeration was achieved using a Pasteur pipette connected to a compressor with purified air.

2.7 *Test substance stock solution*

A test substance stock solution was prepared by weighing out the test substance (0.50091 g) using an analytical balance (Sartorius, 160P, Breukelen, The Netherlands). This was transferred to a separatory funnel filled with 5 liter DSW. This mixture was ultrasonically treated for approximately 20 minutes and subsequently mixed for approximately 20 hours at room temperature using a magnetic stirrer. Thereafter, the liquid was filtered using a glass fibre filter (Schleicher& Schull, GF 92 Glasfaser filter (ref.nr. 421057 X 27358), 1µm Ø) to remove all particles. The clear aqueous filtrate was used as stock solution for preparation of the desired concentration range. This method was chosen from a range of methods (e.g. generator column, ultrasonic treatment) which have been tested for the preparation of a suitable stock solution of the test substance. This preliminary investigation was not performed according to the OECD principles of GLP. The results are stored in the archive of AquaSense Consultancy [4.10]. The tested methods were in accordance with the OECD

203 and EEC C1 guidelines [4.1, 4.2] and/or international guidelines on the preparation of poorly soluble substances for aquatic toxicity testing [4.11].

2.8 *Determination of test parameters*

The pH and oxygen of all test substance samples and controls were measured daily. The pH's were determined with the aid of a pH meter (pH 196, WTW, Weilheim, Germany), and the oxygen concentration was determined using a oxygen monitor (Oxi 196, WTW, Weilheim, Germany) The temperature was measured continuously using a temperature sensor coupled to a data logger (Elbanton, Kerkdriel, The Netherlands). The light intensity in the test chamber was measured at the beginning and end of the test.

2.9 *Test method*

The test concentration range used in this study was derived from the results of preliminary investigations on the toxicity of Phantolid® to fish, which was not performed under GLP [4.10]. In this investigation 100% survival was found at a concentration of 20% of the stock solution and at a concentration of 100% of the stock solution adverse behaviour was observed.

The definitive test was performed as a static test using 10 fish per test concentration and control. The concentration range was made using a stock solution. The fish were exposed to the following nominal concentration range: 0, 41, 51.2, 64, 80 and 100 % of stock solution (equal to 0.462, 0.578, 0.721, 0.902 and 1.127 mg/l Phantolid®). The test was performed in single-fold. The control consisted of DSW alone. The fish were allocated randomly in the test vessels. The test vessels were aerated gently during the test. The pH was not adjusted during the test. The test duration was 96 hours. The fish were not fed during the performance of the test. Mortalities and other abnormalities were recorded within the first 6 hours of testing and subsequently every 24 hours. Those fish which were not able to breathe and which did not react to a gentle mechanical touch were considered to be dead. Dead individuals were removed at each observation and a record was maintained of mortality and abnormal behaviour for each concentration tested.

2.10 *Statistical evaluation*

The nominal and measured concentrations of the test substance were used for the calculation of the LC_{50} values, and to plot the dose-response curve. The LC_{50} values were calculated using the Binomial method using the computer program ToxCalc of Tidepool Scientific Software [4.4] and according to SOP A-360 [4.12]. Confidence limits (95%) were also calculated. The program was validated with a known dataset before use according to SOP A-360 [4.12]. The NOEC was calculated using the threshold values of Williams test [4.5] and the 100% mortality was derived from the results as presented, where possible.

Calculation procedures are based on both nominal values (calculated from the measured concentration of the stock solution), and geometric means of measured values of the concentration range (whenever the recovery was < 80% after 96 hours of testing as described in the OECD 202 Guideline and EEC 67/548 C2 Directive). The geometric means of concentrations which were not measured by chemical analysis, were estimated on the basis of the formula:

estimation of $M_x = N_x * (1 - (0.5 (N_{x-1} - M_{x-1})/N_{x-1} + 0.5(N_{x+1} - M_{x+1})/N_{x+1}))$

Where : x = Concentration step
N = Nominal concentration value
M = Measured concentration value

From these estimated concentrations at the various time intervals the geometric mean was calculated. When measured concentrations were below the detection limit the values were not used for calculation of the geometric means of the non-analytically determined samples. Concentrations which were below the detection limit at T=0 were not used in calculation of EC_n values.

2.11 Validity criteria

The test should be valid if no more than 10% of the control test fish die or exhibit abnormalities during the test period (OECD, EEC). The dissolved oxygen concentration should be > 60% of the air-saturation throughout the test (OECD, EEC. The pH should not vary more than one unit during the test (EEC).

2.12 Chemical analysis

The method including validation, calibration and description of the procedures and apparatus used to determine the concentration of the test substance in the test medium is described in a separate study. The protocol for this study (Study plan T98005A) [4.13] was drawn up by Dr. G. Stegeman (Study director) of Akzo Nobel Central Research, Dept. RGL, Arnhem, The Netherlands. This protocol is stored in the study file and was approved and signed by both the Study director (Akzo Nobel Central Research) and the Study monitor (AquaSense Lab).

The concentration of the test substance in the samples was determined by means of gas chromatography with flame ionization detection. Samples (≈ 20 ml) were withdrawn from the control, lowest , middle and highest concentration. Samples from the other concentrations were also withdrawn and stored for additional analyses, when needed. The concentration of

the test substance in the solution withdrawn directly from the stock solution was also determined. The samples were stored in the refrigerator in the dark until analysis. All samples will be taken in single-fold and analyzed in duplicate. Samples were taken at 0, 24, 48, 72 and 96 hours of testing. The initial samples for analysis (0 hours) were withdrawn from the vessels after addition of the testorganisms and stabilization of the test system. The samples were stored for a maximum period of 4 weeks.

3. RESULTS

3.1 *Test environment*

The pH values during the 96 hours of testing showed a maximum deviation of 0.3 units (see Table I). The oxygen values exceeded the 60% of the saturation values by far as shown in Table II. The temperature during the test varied between 22.3 and 24.7 °C with a mean value of 23.2 °C. The light intensity during the test in the test chamber was between 9.73 and 7.35 µmol/m²/s, which is in agreement with the light intensity of approximately 8 ± 2 µmol/m²/s as recommended in the OECD/EEC guidelines.

3.2 *Test results*

During the test partial mortalities were observed only at the highest concentration tested (1.127 mg/l nominal) . Because the test concentrations dropped below 80% of the initial concentration, the calculation of the effects were also performed using the geometric means of the measured concentrations. Only moderate effects could be determined, and therefore no LC_{50} value could be calculated. The nominal No Observed Effect Concentration (NOEC) found for Phantolid® is 0.902 and based on the measured concentrations the NOEC is 0.075 mg/l. During the test period changes in the behaviour and mortalities were only observed at the highest concentration tested. This observation is in agreement with the results of the chemical analyses which revealed that after 24 hours the test substance was only detected in the medium with the highest dose.

3.3 *Validity criteria*

The test was valid as shown by the oxygen and pH values described in section 3.1 and by the absence of mortality in the control after 96 hours of testing.

3.4 *Chemical analysis*

The results of the chemical analysis are described in the GLP report of Akzo Nobel Central Research RGL F98046 [4.14], and is added in Annex II. The concentrations of Phantolid® measured at the beginning of the test were slightly lower than the nominal values. After the test was initiated the concentrations dropped rapidly and after 24 hours of testing all concentrations were below the detection limit of the analysis, except for the highest dose at 24 h. This rapid decrease may be due to adsorption onto the fish or the test vessel wall. The test substance may also be metabolized by the fish. Based on the observed behavioural effects at the highest dose it is likely that some material remained in solution.

3.5 *Deviations from Study Protocol*

The following deviations were observed during the study :

- The amount of sample taken for chemical analyses was approximately 20 ml at each sampling time. The amount of sample was not mentioned in the Study Protocol.
- The light intensity in the test chamber was measured at the beginning and end of the test. This was not described in the Study Protocol.
- The EEC (see 4.2) guideline used in the study was the Dutch version instead of the English. In the Study Protocol the English version was mentioned.
- LC_{50} values will also be calculated for 24, 48 and 72 hours of observation, where possible. Only LC_{50} for 96 hours was described in the Study Protocol.

3.6 *Conclusions*

The toxicity of Phantolid® to *Brachydanio rerio* was determined over a period of 96 hours. The LC_{50} value could not be calculated due to the low solubility and low toxicity at the tested concentration range. Based on the nominal values the NOEC is 0.902 mg/ . The NOEC value of 0.075 mg/l based on measured values (geometric means) is questionable as all but one measured value was below the detection limit.

4. REFERENCES

4.1 *OECD* (1992) "Fish, acute toxicity test", 203, June 7th, Guidelines for testing of chemicals, Organisation for Economic Cooperation and Development, Paris

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4.3 *OECD* (1996) "The Revised OECD Principles of GLP", Final Report of the OECD Expert Group on Good Laboratory Practice, Paris, France.

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4.14 *Stegeman, G., Hendriks, H.*(1998) Analysis of Phantolid® in test media from toxicity tests. RGL F98046, Akzo Nobel Central Research, Dept. RGL, Arnhem, The Netherlands.

Table I

Measurements of the pH values in the test vessels at the various time intervals.

Nominal concentration (mg/l)	pH				
Time (hours)	0	24	48	72	96
Control	8.3	8.2	8.2	8.1	8.3
0.462	8.2	8.2	8.2	8.1	8.1
0.578	8.2	8.2	8.3	8.2	8.1
0.721	8.2	8.3	8.2	8.2	8.2
0.902	8.1	8.2	8.2	8.2	8.3
1.127	8.1	8.3	8.3	8.1	8.4

Table II

Measurements of the oxygen values in the test vessels at the various time intervals.

Nominal concentration (mg/l)	oxygen (%)[1]				
Time (hours)	0	24	48	72	96
Control	106	96	96	98	96
0.462	106	96	95	96	94
0.578	106	96	96	96	92
0.721	106	96	95	96	93
0.902	107	97	96	97	93
1.127	105	97	96	95	94

[1] = Oxygen concentrations are expressed as percentage of the saturation values at the measured temperature.

able III

esults of the chemical analysis. Concentrations are presented as mean values of duplicate analysis.

lominal oncen- ation ng/l)	**Measured concentration (mg/l) with 95% confidence limits**					**Geometric mean mg/l**	**% of initial concentration**				
time (h)	**0**	**24**	**48**	**72**	**96**	-	**0**	**24**	**48**	**72**	**96**
	< 0.039[1]	< 0.039[1]	< 0.039[1]	< 0.039[1]	< 0.039[1]	-	-	-	-	-	-
.462	0.362 ± 0.032	< 0.039[1]	< 0.039[1]	< 0.039[1]	< 0.039[1]	0.061	100	-	-	-	-
.578	n.d.	n.d.	n.d.	n.d.	n.d.	0.065					
.721	0.650 ± 0.031	< 0.039[1]	< 0.039[1]	< 0.039[1]	< 0.039[1]	0.068	100	-	-	-	-
.902	n.d.	n.d.	n.d.	n.d.	n.d.	0.071					
.127	0.950 ± 0.031	0.063 ± 0.032	< 0.039[1]	< 0.039[1]	< 0.039[1]	0.083	100	7	-	-	-
tock	1.127 ± 0.031	n.d.									

= Concentrations were < detection limit (= 0.039 mg/l)

Table IV

Survivals during the 96 hours of testing.

Nominal concentration (mg/l)	Survivals					
Time (hours)	0	6	24	48	72	96
Control	10	10	10	10	10	10
0.462	10	10	10	10	10	10
0.578	10	10	10	10	10	10
0.721	10	10	10	10	10	10
0.902	10	10	10	10	10	10
1.127	10	7	7[1]	7	7[2]	7[3]

[1] = One fish swimming at the surface, and 7 fish are sluggish.

[2] = Two fish are swimming sluggish.

[3] = One fish is swimming sluggish.

Table V

Summary of the statistical evaluation.

Time (hours)	LC_{50} (mg/l)	95% confidence limits	100% mortality (mg/l)	NOEC (mg/l)
Nominal concentrations				
24	> 1.127	-	-	n.d.
48	> 1.127	-	-	n.d.
72	> 1.127	-	-	n.d.
96	> 1.127	-		0.902
Measured concentrations				
24	> 0.245	-	-	n.d
48	> 0.133	-		n.d
72	> 0.098	-	-	n.d
96	> 0.081	-	-	0.075

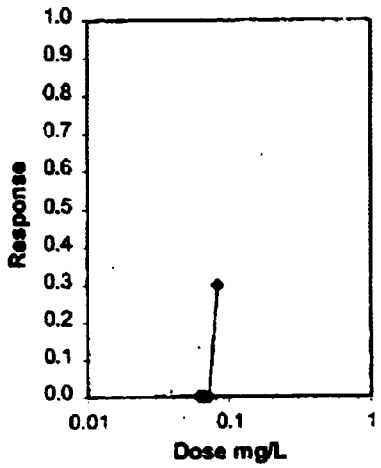

Figure 1

Dose-response curve of Phantolid® calculated from the percentage of inhibition at various test concentrations (measured).

Annex I

PFW Aroma Chemicals B.V.
Nijverheidsweg 80
P.O. Box 414
3770 AK Barneveld
The Netherlands
Telephone: (31) 342 - 41 04 11
Telefax: (31) 342 - 41 06 58

BIS/EL/

January 13, 1998

CERTIFICATE OF ANALYSIS

PHANTOLID 263271

Your order reference	: Dated 13.01.97
Green Card Numbers	: 58058
Lot Number	: 10237
Quantity	: 1 x 30 g
Appearance	: An off-white solid
Odour	: As standard
Melting Range	: 58.6°C - 59.5°C
Purity (GLC)	: 96.7 %

E.J. Swinkels
Manager Quality Assurance Dept.

A member of the Yule Catto group of companies.
Traderegister Arnhem no. 09093508

Annex II

AKZO NOBEL

Central Research
Location Arnhem

Final Research Report

RGL F98046 T 98005 A May 29, 1998

G. Stegeman, H. Henderiks

ANALYSIS OF PHANTOLID IN TEST MEDIA FROM TOXICITY TESTS

Project identification

Client	Aquasense, Amsterdam	Client No	Project No	
Subclient		Subclient No	Task No	9133

Drafted by department General Analytical and Environmental Chemistry

Keywords GC, ecotox, fish toxicity, Daphnia toxicity

AKZO NOBEL

ABSTRACT

To determine toxicity of Phantolid a series of tox tests with fish and daphnids and an algal growth inhibition test were performed by Aquasense Amsterdam. The concentration of Phantolid in the used test media was determined by the General Analytical and Environmental Chemistry Department of Akzo Nobel Central Research. Samples to be analysed were test media with high, medium, low and zero concentration of Phantolid. Samples were taken at the beginning of the test (t=0 hour), at the end of the test and between times after every 24 hours. In this report procedures and results of the chemical analyses are described.

Analyses were carried out in compliance with the OECD Principles of Good Laboratory Practice (GLP). Test media samples were extracted with dibutylether using a so-called in-vial extraction procedure. The ether layer was subsequently analysed by means of gas chromatography (GC) with flame ionisation detection.

The concentration of Phantolid in the test media from the Daphnia magna tests at t=0 hours were between 0 and 1.14 mg/l. In the course of the test the concentrations decreased gradually. At the end of the test, after 48 hours, concentrations were roughly 50% of the initial concentration.

The initial concentration of Phantolid in the algal media ranged between 0 and 0.24 mg/l. The low concentration level was already below the detection limit of 0.04 mg/l. During the 72 hour test the Phantolid concentrations were found to decrease steadily. At the end of the test concentration levels in the test media were all below the detection limit except for the highest dose concentration, which had declined to 0.05 mg/l.

The concentration of Phantolid in test media from the 96 hour fish toxicity study were between 0 and 0.95 at the beginning of the test. Already after 48 hours the Phantolid concentration in all test media had decreased below the detection limit of 0.04 mg/l. This decrease in Phantolid concentration is more pronounced than in the algal and Daphnia test.

AKZO NOBEL

ANALYSIS OF PHANTOLID IN TEST MEDIA FROM TOXICITY TESTS

Sponsor	Aquasense P.O. Box 95125 1090 HC Amsterdam The Netherlands
Study monitor	Drs. Ing. A.G.M. Kroon

STUDY ORGANISATION

Location	Akzo Nobel Central Research Arnhem General Analytical and Environmental Chemistry Dept. Velperweg 76 6824 BM Arnhem The Netherlands
Study director	Dr. G. Stegeman
Quality Assurance Unit	E.H.V. Derks
Management, Head of Department RGL	Dr.Ir. C.J. Groenenboom
Initiation date of the study	1998-05-15
Completion date of the study	1998-05-25

ARCHIVING AND STORAGE

The project file including the final report, amendments to the final report, the study plan, amendments to the study plan, records of quality assurance inspections, all letters, memos and notes and raw data pertaining to the study will be retained in the archives of Akzo Nobel Central Research Arnhem for a period of ten years. Other records including master schedule sheet, laboratory notebooks, logbooks, records of the maintenance and calibration of equipment, summary of training, curricula vitae and job descriptions of the personnel involved in the study, records related to location and storage of the test substance will also be kept in the Akzo Nobel Central Research Arnhem archives for a period of ten years. Test material will be stored deepfrozen under the sample code T 98005 for ten years or only as long as the quality of the test substance permits evaluation.

AKZO NOBEL

GLP COMPLIANCE STATEMENT

The study reported here was carried out according to the study plan in compliance with the OECD Principles of Good Laboratory Practice (5.1). The report contains an accurate description of the results.

Study director
Dr. G. Stegeman

date 1998.05.29

Management, Head of Department RGL
Dr.Ir. C.J. Groenenboom

date 1998-06-02

AKZO NOBEL

QUALITY ASSURANCE STATEMENT

This report was audited by the Quality Assurance Unit of Akzo Nobel Central Research Arnhem. It is considered to be an accurate presentation of the methods and procedures applied in the course of the study and an accurate reproduction of the data recorded.
Listed below are the dates of inspection of this study by the Quality Assurance Unit and the dates on which its findings were reported to Study Director and Management.

Dates of inspection	Dates of reporting
1998-05-20	1998-05-20
1998-05-29	1998-05-29

Quality Assurance Unit
E.H.V. Derks

date 1998.05.29

AKZO NOBEL

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1. INTRODUCTION

To determine toxicity of Phantolid, a substance produced by PFW Aroma Chemicals, a series of tox tests with fish and daphnids and an algal growth inhibition test were performed by Aquasense Amsterdam (project No 1202b). The concentration of Phantolid in the used test media was determined by the General Analytical and Environmental Chemistry Department of Akzo Nobel Central Research. In this report the procedures and results of the chemical analyses are described.

The samples to be analysed were taken from test media at the beginning, at the end and during the toxicity tests at regular time intervals. The samples were extracted "in-vial" with dibutylether. The concentration of Phantolid in the dibutylether layer was determined by means of gas chromatography (GC) with flame ionisation detection. The analyses were carried out in compliance with the OECD Principles of Good Laboratory Practice (5.1) and according to Standard Operation Procedure N 1 (5.2).

2. MATERIALS

2.1 Test substance

The test substance Phantolid (sample code: T98005) was received from Aquasense on 1998.05.08. The following test substance data were submitted by the sponsor, who accepted full responsibility for the validity of these data. Information concerning the test material:

-	name of test substance	Phantolid
-	chemical name	5-acetyl-1,1,2,3,3,6-hexamethylindan
-	Cas reg.No.	15323-35-0
-	batch/lot No.	10237
-	Aquasense sample No	302900
-	purity	96.7 %
-	appearance	an off-white solid (powder)
-	storage until required	in a cool place in the dark
-	stability	stable under storage conditions

2.2 Samples

The samples to be analysed were received from Aquasense on 1998.05.15. On receipt all samples were still refrigerated, which was checked by taking some samples in the palm of the hand. After checking all the samples, they were immediately stored in a refrigerator until analysis. Sample codes and other relevant data supplied by Aquasense were assumed to be correct and were used without further check. Samples were analysed within one week after receipt

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- sample description: aqueous solutions of test substance
- storage until required: in a refrigerator

2.3 Chemicals

Dibutylether, $[CH_3(CH_2)_3]_2$ O: 99% GC-grade

2.4 Deionized water

Deionized water was produced from tap water in a water purification system (Spectrum-Elgastat, Breukelen, The Netherlands). It had a conductivity of less than 5 µ $S.cm^{-1}$, a TOC content of less than 2 mg/l and it contained no more than 0.01 mg Cu per litre (Standard Operation Procedure K 10 (5.3)).

2.5 Apparatus

Description of the apparatus used and some relevant settings:

Gas chromatography system	Carlo Erba GC 8160
Autosampler	Carlo Erba AS 800
	sampling depth: -22 mm
Injection	cold on-column injection
	cool time: 12 seconds
	injection volume: 1 µl
	injection speed: maximum
	syringe : Hamilton microliter, 10 µl AS800
	syringe rinse procedure: standard AS800 procedure (bubble elimination = 5)
GC-column	J&W DB-1 with retention gap
	length: 30 meter
	internal diameter: 0.32 mm
	film thickness: 0.5 µm
Detection	Carlo Erba FID-80 flame ionization detector
	temperature: 300°C
	flame gases: 50 kPa H_2 and 100 kPa air
Carrier gas	Helium 5.0; 100 kPa head pressure
Temperature program	Initial temperature: 150°C (2 minutes)
	temperature rise: 4°C/min
	final temperature 225°C (5 minutes)
Data collection/integration	Fisons Multichrom rev.2 chromatography software
Recorder	Kipp & Zn BD-8, 1 mV

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2.6 Materials

Chromacol 2-ml glass GC autosampler vials with Teflon lined crimp caps.

3. METHODS

3.1 Preparations for GC analysis

The performance of the installed GC column was checked by a blank run (i.e. a chromatographic run without sample injection) and by analysis of a Phantolid standard in dibutylether. After checking column contamination, column bleed and peak shapes, the column was approved for chemical analyses.

3.2 Calibration of chromatographic system

A stock solution of Phantolid was prepared in dibutylether. From this stock solution a series of 6 calibration standards was prepared in dibutylether covering the expected concentration range of the samples.
The GC standards were prepared by adding 0.8 ml of deionized water and 0.8 ml of the standard solution to a GC sample vial. For each concentration level a duplicate GC standard was prepared. The capped vials were shaken vigorously for 2 minutes and were then left standing for at least 5 minutes to settle. The series of duplicate calibration standards were analyzed in single fold starting with the lowest concentration.

3.3 Control samples

To check on errors in the preparation of calibration standards, a second independent stock solution of Phantolid was prepared in dibutylether. From this stock solution a control sample of known Phantolid concentration was prepared within the concentration range of the calibration line. The control sample was prepared in duplicate according to the procedure described in 3.2. The duplicate control samples were analyzed in single fold.

3.4 Analysis of Phantolid samples

The refrigerated samples were brought to room temperature. The samples were shaken vigorously to homogenize and then sonicated for 10 minutes. If aggregates or particles were visible (e.g. algae of fish scales), the samples were centrifuged at 8000 rpm for 10 minutes. When necessary, samples were diluted in deionized water to obtain a nominal concentration within the calibration range.
From each test sample a duplicate sample for GC analysis was prepared. GC samples were prepared by adding 0.8 ml of the aqueous sample and 0.8 ml of dibutylether to a GC sample vial. The capped vials were shaken vigorously for 2 minutes to extract the aqueous layer and were then left standing for at least 5 minutes to settle.

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Each GC sample vial was assayed in single fold (which means that each received test media sample was analyzed in duplicate since GC samples were prepared in duplicate). After a series of ten duplicate samples a calibration standard (except for the highest or lowest standard) was analyzed in single fold. It was checked if results satisfied quality criteria (see 3.6.3, 3.6.4).

3.5 Data processing

The peak area of the Phantolid peak (only one single Phantolid peak was detected) was determined using the Multichrom integration software. Key integration parameters were kept the same for calibration runs and sample analysis. Calibration line(s) were constructed by plotting peak area (in counts) against the concentration of test substance in the calibration standards using Statcal 6.50 software package [5.4]. The optimum polynomial fit of the calibration curve, coefficients of the polynomial (A, B, etc.) and standard deviation of residuals (S_R) were calculated.

For each sample the concentration of Phantolid in the dibutylether layer and the 95% confidence interval were calculated from the measured peak area and the calibration line using Statcal 6.50. To obtain the true Phantolid concentration a correction was made for impurity of the test substance. The concentration of Phantolid in the aqueous test samples were calculated assuming that the distribution coefficient of Phantolid, describing equilibrium distribution of Phantolid between the aqueous and ether phase, is the same for the calibration standards and the samples.

3.6 Data evaluation and quality control

3.6.1 Calibration standards

It was checked if the calibration standards were within the 99% confidence limits of the corresponding calibration line. If one data point was outside the 99% confidence interval this data point was rejected and the regression line was recalculated. If the calibration series contained more than one data point outside the 99% confidence interval the whole calibration series was rejected and a new calibration line was constructed with freshly prepared standards.

3.6.2 Control samples

If the known concentration of the control samples was outside the 99% confidence interval of the measured concentration another control sample, prepared from a fresh stock solution, was analyzed. If the measured concentration of this control sample was still deviating significantly (99% confidence) from the true concentration, the whole calibration line was rejected, a series of freshly prepared standards was analyzed and a new calibration line was constructed.

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3.6.3 *Samples*

Using Statcal 6.50 the width of the 99% confidence interval of the calibration line (= 2 * $t_{99\%}$*S_R, where the t-value is from the statistical two-tailed t-distribution) was calculated. If the difference between measured peak areas (in area counts) of duplicate analyses was larger than the width of the 99% confidence interval of the calibration line, the results for this sample were rejected and the sample was reanalyzed.

3.6.4 *Control standards*

If the measured concentration of the control standards, injected after each series of ten samples, deviated significantly from the true concentration (i.e. if the true concentration was not within the 99% confidence interval of the measured value), the control standard was reanalyzed in duplicate. If the true concentration remained outside the 99% confidence interval, the results for the previous 10 samples were rejected, the system was recalibrated using the original calibration standards and the 10 samples were reanalyzed.

4. RESULTS AND DISCUSSION

4.1 General

GC analysis of pure extraction solvent (dibutylether) and a Phantolid standard solution showed that Phantolid appears as a single peak which is free from interferences from the solvent and solvent impurities.
Analysed samples were test media samples containing high, medium, low and zero Phantolid dose concentrations. The samples were taken at the beginning of the test (t=0 hr), at the end of the test and between times after every 24 hours.
After shaking and sonication, none of the samples contained visible particles or aggregates so none of the samples was centrifuged.
Results of the analyses of the test media are summarized in table 6.1-6.3. The first three columns of each table ("Aquasense sample code", "Sampling date" and "Nominal sampling time") contain data provided by Aquasense. These data were taken from the sample dispatch form which was sent along with the samples. Reported Phantolid concentrations are corrected for impurity of the test substance and thus represent the concentration of pure Phantolid.

4.2 Samples from toxicity test with Daphnia magna.

The concentration of Phantolid in the test media from the Daphnia magna tests at t=0 hours were between 0 and 1.14 mg/l. In the course of the test the concentrations decreased gradually. At the end of the test, after 48 hours, concentrations were roughly 50% of the initial concentration. The current chemical analyses do not give a clue for the cause of the Phantolid losses.

4.3 Samples from algal growth inhibition test

The initial concentration of Phantolid in the algal media was between 0 and 0.24 mg/l. The low dose concentration was already below the detection limit of 0.04 mg/l. During the 72 hour test the Phantolid concentrations were found to decrease steadily. At the end of the test concentration levels in the test media were all below the detection limit except for the highest dose concentration, which had declined to 0.05 mg/l.

4.4 Samples from toxicity tests with fish

The concentration of Phantolid in test media from the 96 hour fish toxicity study were between 0 and 0.95 at the beginning of the test. Already after 48 hours the Phantolid concentration in all test media had decreased below the detection limit of 0.04 mg/l. The observed decrease in Phantolid concentration is more pronounced than in the algal and Daphnia test.

5. REFERENCES

5.1 OECD, 1981. Principles of Good Laboratory Practice, C(81)30(final)

5.2 Standard Operation Procedure (SOP) N 1: Procedures and quality assurance of chemical analyses, procedure.

5.3 Standard Operation Procedure (SOP) K 10: Deionisation, method.

5.4 Statistics software package: Statcal 6.50, J. Kragten, University of Amsterdam, Amsterdam, 1996

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6. TABLES

Table 6.1 Analysis of Phantolid in test media from Daphnia magna toxicity test Aquasense project 1202B

Aquasense sample code	Sampling date	Nominal sampling time	Phantolid concentration and 95% confidence interval (mg/l)
DM stock, wit	1998-05-11	t= 0 hr	1.127 ± 0.031
DM [0], wit	1998-05-11	t= 0 hr	< 0.039 [a]
DM [1], wit	1998-05-11	t= 0 hr	0.267 ± 0.032
DM [3], wit	1998-05-11	t= 0 hr	0.640 ± 0.031
DM [5], wit	1998-05-11	t= 0 hr	1.143 ± 0.031
DM [0], groen	1998-05-12	t= 24 hr	< 0.039 [a]
DM [1], groen	1998-05-12	t= 24 hr	0.144 ± 0.032
DM [3], groen	1998-05-12	t= 24 hr	0.362 ± 0.032
DM [5], groen	1998-05-12	t= 24 hr	0.840 ± 0.031
DM [0], rood	1998-05-13	t= 48 hr	< 0.039 [a]
DM [1], rood	1998-05-13	t= 48 hr	0.123 ± 0.032
DM [3], rood	1998-05-13	t= 48 hr	0.337 ± 0.032
DM [5], rood	1998-05-13	t= 48 hr	0.659 ± 0.031

[a] concentration below limit of detection

Table 6.2 Analysis of Phantolid in test media from the algal growth inhibition test Aquasense project 1202B

Aquasense sample code	Sampling date	Nominal sampling time	Phantolid concentration and 95% confidence interval (mg/l)
A stock, wit	1998-05-12	t= 0 hr	0.741 ± 0.031
A [0], wit	1998-05-12	t= 0 hr	< 0.039 [a]
A [1], wit	1998-05-12	t= 0 hr	< 0.039 [a]
A [3], wit	1998-05-12	t= 0 hr	0.059 ± 0.032
A [5], wit	1998-05-12	t= 0 hr	0.232 ± 0.032
A [0], groen	1998-05-13	t= 24 hr	< 0.039 [a]
A [1], groen	1998-05-13	t= 24 hr	< 0.039 [a]
A [3], groen	1998-05-13	t= 24 hr	< 0.039 [a]
A [5], groen	1998-05-13	t= 24 hr	0.151 ± 0.032
A [0], rood	1998-05-14	t= 48 hr	< 0.039 [a]
A [1], rood	1998-05-14	t= 48 hr	< 0.039 [a]
A [3], rood	1998-05-14	t= 48 hr	< 0.039 [a]
A [5], rood	1998-05-14	t= 48 hr	0.075 ± 0.032
A [0], blauw	1998-05-15	t= 72 hr	< 0.039 [a]
A [1], blauw	1998-05-15	t= 72 hr	< 0.039 [a]
A [3], blauw	1998-05-15	t= 72 hr	< 0.039 [a]
A [5], blauw	1998-05-15	t= 72 hr	0.051 ± 0.032

[a] concentration below limit of detection

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Table 6.3 Analysis of Phantolid in test media from fish toxicity test Aquasense project 1202B

Aquasense sample code	Sampling date	Nominal sampling time.	Phantolid concentration and 95% confidence interval (mg/l)
V stock, wit [b]	1998-05-11	t= 0 hr	1.127 ± 0.031
V [0], wit	1998-05-11	t= 0 hr	< 0.039 [a]
V [1], wit	1998-05-11	t= 0 hr	0.362 ± 0.032
V [3], wit	1998-05-11	t= 0 hr	0.650 ± 0.031
V [5], wit	1998-05-11	t= 0 hr	0.950 ± 0.031
V [0], groen	1998-05-12	t= 24 hr	< 0.039 [a]
V [1], groen	1998-05-12	t= 24 hr	< 0.039 [a]
V [3], groen	1998-05-12	t= 24 hr	< 0.039 [a]
V [5], groen	1998-05-12	t= 24 hr	0.063 ± 0.032
V [0], rood	1998-05-13	t= 48 hr	< 0.039 [a]
V [1], rood	1998-05-13	t= 48 hr	< 0.039 [a]
V [3], rood	1998-05-13	t= 48 hr	< 0.039 [a]
V [5], rood	1998-05-13	t= 48 hr	< 0.039 [a]
V [0], blauw	1998-05-14	t= 72 hr	< 0.039 [a]
V [1], blauw	1998-05-14	t= 72 hr	< 0.039 [a]
V [3], blauw	1998-05-14	t= 72 hr	< 0.039 [a]
V [5], blauw	1998-05-14	t= 72 hr	< 0.039 [a]
V [0], geel	1998-05-15	t= 96 hr	< 0.039 [a]
V [1], geel	1998-05-15	t= 96 hr	< 0.039 [a]
V [3], geel	1998-05-15	t= 96 hr	< 0.039 [a]
V [5], geel	1998-05-15	t= 96 hr	< 0.039 [a]

[a] concentration below limit of detection
[b] V stock.,wit = DM stock, wit (see table 6.1)

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Evaluation of the toxic effects of Phantolid® to *Daphnia magna*.

02

Evaluation of the toxic effects of Phantolid® to *Daphnia magna.*

Final Report

CONFIDENTIAL

Study started	11-May-98
Study completed	15-May-98
Study number	1202B.GLP.DM-02
Report number	98.1202-16 (re-issue of 98.1202-9)
Performing laboratory	AquaSense Lab, P.O. Box 95125, 1090 HC Amsterdam, The Netherlands
Study Director	Drs.Ing. A.G.M. Kroon
Approved by	Dr. J.T. Meulemans

Sponsor	PFW Aroma Chemicals BV, Barneveld, The Netherlands
Study Monitor	Dr. H-D. Gaisser

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CONFIDENTIALITY STATEMENT

This document contains information and unpublished results of research which may be confidential, and therefore it should not be photocopied or microfilmed. It should also not be released in any form to an outside party, nor should information contained herein be used by any authority without the written permission of the Sponsor.

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with the Principles of Good Laboratory Practice as described in the OECD Principles of Good Laboratory Practice (1998). All data on the test substance such as , characterization, verification and properties were submitted by the Sponsor who accepted full responsibility for the validity thereof.

The study is valid for the purpose for which it was conducted and this report is a true reflection of the raw data generated.

All persons involved in the performance of the study were experienced and qualified, able to conduct the experimental tasks assigned to them.

Drs.Ing. A.G.M Kroon,
Study Director

14/01/99

Date,

I have reviewed this report and concur with its contents.

Dr. J.T. Meulemans,
Manager Aquasense Lab

15-1-99
Date.

QUALITY ASSURANCE STATEMENT

The testing facilities utilized in this study have been inspected regularly in accordance with the Principles of Good Laboratory Practice.

This study was inspected and audited and the findings were reported to the Study Director and the Management on the dates as shown below. Inspections were performed according to the Standard Operating Procedures (SOP) of the testing facility. The final report was audited in detail against the approved Study Protocol and all pertinent raw data.

It is considered to be an accurate presentation of the methods and procedures applied in the course of the study and an accurate reproduction of the data recorded.

Inspection date	Inspection/Audit	Date of QA report
12-05-98	Study protocol inspection	13-05-98
07-05-98	Study-based inspection	08-05-98
05-06-98	Report inspection	05-06-98
18-08-98	Re-issue inspection	18-08-98
04-11-98	Report Amendment inspection	05-11-98
14-12-98	Re-issue inspection	14-12-98

Ir. G.M. van Eijnatten,
Quality Assurance manager

14/01/99
Date,

STUDY PERSONNEL AQUASENSE LAB

Study Director	Drs.Ing. A.G.M. Kroon
Technician(s)	Ing. A. van Mullem, Ing. S. de Valk S. Rooijakkers
Management	Dr. J.T. Meulemans

ARCHIVING OF RECORDS AND SAMPLES

The study file including the Final Report, Study Protocol, ammendments, QA records, and all raw data pertaining to the study is retained in the archives of Aquasense Lab for a period of ten years. The test material is stored deepfrozen under the sample number (302900) for ten years, or as long as the quality of the test substance permits evaluation. All other records connected with the study are also being registered in the Aquasense Lab archives for a period of at least ten years.

Summary

In order to predict effects of chemicals in an aquatic environment, the toxicity of Phantolid® to *Daphnia magna* was assessed. The toxicity was determined in an acute toxicity test which is described in the OECD Guideline 202 (1984) and EEC Directive 67/548 C2 (1992), and was performed in accordance with the principles of Good Laboratory Practice (GLP).
The toxicity was determined over an exposure period of 48 hours under defined conditions. During the test the pH varied no more than 0.2 units, and the oxygen concentrations were all above 60% of the saturation values. The temperature varied between 19.8 and 20.6 °C throughout the test. Therefore the test meets the requirements and is valid as shown by the absence of immobility in the controls. The EC_{50} value calculated after 48 hours of testing based on the nominal values is 0.396 mg/l (0.345 - 0.453 95% confidence limits) and the NOEC derived from the results is 0.223 mg/l. Based on the measured concentrations the EC_{50} is 0.321 mg/l (0.275 - 0.370 95% confidence limits), and the NOEC is 0.168 mg/l. Chemical analyses showed that the initial concentrations were substantially achieved, as shown by the percentage recovery at t=0, but the concentration gradually decreased during the 48 hours of testing. This decrease is not an inherent property of the test substance and may be attributed, to some extent, to adsorption onto the test vessel wall or onto the Daphnids.

1. INTRODUCTION

The objective of this study was to assess the acute toxicity of Phantolid® to *Daphnia magna* under static test conditions. The Daphnids were exposed to five concentrations of the test substance during 48 hours of testing. The test solutions were not renewed during the test duration, and mortalities were recorded every 24 hours. The acute toxicity is expressed as the median effective concentration (EC_{50}), that is the concentration which immobilizes 50% of the Daphnids within the period of testing. The No Observed Effect Concentration (NOEC) and the lowest concentration causing 100% immobility are also determined.

The methods employed were based on the OECD test guideline 202 [4.1] and the EEC Directive 67/548 C2 [4.2], and in compliance with the OECD principles of Good Laboratory Practice [4.3].

2. MATERIALS AND METHODS

2.1 Test substance

The sample of Phantolid® was received at Aquasense Lab on 15-jan-98 and assigned the sample number 302900. The following test substance data were submitted by the Sponsor. The Sponsor accepted full responsibility for the validity of these data.

• name/code	Phantolid®
• chemical name	5-acetyl-1,1,2,3,3,6-hexamethylindan
• batch/lot No.	10237
• CAS nr.	15323-35-0
• purity	96.7 %
• appearance	off-white powder
• water solubility	estimated at 0.1 - 1 mg/l
• vapour pressure	not known
• storage conditions	at room temperature in the dark
• stability	stable for at least 12 months under storage conditions
• study sample number	302900

The nominal concentrations as referred in this report are the concentrations based on the 96.7% purity of the active ingredient. The Certificate of Analysis is presented in Annex I.

2.2 Test organism

Daphnia magna (Straus, clone IV, RIZA,. The Netherlands) was obtained from the laboratory parthenogenetic culture. This culture was kept as a synchronuous brood stock at the GLP cluster of Aquasense Lab. The Daphnids were cultivated and maintained according to the Standard Operating Procedure A-321 [4.6] used at AquaSense Lab. The Daphnids were maintained in the synthetic culture medium (Elendt M-4 which is a reconstituted water and prepared according to the Standard Operating Procedure A-302 [4.7]. The culture method and the production of neonates were also in accordance with these procedures. During culturing the Daphnids were fed with algae (*Scenedesmus*), which were cultivated according to Standard Operating Procedure A-320 [4.8]. The neonates used for test performance were between 0 and 24 hours old. The quality of the Daphnids is checked every 6 months, and compared to inter-laboratory test data.

2.3 *Chemicals*

The chemicals for preparation of the test medium were all of analytical grade and obtained from established commercial suppliers. (Merck, Darmstadt, Germany, main supplier).

2.4 *Glassware*

The test were performed in small vessels of approximately 70 ml total volume. The test vessels for each test were placed in separate racks, and were kept open to the air. All other equipment was made of glass. All glassware used was cleaned prior to testing according to Standard Operating Procedure A-340 [4.10].

2.5 *Dilution medium*

The dilution water used for testing and dilutions was Dutch Standard Water (DSW), having a pH of 7.8 ± 0.5. The DSW was prepared using deionized water, which has a conductivity of less than 0.5 µS/mm, and according to Standard Operating Procedure A-301 [4.9]. The dilution water was aerated before being used in the test. The air was purified by active coal, cotton filter and water. The deionized water was prepared using a water purification system (Milli-Q, Millipore, Breda, The Netherlands). The water contained the following minerals per litre: 100 mg $NaHCO_3$, 20 mg $KHCO_3$, 200 mg $CaCl_2 \cdot 2H_2O$ and 180 mg $MgSO_4 \cdot 7H_2O$.

2.6 *Apparatus and test conditions*

The test were performed in glass vessels containing 50 ml of medium. The fluids were not aerated during the test period, and the Daphnids were not fed. The temperature in the incubator was kept constant between 18 and 22 °C ± 1°C (OECD, EEC[1]) with a light regime of 16 hours of artificial light per day (8 ± 2 µmol/m²/s). The test was static with the fluids not being renewed during the test. The Daphnids were randomly placed in the test vessels.

[1] = Temperature deviation is 1°C instead of 0.1°C as mentioned in the EEC guideline, which is a typographical error in the Dutch version.

2.7 *Test substance stock solution*

A test substance stock solution was prepared by weighing out the test substance (0.50091 gram) using a analytical balance (Sartorius, 160P, Breukelen, The Netherlands). This was transferred to a separatory funnel filled with 5 liter DSW. This mixture was ultrasonically treated for approximately 20 minutes and subsequently mixed for approximately 20 hours at

room temperature using a magnetic stirrer. Thereafter, the liquid was filtered using a glass fibre filter (Schleicher& Schull, GF 92 Glasfaser filter (ref.nr. 421057 X 27358), 1µm ∅) to remove all particles. The clear aqueous filtrate was used as stock solution for preparation of the desired concentration range. This method was chosen from a range of methods (e.g. generator column, ultrasonic treatment) which have been tested for the preparation of a suitable stock solution of the test substance. This preliminary investigation was not performed according to the OECD principles of GLP. The results are stored in the archive of AquaSense Consultancy [4.11]. The tested methods were in accordance with the OECD 202 and EEC Directive 67/548 C2 [4.1, 4.2] and international guidelines on the preparation of poorly soluble substances for aquatic toxicity testing [4.12].

2.8 *Determination of test parameters*

The pH and oxygen of all test substance samples and controls were measured at the beginning (in pooled samples of each concentration) and end of the test (in all test concentrations). The pH values were determined with the aid of a pH meter (pH 196, WTW, Weilheim, Germany), and the oxygen concentration were determined using anoxygen monitor (Oxi 196, WTW, Weilheim, Germany) The temperature were measured continuously using a temperature sensor coupled to a data logger (Elbanton, Kerkdriel, The Netherlands). The light intensity was measured at the beginning and end of the test.

2.9 *Test method*

The test concentration range used in this study was derived from the results of preliminary investigations on the toxicity of Phantolid® to Daphnids, which were not performed according to the principles of GLP [4.11]. In this investigation 100% immobility was found at a concentration of 100% of the stock solution and no immobility at concentrations of 20% or lower.

The definitive test was performed as a static test using 20 Daphnids per test concentration and control. The concentration range was made using the stock solution. The test were initiated by exposing 20 Daphnids divided into 4 groups of five organisms. The Daphnids were exposed to the following nominal concentration range: 19.8, 29.7, 44.5, 66.7 and 100% of the stock solution concentration (equal to 0.223, 0.335, 0.502, 0.752 and 1.127 mg/l). The control consisted of DSW alone. The Daphnids were observed every 24 hours for immobilisation and other adverse behavioral effects. Daphnids which were not able to swim for 15 seconds after gentle agitation of the test vessel were considered immobile.

2.10 Statistical evaluation

The nominal concentrations of the test substance were used to plot the dose-response curve. The EC_{50} values were calculated according to SOP A-360 [4.13] using the Maximum Likelihood Probit method using the computer program ToxCalc of Tidepool Scientific Software [4.4]. Confidence limits (95%) were calculated for each EC_{50} value. The program was validated with a known dataset before use, according to Standard Operating Procedure A-360 [4.13]. The NOEC was calculated using the threshold values of Williams test [4.5] and the 100% mortality was derived from the results as presented.

Calculation procedures are based on both nominal values (calculated from the measured concentration of the stock solution), and geometric means of measured values of the concentration range (whenever the recovery was < 80% after 48 hours of testing as described in the OECD 202 Guideline and EEC 67/548 C2 Directive). The geometric means of concentrations which were not measured by chemical analysis, were estimated on the basis of the formula:

$$\text{estimation of } M_x = N_x * (1 - (0.5 (N_{x-1} - M_{x-1})/N_{x-1} + 0.5(N_{x+1} - M_{x+1})/N_{x-1}))$$

Where : x = Concentration step
N = Nominal concentration value
M = Measured concentration value

From these estimated concentrations at the various time intervals the geometric mean was calculated. When measured concentrations were below the detection limit the values were not used for calculation of the geometric means of the non-analytically determined samples. Concentrations which were below the detection limit at T=0 were not used in calculation of EC_n values.

2.11 Validity criteria

For the test to be valid, the number of floating, and /or immobile Daphnids in the control group must not exceed 10% at the end of the test, and the Daphnids should not be trapped at the surface (OECD/EEC). The dissolved oxygen concentration should be > 60% of the air-saturation throughout the test (OECD/EEC). The pH of the test medium should not vary more than one unit during the test (EEC).

2.12 *Chemical analysis*

The method including validation, calibration and description of the procedures and apparatus used to determine the concentration of the test substance in the test medium is described in a separate study. The protocol for this study (Study plan T98005A) [4.14] was drawn up by Dr. G. Stegeman (Study director) of Akzo Nobel Central Research, Dept. RGL, Arnhem, The Netherlands. This protocol is stored in the study file and was approved and signed by both the Study director (Akzo Nobel Central Research) and the Study monitor (AquaSense Lab).

The concentration of the test substance in the samples was determined by means of gas chromatography with flame ionization detection. Samples (≈ 20 ml) were withdrawn from the control, lowest , middle and highest concentration. Samples from the other concentrations were also withdrawn and stored for additional analyses, if needed. The concentration of the test substance in the soiution withdrawn directly from the stock solution was also determined. The samples were stored in the refrigerator in the dark until analysis. All samples were taken in single-fold and analyzed in duplicate. Samples were taken at 0, 24 and 48 hours of testing. The initial samples for analysis (0 hours) were withdrawn from the vessels after addition of the testorganisms and stabilization of the testsystem. The samples were stored for a maximum period of 4 weeks.

3. RESULTS

3.1 *Test environment*

The pH values during the 48 hours of testing showed a maximum deviation of 0.2 units (see Table I). The oxygen values exceeded the 60% of the saturation values by far as shown in Table I. The temperature during the test varied between 19.8 and 20.6 °C with a mean value of 20.3 °C. The light intensity measured at the beginning and end of the test in the test chamber was between 8.30 and 8.90 µmol/m²/s, which is in agreement with the light intensity of 8 ± 2 µmol/m²/s as recommended in the OECD/EEC guidelines.

3.2 *Test results*

All Daphnids survived after 24 hours of testing at the concentrations 0.223 and 0.335 mg/l. The highest two concentrations showed 100% mortality after 48 hours of testing, and partial immobilities were found at all other concentrations after 48 hours of testing. Because the testconcentrations dropped below 80% of the initial concentration, the calculation of the effects were also performed using the geometric means of the measured concentrations. The EC_{50} value calculated after 48 hours of testing based on the nominal values is 0.396 mg/l (0.345 - 0.455 95% confidence limits) and the NOEC derived from the results is 0.223 mg/l. Based on the measured concentrations the EC_{50} is 0.321 mg/l (0.275 - 0.370 95% confidence limits), and the NOEC is 0.168 mg/l. No other deviations, such as changes in the behaviour or other effects have been observed.

3.3 *Validity of test*

The test is valid as shown by the oxygen and pH values described in section 3.1 and by the absence of immobility in the controls after 48 hours of testing, which is less than the maximum acceptable value of 10% as described in the OECD/EEC guidelines. The results of the quality control test with potassiumdichromate (24-Feb-1998: 24Hr-EC_{50}, 2.0 mg/l) are in agreement with the criteria mentioned in the Study Protocol, indicating that the sensitivity of the Daphnids has not changed significantly.

3.4 *Chemical analysis*

The results of the chemical analysis are described in the GLP report of Akzo Nobel Central Research RGL F98046 [4.15], and is added in Annex II. The results of the chemical analyses of the concentration range with Phantolid® showed that the initial values were

substantially achieved. Some of these values are above 100%, which was probably due to variations during preparation of the test substance test series. During the test the concentrations dropped gradually to approximately 50% of the initial values. This decrease could not be fully caused by photodegradation or adsorption onto the test vessel wall, based on an investigation of the photodegradation of Phantolid® under light conditons [4.16]. The results of this investigation indicated that the decrease in the concentration was almost identical in samples incubated in the light and dark. Additionally, it was found that only 4.5% of the total amount of Phantolid® was adsorbed onto the test vessel wall during an incubation period of 72 hours.

3.5 Deviations from Study Protocol

The following deviations from the Study Protocol were observed during the study:

- The amount of sample taken for chemical analyses was approximately 20 ml at each sampling time, with equal amounts from each four vessels. This procedure was not described in the Study Protocol.
- The light intensity in the test chamber was measured at the beginning and end of the test. This was not described in the Study Protocol.
- The test vessels were approximately 70 ml total volume instead of 100 ml.
- Test vessels were not placed in a random way in the incubator, as mentioned in the Study Protocol.
- The EEC 67/548 C2 guideline used in the study was the Dutch version instead of the English.

3.6 Conclusions

The toxicity of Phantolid® to *Daphnia magna* was determined over a period of 48 hours. The EC_{50} value calculated after 48 hours of testing based on the nominal values is 0.396 mg/l (0.345 - 0.453 95% confidence limits) and the NOEC derived from the results is 0.223 mg/l. Based on the measured concentrations the EC_{50} is 0.321 mg/l (0.275 - 0.370 95% confidence limits), and the NOEC is 0.168 mg/l.

4. REFERENCES

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Table I

Measurements of the pH and oxygen values in the test vessels at the various time intervals.

Nominal concentration (mg/l)	pH		O_2 [1]	
Time (hours)	0	48	0	48
Control	8.3	8.1	100	95
	8.3	8.1	100	95
	8.3	8.1	100	97
	8.3	8.1	100	98
0.223	8.2	8.1	98	95
	8.2	8.1	98	94
	8.2	8.1	98	94
	8.2	8.1	98	96
0.335	8.2	8.1	101	94
	8.2	8.1	101	96
	8.2	8.1	101	94
	8.2	8.1	101	96
0.502	8.2	8.1	101	95
	8.2	8.1	101	94
	8.2	8.1	101	94
	8.2	8.1	101	96
0.752	8.1	8.1	102	95
	8.1	8.1	102	94
	8.1	8.1	102	94
	8.1	8.1	102	95
1.127	8.1	8.1	104	96
	8.1	8.1	104	95
	8.1	8.1	104	95
	8.1	8.1	104	96

[1] = Oxygen concentrations are expressed as percentage of the saturation values at the measured temperature.

Table II

Number of mobile Daphnids during the 48 hours of testing.

Nominal concentration (mg/l)	Number of mobile Daphnids		
Time (hours)	0	24	48
Control	5	5	5
	5	5	5
	5	5	5
	5	5	5
total	**20**	**20**	**20**
0.223	5	5	5
	5	5	4
	5	5	5
	5	5	4
total	**20**	**20**	**18**
0.335	5	5	4
	5	5	4
	5	5	3
	5	5	3
total	**20**	**20**	**14**
0.502	5	5	1
	5	5	2
	5	5	1
	5	3	3
total	**20**	**18**	**7**
0.752	5	2	0
	5	2	0
	5	1	0
	5	1	0
total	**20**	**6**	**0**
1.127	5	0	0
	5	0	0
	5	1	0
	5	1	0
total	**20**	**2**	**0**

Table III

Results of the chemical analysis. Concentrations are presented as mean values of duplicate analysis.

Nominal concentration (mg/l)	Measured concentration (mg/l) with 95% confidence limits			Geometric mean (mg/l)	% of initial concentration		
time (h)	0	24	48	-	0	24	48
0	< 0.039[1]	< 0.039[1]	< 0.039[1]	-	-	-	-
0.223	0.267 ± 0.032	0.144 ± 0.032	0.123 ± 0.032	0.168	100	54	46
0.335	n.d.	n.d.	n.d.	0.270			
0.502	0.640 ± 0.031	0.362 ± 0.032	0.337 ± 0.032	0.427	100	57	53
0.752	n.d.	n.d.	n.d.	0.606			
1.127	1.143 ± 0.031	0.840 ± 0.031	0.659 ± 0.031	0.858	100	73	58
Stock	1.127 ± 0.031	n.d.	n.d.				

[1] = Concentrations were below detection limit (= 0.039 mg/l)

Table IV

Summary of the statistical evaluation.

Time (hours)	EC_{50} (mg/l)	95% confidence limits	100% immobility	NOEC (mg/l)
Nominal concentrations				
24	0.700	0.621 - 0.793		-
48	0.396	0.345 - 0.453	0.752	0.223
Measured concentrations				
24	0.644	0.576 - 0.719		-
48	0.321	0.275 - 0.370	0.610	0.168

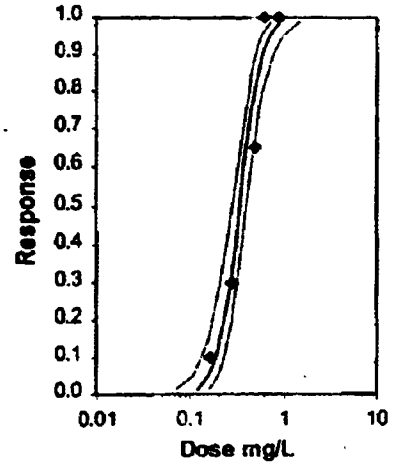
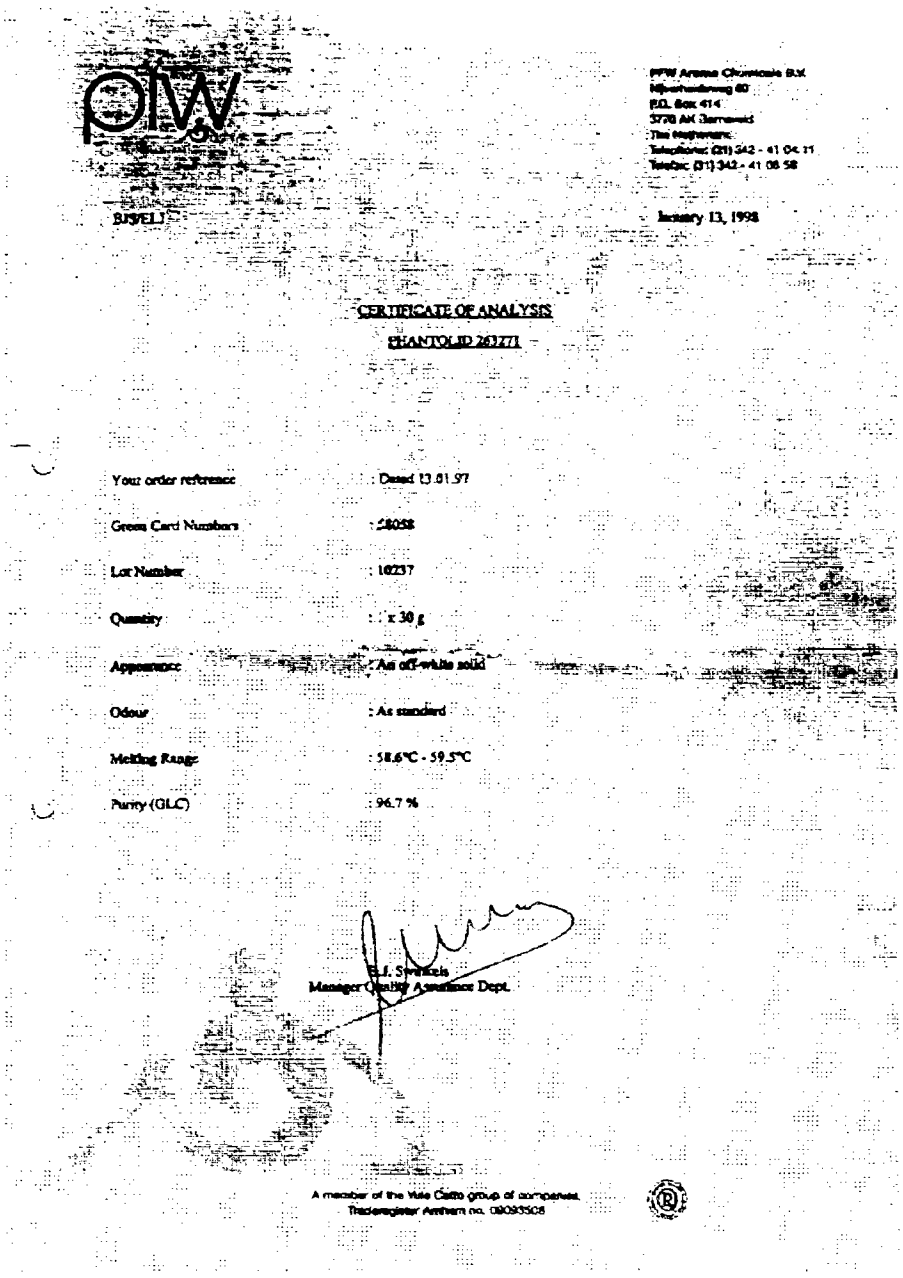


Figure 1

Dose-response curve and the 95 % confidence limits of Phantolid® calculated from the percentage of inhibition at various test concentrations (measured values) after 48 hours of testing.

Annex I



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BJS/ELJ

January 13, 1998

CERTIFICATE OF ANALYSIS

PHANTOLID 263271

Your order reference	: Dated 13.01.97
Green Card Numbers	: 58058
Lot Number	: 10237
Quantity	: [illegible] x 30 g
Appearance	: An off-white solid
Odour	: As standard
Melting Range	: 58.6°C - 59.5°C
Purity (GLC)	: 96.7 %

E.J. Swinkels
Manager Quality Assurance Dept.

A member of the Vale Catto group of companies.
Traderegister Arnhem no. 09093508

Annex II

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Central Research
Location Arnhem

Final Research Report

RGL F98046 T 98005 A May 29, 1998

G. Stegeman, H. Henderiks

ANALYSIS OF PHANTOLID IN TEST MEDIA FROM TOXICITY TESTS

Project identification

Client	Aquasense, Amsterdam	Client No	Project No	
Subclient		Subclient No	Task No	9133

Drafted by department General Analytical and Environmental Chemistry

Keywords GC, ecotox, fish toxicity, Daphnia toxicity

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ABSTRACT

To determine toxicity of Phantolid a series of tox tests with fish and daphnids and an algal growth inhibition test were performed by Aquasense Amsterdam. The concentration of Phantolid in the used test media was determined by the General Analytical and Environmental Chemistry Department of Akzo Nobel Central Research. Samples to be analysed were test media with high, medium, low and zero concentration of Phantolid. Samples were taken at the beginning of the test (t=0 hour), at the end of the test and between times after every 24 hours. In this report procedures and results of the chemical analyses are described.

Analyses were carried out in compliance with the OECD Principles of Good Laboratory Practice (GLP). Test media samples were extracted with dibutylether using a so-called in-vial extraction procedure. The ether layer was subsequently analysed by means of gas chromatography (GC) with flame ionisation detection.

The concentration of Phantolid in the test media from the Daphnia magna tests at t=0 hours were between 0 and 1.14 mg/l. In the course of the test the concentrations decreased gradually. At the end of the test, after 48 hours, concentrations were roughly 50% of the initial concentration.

The initial concentration of Phantolid in the algal media ranged between 0 and 0.24 mg/l. The low concentration level was already below the detection limit of 0.04 mg/l. During the 72 hour test the Phantolid concentrations were found to decrease steadily. At the end of the test concentration levels in the test media were all below the detection limit except for the highest dose concentration, which had declined to 0.05 mg/l.

The concentration of Phantolid in test media from the 96 hour fish toxicity study were between 0 and 0.95 at the beginning of the test. Already after 48 hours the Phantolid concentration in all test media had decreased below the detection limit of 0.04 mg/l. This decrease in Phantolid concentration is more pronounced than in the algal and Daphnia test.

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ANALYSIS OF PHANTOLID IN TEST MEDIA FROM TOXICITY TESTS

Sponsor	Aquasense P.O. Box 95125 1090 HC Amsterdam The Netherlands
Study monitor	Drs. Ing. A.G.M. Kroon

STUDY ORGANISATION

Location	Akzo Nobel Central Research Arnhem General Analytical and Environmental Chemistry Dept Velperweg 76 6824 BM Arnhem The Netherlands
Study director	Dr. G. Stegeman
Quality Assurance Unit	E.H.V. Denks
Management, Head of Department RGL	Dr.Ir. C J. Groenenboom
Initiation date of the study	1998-05-15
Completion date of the study	1998-05-25

ARCHIVING AND STORAGE

The project file including the final report, amendments to the final report, the study plan, amendments to the study plan, records of quality assurance inspections, all letters, memos and notes and raw data pertaining to the study will be retained in the archives of Akzo Nobel Central Research Arnhem for a period of ten years. Other records including master schedule sheet, laboratory notebooks, logbooks, records of the maintenance and calibration of equipment, summary of training, curricula vitae and job descriptions of the personnel involved in the study, records related to location and storage of the test substance will also be kept in the Akzo Nobel Central Research Arnhem archives for a period of ten years. Test material will be stored deepfrozen under the sample code T 98005 for ten years or only as long as the quality of the test substance permits evaluation.

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GLP COMPLIANCE STATEMENT

The study reported here was carried out according to the study plan in compliance with the OECD Principles of Good Laboratory Practice (5.1). The report contains an accurate description of the results.

Study director
Dr. G. Stegeman

date 1998.05.29

Management, Head of Department RGL
Dr.Ir. C.J. Groenenboom

date 1998-06-02

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QUALITY ASSURANCE STATEMENT

This report was audited by the Quality Assurance Unit of Akzo Nobel Central Research Arnhem. It is considered to be an accurate presentation of the methods and procedures applied in the course of the study and an accurate reproduction of the data recorded.
Listed below are the dates of inspection of this study by the Quality Assurance Unit and the dates on which its findings were reported to Study Director and Management.

Dates of inspection	Dates of reporting
1998-05-20	1998-05-20
1998-05-29	1998-05-29

Quality Assurance Unit
E.H.V. Derks

date 1998.05.29

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1. INTRODUCTION

To determine toxicity of Phantolid, a substance produced by PFW Aroma Chemicals, a series of tox tests with fish and daphnids and an algal growth inhibition test were performed by Aquasense Amsterdam (project No 1202B). The concentration of Phantolid in the used test media was determined by the General Analytical and Environmental Chemistry Department of Akzo Nobel Central Research. In this report the procedures and results of the chemical analyses are described.

The samples to be analysed were taken from test media at the beginning, at the end and during the toxicity tests at regular time intervals. The samples were extracted "in-vial" with dibutylether. The concentration of Phantolid in the dibutylether layer was determined by means of gas chromatography (GC) with flame ionisation detection. The analyses were carried out in compliance with the OECD Principles of Good Laboratory Practice (5.1) and according to Standard Operation Procedure N 1 (5.2).

2. MATERIALS

2.1 Test substance

The test substance Phantolid (sample code: T98005) was received from Aquasense on 1998.05.08. The following test substance data were submitted by the sponsor, who accepted full responsibility for the validity of these data. Information concerning the test material:

-	name of test substance	Phantolid
-	chemical name	5-acetyl-1,1,2,3,3,6-hexamethylindan
-	Cas reg.No.	15323-35-0
-	batch/lot No.	10237
-	Aquasense sample No	302900
-	purity	96.7 %
-	appearance	an off-white solid (powder)
-	storage until required	in a cool place in the dark
-	stability	stable under storage conditions

2.2 Samples

The samples to be analysed were received from Aquasense on 1998.05.15. On receipt all samples were still refrigerated, which was checked by taking some samples in the palm of the hand. After checking all the samples, they were immediately stored in a refrigerator until analysis. Sample codes and other relevant data supplied by Aquasense were assumed to be correct and were used without further check. Samples were analysed within one week after receipt.

-	sample description	aqueous solutions of test substance
-	storage until required	in a refrigerator

2.3 Chemicals

Dibutylether, $[CH_3(CH_2)_3]_2$ O	99% GC-grade

2.4 Deionized water

Deionized water was produced from tap water in a water purification system (Spectrum-Elgastat, Breukelen, The Netherlands). It had a conductivity of less than 5 µ $S.cm^{-1}$, a TOC content of less than 2 mg/l and it contained no more than 0.01 mg Cu per litre (Standard Operation Procedure K 10 (5.3)).

2.5 Apparatus

Description of the apparatus used and some relevant settings:

Gas chromatography system	Carlo Erba GC 8160
Autosampler	Carlo Erba AS 800
	sampling depth: -22 mm
Injection	cold on-column injection
	cool time: 12 seconds
	injection volume: 1 µl
	injection speed: maximum
	syringe : Hamilton microliter, 10 µl AS800
	syringe rinse procedure: standard AS800 procedure (bubble elimination = 5)
GC-column	J&W DB-1 with retention gap
	length: 30 meter
	internal diameter: 0.32 mm
	film thickness: 0.5 µm
Detection	Carlo Erba FID-80 flame ionization detector
	temperature: 300°C
	flame gases: 50 kPa H_2 and 100 kPa air
Carrier gas	Helium 5.0; 100 kPa head pressure
Temperature program	Initial temperature: 150°C (2 minutes)
	temperature rise: 4°C/min
	final temperature 225°C (5 minutes)
Data collection/integration	Fisons Multichrom rev.2 chromatography software
Recorder	Kipp & Zn BD-8, 1 mV

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2.6 Materials

Chromacol 2-ml glass GC autosampler vials with Teflon lined crimp caps.

3. METHODS

3.1 Preparations for GC analysis

The performance of the installed GC column was checked by a blank run (i.e. a chromatographic run without sample injection) and by analysis of a Phantolid standard in dibutylether. After checking column contamination, column bleed and peak shapes, the column was approved for chemical analyses.

3.2 Calibration of chromatographic system

A stock solution of Phantolid was prepared in dibutylether. From this stock solution a series of 6 calibration standards was prepared in dibutylether covering the expected concentration range of the samples.
The GC standards were prepared by adding 0.8 ml of deionized water and 0.8 ml of the standard solution to a GC sample vial. For each concentration level a duplicate GC standard was prepared. The capped vials were shaken vigorously for 2 minutes and were then left standing for at least 5 minutes to settle. The series of duplicate calibration standards were analyzed in single fold starting with the lowest concentration.

3.3 Control samples

To check on errors in the preparation of calibration standards, a second independent stock solution of Phantolid was prepared in dibutylether. From this stock solution a control sample of known Phantolid concentration was prepared within the concentration range of the calibration line. The control sample was prepared in duplicate according to the procedure described in 3.2. The duplicate control samples were analyzed in single fold.

3.4 Analysis of Phantolid samples

The refrigerated samples were brought to room temperature. The samples were shaken vigorously to homogenize and then sonicated for 10 minutes. If aggregates or particles were visible (e.g. algae of fish scales), the samples were centrifuged at 8000 rpm for 10 minutes. When necessary, samples were diluted in deionized water to obtain a nominal concentration within the calibration range.
From each test sample a duplicate sample for GC analysis was prepared. GC samples were prepared by adding 0.8 ml of the aqueous sample and 0.8 ml of dibutylether to a GC sample vial. The capped vials were shaken vigorously for 2 minutes to extract the aqueous layer and were then left standing for at least 5 minutes to settle.

Each GC sample vial was assayed in single fold (which means that each received test media sample was analyzed in duplicate since GC samples were prepared in duplicate). After a series of ten duplicate samples a calibration standard (except for the highest or lowest standard) was analyzed in single fold. It was checked if results satisfied quality criteria (see 3.6.3, 3.6.4).

3.5 Data processing

The peak area of the Phantolid peak (only one single Phantolid peak was detected) was determined using the Multichrom integration software. Key integration parameters were kept the same for calibration runs and sample analysis. Calibration line(s) were constructed by plotting peak area (in counts) against the concentration of test substance in the calibration standards using Statcal 6.50 software package [5.4]. The optimum polynomial fit of the calibration curve, coefficients of the polynomial (A, B, etc.) and standard deviation of residuals (S_R) were calculated.

For each sample the concentration of Phantolid in the dibutylether layer and the 95% confidence interval were calculated from the measured peak area and the calibration line using Statcal 6.50. To obtain the true Phantolid concentration a correction was made for impurity of the test substance. The concentration of Phantolid in the aqueous test samples were calculated assuming that the distribution coefficient of Phantolid, describing equilibrium distribution of Phantolid between the aqueous and ether phase, is the same for the calibration standards and the samples.

3.6 Data evaluation and quality control

3.6.1 Calibration standards

It was checked if the calibration standards were within the 99% confidence limits of the corresponding calibration line. If one data point was outside the 99% confidence interval this data point was rejected and the regression line was recalculated. If the calibration series contained more than one data point outside the 99% confidence interval the whole calibration series was rejected and a new calibration line was constructed with freshly prepared standards.

3.6.2 Control samples

If the known concentration of the control samples was outside the 99% confidence interval of the measured concentration another control sample, prepared from a fresh stock solution, was analyzed. If the measured concentration of this control sample was still deviating significantly (99% confidence) from the true concentration, the whole calibration line was rejected, a series of freshly prepared standards was analyzed and a new calibration line was constructed.

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3.6.3 Samples

Using Statcal 6.50 the width of the 99% confidence interval of the calibration line (= 2 * $t_{99\%}$*S_R, where the t-value is from the statistical two-tailed t-distribution) was calculated. If the difference between measured peak areas (in area counts) of duplicate analyses was larger than the width of the 99% confidence interval of the calibration line, the results for this sample were rejected and the sample was reanalyzed.

3.6.4 Control standards

If the measured concentration of the control standards; injected after each series of ten samples, deviated significantly from the true concentration (i.e. if the true concentration was not within the 99% confidence interval of the measured value), the control standard was reanalyzed in duplicate. If the true concentration remained outside the 99% confidence interval, the results for the previous 10 samples were rejected, the system was recalibrated using the original calibration standards and the 10 samples were reanalyzed.

4. RESULTS AND DISCUSSION

4.1 General

GC analysis of pure extraction solvent (dibutylether) and a Phantolid standard solution showed that Phantolid appears as a single peak which is free from interferences from the solvent and solvent impurities.

Analysed samples were test media samples containing high, medium, low and zero Phantolid dose concentrations. The samples were taken at the beginning of the test (t=0 hr), at the end of the test and between times after every 24 hours.

After shaking and sonication, none of the samples contained visible particles or aggregates so none of the samples was centrifuged.

Results of the analyses of the test media are summarized in table 6.1-6.3. The first three columns of each table ("Aquasense sample code", "Sampling date" and "Nominal sampling time") contain data provided by Aquasense. These data were taken from the sample dispatch form which was sent along with the samples. Reported Phantolid concentrations are corrected for impurity of the test substance and thus represent the concentration of pure Phantolid.

4.2 Samples from toxicity test with Daphnia magna.

The concentration of Phantolid in the test media from the Daphnia magna tests at t=0 hours were between 0 and 1.14 mg/l. In the course of the test the concentrations decreased gradually. At the end of the test, after 48 hours, concentrations were roughly 50% of the initial concentration. The current chemical analyses do not give a clue for the cause of the Phantolid losses.

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4.3 Samples from algal growth inhibition test

The initial concentration of Phantolid in the algal media was between 0 and 0.24 mg/l. The low dose concentration was already below the detection limit of 0.04 mg/l. During the 72 hour test the Phantolid concentrations were found to decrease steadily. At the end of the test concentration levels in the test media were all below the detection limit except for the highest dose concentration, which had declined to 0.05 mg/l.

4.4 Samples from toxicity tests with fish

The concentration of Phantolid in test media from the 96 hour fish toxicity study were between 0 and 0.95 at the beginning of the test. Already after 48 hours the Phantolid concentration in all test media had decreased below the detection limit of 0.04 mg/l. The observed decrease in Phantolid concentration is more pronounced than in the algal and Daphnia test.

5. REFERENCES

5.1 OECD, 1981. Principles of Good Laboratory Practice, C(81)30(final)

5.2 Standard Operation Procedure (SOP) N 1: Procedures and quality assurance of chemical analyses, procedure.

5.3 Standard Operation Procedure (SOP) K 10: Deionisator, method.

5.4 Statistics software package: Statcal 6.50, J. Kragten, University of Amsterdam, Amsterdam, 1996

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6. TABLES

Table 6.1 Analysis of Phantolid in test media from Daphnia magna toxicity test Aquasense project 1202B

Aquasense sample code	Sampling date	Nominal sampling time	Phantolid concentration and 95% confidence interval (mg/l)
DM stock, wit	1998-05-11	t= 0 hr	1.127 ± 0.031
DM [0], wit	1998-05-11	t= 0 hr	< 0.039 [a]
DM [1], wit	1998-05-11	t= 0 hr	0.267 ± 0.032
DM [3], wit	1998-05-11	t= 0 hr	0.640 ± 0.031
DM [5], wit	1998-05-11	t= 0 hr	1.143 ± 0.031
DM [0], groen	1998-05-12	t= 24 hr	< 0.039 [a]
DM [1], groen	1998-05-12	t= 24 hr	0.144 ± 0.032
DM [3], groen	1998-05-12	t= 24 hr	0.362 ± 0.032
DM [5], groen	1998-05-12	t= 24 hr	0.840 ± 0.031
DM [0], rood	1998-05-13	t= 48 hr	< 0.039 [a]
DM [1], rood	1998-05-13	t= 48 hr	0.123 ± 0.032
DM [3], rood	1998-05-13	t= 48 hr	0.337 ± 0.032
DM [5], rood	1998-05-13	t= 48 hr	0.659 ± 0.031

[a] concentration below limit of detection

Table 6.2 Analysis of Phantolid in test media from the algal growth inhibition test Aquasense project 1202B

Aquasense sample code	Sampling date	Nominal sampling time	Phantolid concentration and 95% confidence interval (mg/l)
A stock, wit	1998-05-12	t= 0 hr	0.741 ± 0.031
A [0], wit	1998-05-12	t= 0 hr	< 0.039 [a]
A [1], wit	1998-05-12	t= 0 hr	< 0.039 [a]
A [3], wit	1998-05-12	t= 0 hr	0.059 ± 0.032
A [5], wit	1998-05-12	t= 0 hr	0.232 ± 0.032
A [0], groen	1998-05-13	t= 24 hr	< 0.039 [a]
A [1], groen	1998-05-13	t= 24 hr	< 0.039 [a]
A [3], groen	1998-05-13	t= 24 hr	< 0.039 [a]
A [5], groen	1998-05-13	t= 24 hr	0.151 ± 0.032
A [0], rood	1998-05-14	t= 48 hr	< 0.039 [a]
A [1], rood	1998-05-14	t= 48 hr	< 0.039 [a]
A [3], rood	1998-05-14	t= 48 hr	< 0.039 [a]
A [5], rood	1998-05-14	t= 48 hr	0.075 ± 0.032
A [0], blauw	1998-05-15	t= 72 hr	< 0.039 [a]
A [1], blauw	1998-05-15	t= 72 hr	< 0.039 [a]
A [3], blauw	1998-05-15	t= 72 hr	< 0.039 [a]
A [5], blauw	1998-05-15	t= 72 hr	0.051 ± 0.032

[a] concentration below limit of detection

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Table 6.3 Analysis of Phantolid in test media from fish toxicity test Aquasense project 1202B

Aquasense sample code	Sampling date	Nominal sampling time	Phantolid concentration and 95% confidence interval (mg/l)
V stock, wit [b]	1998-05-11	t= 0 hr	1.127 ± 0.031
V [0], wit	1998-05-11	t= 0 hr	< 0.039 [a]
V [1], wit	1998-05-11	t= 0 hr	0.362 ± 0.032
V [3], wit	1998-05-11	t= 0 hr	0.650 ± 0.031
V [5], wit	1998-05-11	t= 0 hr	0.950 ± 0.031
V [0], groen	1998-05-12	t= 24 hr	< 0.039 [a]
V [1], groen	1998-05-12	t= 24 hr	< 0.039 [a]
V [3], groen	1998-05-12	t= 24 hr	< 0.039 [a]
V [5], groen	1998-05-12	t= 24 hr	0.063 ± 0.032
V [0], rood	1998-05-13	t= 48 hr	< 0.039 [a]
V [1], rood	1998-05-13	t= 48 hr	< 0.039 [a]
V [3], rood	1998-05-13	t= 48 hr	< 0.039 [a]
V [5], rood	1998-05-13	t= 48 hr	< 0.039 [a]
V [0], blauw	1998-05-14	t= 72 hr	< 0.039 [a]
V [1], blauw	1998-05-14	t= 72 hr	< 0.039 [a]
V [3], blauw	1998-05-14	t= 72 hr	< 0.039 [a]
V [5], blauw	1998-05-14	t= 72 hr	< 0.039 [a]
V [0], geel	1998-05-15	t= 96 hr	< 0.039 [a]
V [1], geel	1998-05-15	t= 96 hr	< 0.039 [a]
V [3], geel	1998-05-15	t= 96 hr	< 0.039 [a]
V [5], geel	1998-05-15	t= 96 hr	< 0.039 [a]

[a] concentration below limit of detection
[b] V stock, wit = DM stock, wit (see table 6.1)

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Evaluation of the toxic effects of Phantolid® on the growth of the freshwater alga *Selenastrum capricornutum*

02

Evaluation of the toxic effects of Phantolid® on the growth of the freshwater alga *Selenastrum capricornutum*

Final Report

CONFIDENTIAL

Study started	11-May-1998
Study completed	15-May-1998
Study number	1202B.GLP.ALG03
Report number	98.1202-15 (re-issue of 98.1202-8)
Performing laboratory	AquaSense Lab, P.O. Box 95125, 1090 HC Amsterdam, The Netherlands
Study Director	Drs.Ing. A.G.M. Kroon
Approved by	Dr. J.T. Meulemans

Sponsor	PFW Aroma Chemicals BV, Barneveld, The Netherlands
Study Monitor	Dr. H-D. Gaisser

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CONFIDENTIALITY STATEMENT

This document contains information and unpublished results of research which may be confidential, and therefore it should not be photocopied or microfilmed. It should also not be released in any form to an outside party, nor should information contained herein be used by any authority without the written permission of the Sponsor.

05

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with the Principles of Good Laboratory Practice as described in the OECD Principles of Good Laboratory Practice (1998). All data on the test substance such as , characterization, verification and properties were submitted by the Sponsor who accepted full responsibility for the validity thereof.

The study is valid for the purpose for which it was conducted and this report is a true reflection of the raw data generated.

All persons involved in the performance of the study were experienced and qualified, able to conduct the experimental tasks assigned to them.

Drs. Ing. A.G.M Kroon,
Study Director

14/01/99
Date,

I have reviewed this report and concur with its contents.

Dr. J T. Meulemans,
Manager Aquasense Lab

15 - 1 - 99
Date.

QUALITY ASSURANCE STATEMENT

The testing facilities utilized in this study have been inspected regularly in accordance with the Principles of Good Laboratory Practice.

This study was inspected and audited and the findings were reported to the Study Director and the Management on the dates as shown below. Inspections were performed according to the Standard Operating Procedures (SOP) of the testing facility. The final report was audited in detail against the approved Study Protocol and all pertinent raw data.

It is considered to be an accurate presentation of the methods and procedures applied in the course of the study and an accurate reproduction of the data recorded.

Inspection date	Inspection/Audit	Date of QA report
07-05-98	Study protocol inspection	08-05-98
12-05-98	Study-based inspection	13-05-98
05-06-98	Report inspection	05-06-98
18-08-98	Re-issue inspection	18-08-98
04-11-98	Report Amendment inspection	05-11-98
14-12-98	Re-issue inspection	14-12-98

Ir. G.M. van Eijnatten,
Quality Assurance manager

14/01/99
Date,

STUDY PERSONNEL AQUASENSE LAB

Study Director	Drs.Ing. A.G.M. Kroon
Technician(s)	Ing. A. van Mullem, Ing. S. de Valk S. Rooijakkers
Management	Dr. J.T. Meulemans

ARCHIVING OF RECORDS AND SAMPLES

The study file including the Final Report, Study Protocol, ammendments, QA records, and all raw data pertaining to the study is retained in the archives of Aquasense Lab for a period of ten years. The test material is stored deepfrozen under the sample number (302900) for ten years, or as long as the quality of the test substance permits evaluation. All other records connected with the study are also being registered in the Aquasense Lab archives for a period of at least ten years.

Summary

In order to predict effects of chemicals in an aquatic environment, the toxicity of Phantolid® to *Selenastrum capricornutum* was assessed. The toxicity was determined in an acute toxicity test which is described in the OECD and EEC test guidelines, and was performed in accordance with the principles of Good Laboratory Practice (GLP). The toxicity was determined over an exposure period of 72 hours under defined conditions. During the test the pH varied no more than 0.5 units, and the cell density increased with a factor of 120. The temperature varied between 22.2 and 23.1 °C, and the light intensity between 86.13 and 89.14 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Almost complete inhibition of growth at the highest concentration and no significant inhibition of growth at the lowest concentration was observed during the test period. These data indicates that the validity criteria of the test was fulfilled. The E_bC_{50} and E_rC_{50} of Phantolid® based on the nominal values are 0.081 (0.069 - 0.095 95% confidence limits) and 0.200 (0.179 - 0.221 95% confidence limits). The NOEC based on the growth rate derived from the results is 0.044 mg/l Phantolid®. The calculation of both the EC_n values and the NOEC's using the measured concentrations are based on very limited data and all the data used were all near the detection limit of the analysis, and therefore the results are very questionable. The chemical analyses indicated a rapid decrease of the concentration during the test period, which may for a small part be attributed to adsorption onto the test vessel wall.

1. INTRODUCTION

The objective of this study was to assess the toxicity of Phantolid® to cultures of the freshwater green algae *Selenastrum capricornutum*. In the present toxicity test exponentially growing cultures of *Selenastrum capricornutum* were exposed to various concentrations of the test substance over several generations under defined conditions. The inhibition of growth in relation to control cultures was determined over a testing period 72 hours. The toxicity was assessed by determining cell counts for each test concentration in time. These cell numbers were used to determine the $EC_{20,50,80}$ values together with the 95% confidence limits based on their average growth rate (E_r) and mean biomass (E_b). The no observed effect concentration (NOEC) is derived from the results, where possible.

The methods employed were based on the OECD test guideline 201 [4.1] and the EEC directive 67/548 C3 [4.2], and in compliance with the OECD principles of Good Laboratory Practice [4.3].

1. INTRODUCTION

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2. MATERIALS AND METHODS

2.1 *Test substance*

The sample of Phantolid® was received at Aquasense Lab on 15-jan-98 and assigned the sample number 302900. The following test substance data were submitted by the Sponsor. The Sponsor accepted full responsibility for the validity of these data.

• name/code	Phantolid®
• chemical name	5-acetyl-1,1,2,3,3,6-hexamethylindan
• batch/lot No.	10237
• CAS nr.	15323-35-0
• purity	96.7 %
• appearance	off-white powder
• water solubility	estimated at 0.1 - 1 mg/l
• vapour pressure	not known
• storage conditions	at room temperature in the dark
• stability	stable for at least 12 months under storage conditions
• study sample number	302900

The nominal concentrations as referred in this report are the concentrations based on the 96.7% purity of the active ingredient. The Certificate of Analysis is presented in Annex I.

2.2 *Test organism*

The test was carried out using the freshwater unicellular green algae *Selenastrum capricornutum* (new name: *Raphidocelis subcapitata*), purchased form Charles River Aquatic, Someren, The Netherlands). The algae was maintained on mineral medium and regularly transferred to a fresh mineral medium to act as inoculum. The procedures for cultivation of the algae were in accordance with Standard Operating Procedure A-323 [4.6]. Reference tests with potassium dichromate will be performed every 6 months and compared with international inter-laboratory test data to confirm that the algae has not changed.

2.3 *Chemicals*

The chemicals for preparation of the test medium were all of analytical grade and obtained from established commercial suppliers. (Merck, Darmstadt, Germany, main supplier).

2.4 *Glassware*

The test was performed in 300 ml Erlenmeyers containing 100 ml of mineral salts medium. The test flasks were closed with cotton-wool stoppers. All other equipment was made out of glass. All glassware used was cleaned prior to testing according to Standard Operating Procedure A-340 [4.12].

2.5 *Apparatus and conditions*

The culturing cabinet was a temperature-controlled illuminated orbital incubator (IOI-400, Sanyo, Breda, The Netherlands) in which the temperature was maintained at 23 ± 2°C, and a continuous uniform illumination was provided in the spectral range of 400 to 700 nm by using 30 W fluorescent lamps of the type 'universal white' (colour temperature of approximately 4000 K), at a distance of 0.35 m from the algal cultures. The light intensity was in the range of 60 to 120 µmol (=µE)•m^{-2}•s^{-1}. The culture flasks were rotated continuously at approximately 100 rev/min to prevent sedimentation of the algae. The pH's were determined with the aid of a pH meter (pH 196, WTW, Weilheim, Germany) . The temperature was measured continuously using a temperature sensor coupled to a datalogger (Elbanton, Kerkdriel, The Netherlands). The light intensity was measured with a light intensity meter (Li-Cor, LI-189, Rijswijk, The Netherlands)

2.6 *Test substance stock solution*

A test substance stock solution was prepared by weighing out the test substance (0.05000 gram) using a analytical balans (Sartorius, 160P, Breukelen, The Netherlands). This was transferred to a separatory funnel filled with 500 ml test medium. This mixture was ultrasonically treated for approximately 20 minutes and subsequently mixed for approximately 20 hours at room temperature using a magnetic stirrer. Thereafter, the liquid was filtered using a glass fibre filter (Schleicher& Schull, GF 92 Glasfaser filter (ref.nr. 421057 X 27358), 1µm Ø) to remove all particles. The clear aqueous filtrate was used as stock solution for preparation of the desired concentration range. This method was chosen from a range of methods (e.g. generator column, ultrasonic treatment) which have been tested for the preparation of a suitable stock solution of the test substance. This preliminary investigation was not performed according to the OECD principles of GLP. The results are stored in the archive of AquaSense Consultancy [4.10]. The tested methods were in accordance with the OECD 201 and EEC Directive 67/548 C3 guidelines [4.1, 4.2] and/or international guidelines on the preparation of poorly soluble substances for aquatic toxicity testing [4.11].

2.7 *Test inoculum*

The initial stock culture was inoculated with *Selenastrum capricornutum* from a pre-culture and checked for purity by microscopic means (1000 times magnification) using an inverted microscope (Olympus IMT-2, Paes, Zoeterwoude, The Netherlands). Before the beginning of the test the extinction of an exponentially growing stock culture was measured. The cell density was determined using a calibration curve (see section 2.9). From this algal culture a dilution was prepared to obtain an initial cell density of approximately $1 \cdot 10^4$ cells/ml in the medium of the test.

2.8 *Test medium*

The test medium (mineral medium) was prepared and contained the following nutrients:

nutrients	mg/l
Macro-nutrients	
NH_4Cl	15
KH_2PO_4	1,6
$CaCl_2(H_2O)_2$	18
$MgSO_4(H_2O)_7$	15
$MgCl_2(H_2O)_6$	12
Fe-EDTA	
$FeCL_3\ (H_2O)_6$	0.08
$Na_2EDTA(H_2O)_2$	0.1
Trace elements	
H_3BO_3	0.185
$ZnCl_2$	0.003
$MnCl_2(H_2O)_2$	0.340[1]
$CoCl_2(H_2O)_6$	0.0015
$CuCl_2(H_2O)_2$	$1x10^{-6}$
$Na_2MoO_4(H_2O)_2$	0.007
$NaHCO_3$	
$NaHCO_3$	50

[1] = $MnCl_2 \cdot 2H_2O$ instead of $MnCl_2 \cdot 4H_2O$ was used, concentration was adjusted.

The pH of the medium was approximately 8. All solutions were made using deionized water with a conductivity of less than 0.5 μSmm^{-1}. The deionized water was prepared using a water purification system (Milli-Q, Milipore, Breda, The Netherlands).

2.9 *Determination of test parameters*

Cell concentrations were determined photometrically, using a UV/VIS spectrophotometer (Shimadzu UV 210-PC, 's-Hertogenbosch, The Netherlands). Measurements were carried out at 685 nm in a cuvette with a light path of 10 cm (initially) after thoroughly mixing. To establish the relation between absorption and cell density, a calibration curve was made. Several dilutions were prepared from an exponentially growing algal culture. Of these diluted cultures the absorptions were measured at 685 nm using the above mentioned spectrophotometer. The cell density of the same samples (at least two) were determined using a microscope and a counting chamber. From the relation between absorption (A) and counted cell number (N in cells/µl) a calibration curve was calculated on the basis of the equation (dd: 06-feb-98) :

$N = 780.1 \cdot A - 8.433$ $(\chi^2 = 0.00202)$

The calibration curve is checked for accuracy and linearity every 12 months.

The pH of all test substance samples and controls were measured at the beginning (t=0h, in pooled samples for each concentration) and at the end of the test (t=72h, in all samples). During the test the temperature in the cabinet was monitored continuously and the light intensity was measured every 24 hours. At the end of the test 6 random samples (from each concentration one) were checked for purity and for possible microbial contamination by microscopical means at 1000 times magnification.

2.10 *Test methods*

The test concentration range used in this study was derived from the results of preliminary investigations on the toxicity of Phantolid® to algae, which were not performed according to the principles of GLP. In this investigation 100% growth inhibition was found at a concentration of 20% and a significant inhibition was still found at 0.8 % of the stock solution.

In the definitive test the algae were exposed to the following nominal concentration range : 3, 6, 12, 24 and 48 % of the nominal concentration (equal to 0.022, 0.044, 0.089, 0.178 and 0.356 mg/l Phantolid,respectively) . The mineral medium (299 ml for each concentration) was prepared with filter sterilized (0.2 µm) deionized water, and subsequently inoculated (1 ml) with an exponentially growing culture of *Selenastrum capricornutum* to obtain a final density of $1 \cdot 10^4$ cells/ml. This medium was divided into three separate vessels. The test was performed using 3 replicates of each concentration and six replicates of a control

culture, where no test substance was added. The absorption of all samples and controls were measured at 685 nm after approximately 0, 24, 48 and 72 hours of testing. Mineral medium was used as blanc in the spectrophotometer.

2.11 *Validity criteria*

For the test to be valid, the cell density in the control cultures should have increased by a factor of at least 16 within 72 hours (OECD,EEC). The pH in the test medium should not deviate more than 1.5 pH unit (EEC). The lowest concentration tested should have no significant effect on the growth of the algae, and the highest concentration should inhibit growth by at least 50% relative to the control (OECD, EEC).

2.12 *Statistical evaluation*

The $EC_{20,50,80}$ values of the test substance were determined by comparing the relative growth (biomass) and growth rate (μ) of the algae at various concentrations of the test substance to the control cultures. Calculations were based on counted cell numbers (measured photometrically). The absorptions measured for each concentration were converted to cell numbers using the calibration curve as described in SOP A-361 [4.8]. A correction for coloured samples was applied when necessary. Whenever the measured cell density at t=0 was below $1 \cdot 10^4$ cells/ml (due to extreme low extinction values at t=0) the density was corrected to this value for calculation. The initial growth and growth rate were determined by two different methods, as described below:

Calculation of growth

Effects on biomass were calculated by determining the areas under the growth curve (A) of both control and test cultures according to.

$$A_t = \frac{N_1 - N_0}{2} \bullet t_1 + \frac{N_1 + N_2 - 2N_0}{2} \bullet (t_2 - t_1) + \frac{N_2 + N_3 - 2N_0}{2} \bullet (t_3 - t_2)$$

Where ;

A_t area under the growth curve at concentration t
N_0 cell counts at time t_0
N_i cell counts at time t_i
t_i time of the i^e measurement after start of test

The percentage inhibition at concentration t can be calculated as follows:

$$I_{A_t} = \frac{A_c - A_t}{A_c} \bullet 100$$

Where ;
IA_t percentge inhibition at concentration t in comparison with control
A_c area under growth curve control
A_t area under growth curve at concentration t

Calculaiion of growth rates

The average specific growth rates (μ) for the individual cultures were calculated from the following relationship:

$$\mu = \frac{Ln_{N_n} - Ln_{N_0}}{t_n - t_0}$$

Where ;

μ	specific growth rate (h^{-1})
LnN_0	natural logarithm of the cell counts at t=0
LnN_n	natural logarithm of the cell counts at t=n
t_0	time at start test
t_n	time after n hours of testing

The EC_n values for growth (E_bC_{50}) and growth rate (E_rC_{50}) were computed using a non-parametric (Spearman-Karber analysis) method (nominal concentrations) and the Probit analyses (measured concentrations) according to Standard Operating Procedure A-360 [4.9]. The values together with the 95% confidence limits were calculated using the computer program ToxCalc [4.4]. Statistical comparisons of the growth and growth rate in control and test cultures for determination of the NOEC were carried out using the treshold values of the William's test [4.5]. The computer program was validated using a known dataset before use according to Standard Operating Procedure A-360 [4.9].
Calculation procedures are based on both nominal values (calculated from the measured concentration of the stock solution), and geometric means of measured values of the concentration range (whenever the recovery was < 80% after 72 hours of testing). The

geometric means of concentrations which were not measured by chemical analysis, were estimated on the basis of the formula:

estimation of $M_x = N_x * (1 - (0.5 (N_{x-1} - M_{x-1})/N_{x-1} + 0.5(N_{x+1} - M_{x+1})/N_{x+1}))$

Where : x = Concentration step
N = Nominal concentration value
M = Measured concentration value

From these estimated concentrations at the various time intervals the geometric mean was calculated. When measured concentrations were below the detection limit the values were not used for calculation of the geometric means of the non-analytically determined samples. Concentrations which were below the detection limit at T=0 were not used in calculation of EC_n values.

2.13 *Chemical analysis*

The method including validation, calibration and description of the procedures and apparatus used to determine the concentration of the test substance in the test medium is described in a separate study. The protocol for this study (Study plan T98005A) [4.13] was drawn up by Dr. G. Stegeman (Study director) of Akzo Nobel Central Research, Dept. RGL, Arnhem, The Netherlands. This protocol is stored in the study file and was approved and signed by both the Study director (Akzo Nobel Central Research) and the Study monitor (AquaSense Lab).

The concentration of the test substance in the samples was determined by means of gas chromatography with flame ionization detection. Samples (≈ 20 ml) were withdrawn from the control, lowest , middle and highest concentration tested. Samples from the other concentrations were also withdrawn and stored for additional analyses, when needed. The concentration of the test substance in the solution withdrawn directly from the stock solution was also determined. The samp'es were stored in the refrigerator in the dark until analysis. All samples will be taken in single-fold and analyzed in duplicate. Samples were taken at 0, 24, 48 and 72 hours of testing. The initial samples for analysis (0 hours) were withdrawn from the vessels after addition of the testorganisms and stabilization of the testsystem. The samples were stored for a maximum period of 4 weeks.

3. RESULTS

3.1 *Test environment*

The cell density of the algae in the control cultures increased with a factor 120 within 72 hours. The pH measurements in Table I show a maximum increase of 0.5 units. The temperature was measured continuously during the test and varied between 22.2 and 23.1 °C, with an average value of 22.9 °C. The light intensity was measured daily, and varied between 86.13 and 89.14 $\mu mol \cdot m^{-2} \cdot s^{-1}$ during the test period. At the end of the test six samples were checked microscopically and all were pure and not contaminated with bacteria or other algae.

3.2 *Test results*

The results of the algae growth measurements are presented in Table II and III. Almost complete inhibition of both growth and growth rate of *Selenastrum capricornutum* was observed at 0.356 mg/l of the test substance, and no significant inhibition was observed at 0.044 mg/l test substance. There was however a substantial effect observed at the lowest concentration tested (0.022 mg/l). Due to this anomaly, the EC_{50} values based on the nominal values were calculated by the non-parametric Spearman-Karber [4.7] method instead of the parametric Probit method, because the Spearman-Karber method provides fail safe EC_{50} values with 95% confidence limits and is resistant to anomalies as observed. The Trimmed Spearman-Karber method provides only EC_{50} values (with 95% confidence limits), and therefore the EC_{20} and EC_{80} could not be determined. Because the testconcentrations dropped below 80% of the initial concentration, the calculation of the effects were also performed using the geometric means of the measured concentrations . For the calculation of the EC_n values using the measured concentrations the Probit method was used (lowest concentrations (0.022 and 0.044 mg/l) were not included in calculation because the measured values were below the detection limit of the chemical analysis). The E_bC_{50} derived from the results using the nominal values is 0.081 mg/l (0.069 - 0.095 95% confidence limits). The nominal E_rC_{50} calculated from the results is 0.200 mg/l (0.179 - 0.221 95% confidence limits). The nominal NOEC determined using the treshold values of the Williams test is < 0.022 mg/l for biomass and 0.044 mg/l based on growth rate. The anomaly observed at the lowest concentration was most apparent for the inhibition of growth and less evident for the inhibition of the growth rate. Therefore the NOEC based on the growth rate is probably more reliable.

The E_bC_{50} value based on the measured concentrations is 0.038 mg/l (0.032 - 0.042 95% confidence limits) and the E_rC_{50} is 0.063 mg/l (0.059 - 0.069 95% confidence limits). The

NOEC based on the measured concentrations was < 0.043 mg/l for biomass and 0.039 mg/l based on the growth rate. The calculation of both the EC_n values and the NOEC's using the measured concentrations are based on a very limited data (only 3 values were used for calculation) and all the data used were all near the detection limit of the analysis, and therefore the results are very questionable.

3.3 *Validity of test*

The test was found valid by the increase of cell density of a factor of 120, a maximum pH deviation of 0.5 units and a inhibition of more than 50% at the highest concentration tested and no significant inhibition at the lowest concentration tested. The results of the last quality control test with potassiumdichromate (06-Feb-1998: E_bC_{50}, 0.51 mg/l - E_rC_{50}, 0.98 mg/l) are in agreement with the criteria mentioned in the EEC Directive 67/548 C3, indicating that the sensitivity of the algae has not changed significantly.

3.4 *Chemical analysis*

The results of the chemical analysis are described in the GLP report of Akzo Nobel Central Research RGL F98046 [4.14], which is added in Annex II. The analyses of the tested concentration range of Phantolid® showed a substantial decrease in concentration during the test period. The initial recoveries (t=0) were all below the nominal values, and decreased rapidly further during the test period. More than 80% of the compound disappeared at all concentrations after 72 hours of testing. Extraction procedures (see Annex II) for chemical analysis were performed using samples *including* algae, therefore the decrease could not be attributed to adsorption onto the algae. Moreover, adsorption onto the vessel wall is not likely to be the main cause of the decrease because the results of a photodegradation study on Phantolid® [4.15] showed a maximum adsorption of 4.5% of the test substance after 72 hours of incubation.

3.5 *Deviations from Study Protocol*

The following deviations were observed during the study :

- The amount of sample taken for chemical analyses was approximately 20 ml at each sampling time, with equal amounts from each triplicate vessel. This was not mentioned in the Study Protocol.
- The statistical method used for calculation of the effect concentrations using the nominal values was the non-parametric Spearman-Karber analysis, instead of the parametric Probit method.

- $EC_{20,80}$ values were not determined for the nominal values. The Spearman-Karber yields only EC_{50} values.
- The algal cell density was measured in all concentrations at t=0 instead of only 24, 48 and 72 hours. This was necessary for correction of turbidity of samples. The test dilutions were slightly coloured at the highest concentrations due to the presence of the test substance.

3.6 Conclusions

The toxicity of Phantolid® to the green algae *Selenastrum capricornutum* was determined over a period of 72 hours. The E_bC_{50} and E_rC_{50} of Phantolid® based on the nominal values are 0.081 (0.069 - 0.095 with 95% confidence limits) and 0.200 mg/l (0.179 - 0.221 with 95% confidence limits). The nominal NOEC derived from the results based on the growth rate is 0.044 mg/l Phantolid®. The EC_n values and NOEC's determined using the measured concentrations are questionable, based on the limited data used. The chemical analyses indicated a rapid decrease of the concentration during the test period, which may for a small part be attributed to adsorption onto the test vessel wall.

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Table I

Measurements of the pH values in the test flasks at the beginning and end of the test.

Nominal concentration (mg/l)	Time (hours)	
	0	72
control	8.2	7.9
	8.2	7.7
	8.2	7.7
	8.2	7.9
	8.2	7.9
	8.2	7.9
0.022	8.1	7.7
	8.1	7.8
	8.1	7.7
0.044	8.1	7.8
	8.1	7.8
	8.1	7.8
0.089	8.1	7.8
	8.1	7.9
	8.1	7.8
0.178	8.1	7.8
	8.1	7.8
	8.1	7.8
0.356	8.0	7.8
	8.0	7.9
	8.0	7.9

Table II

Cell counts of the various concentrations and control at each measurement during the 72 hours of testing.

Nominal (mg/l) concentration	Time (hours) Cell counts (cells/ml)[1, 2]			
	0	24	48	72
control	9128	40403	264460	1415140
	9525	37766	232420	1215420
	10142	44027	260610	1222920
	10532	36802	205630	1156940
	9908	33778	202260	1092480
	10766	32446	179810	1096140
mean	**10000**	**37537**	**224198**	**1199840**
std.dev.	614	4266	34090	119373
0.022	10454	26981	157740	817160
	10844	21839	143580	729000
	10766	26112	166050	800520
mean	**10688**	**24977**	**155790**	**782227**
std.dev.	206	2752	11361	46840
0.044	12248	30267	192810	1012960
	10922	37683	252710	1254840
	10844	33576	216100	1153820
mean	**11338**	**33842**	**220540**	**1140540**
std.dev.	789	3715	30196	121486
0.089	12561	18958	81346	529560
	11859	18827	83477	485920
	13341	24946	110660	590320
mean	**12587**	**20910**	**91828**	**535267**
std.dev.	741	3496	16344	52433
0.178	16851	22160	58657	274260
	16227	22303	69918	259360
	15977	19303	56205	199332
mean	**16352**	**21255**	**61593**	**244317**
std.dev.	450	1692	7313	39664
0.356	20830	19303	23743	40202
	19870	16446	15268	19846
	20441	19077	24446	29822
mean	**20380**	**18275**	**21152**	**29957**
std.dev.	483	1588	5108	10179

[1] = Measured cell counts which were below the initial value (inoculum) were corrected to the initial value in the calculation procedure of the inhibition of the growth and growth rate.

[2] = Absorption data of all test concentrations were corrected for colour and/or turbidity caused by the test substance before calculation of EC_n values.

Table III

Biomass increase (A) after 72 hours of testing and average specific growth rates (μ) of individual measurements and inhibition of growth (A) and growth rates (μ).

			Biomass (A)			
Nominal Concentration (mg/l)	**control**	**0.022**	**0.044**	**0.089**	**0.178**	**0.356**
replicas						
A	23750712	13611984	16774488	8008356	4219668	302376
B	20498004	12067416	21372192	7574796	4352004	0[1]
C	21377808	13572168	19187424	9537924	3245556	208692
D	19069728					
E	18180192					
F	17601864					
average biomass (A)	20079718	13083856	19111368	8373692	3939076	170356
inhibition (%)	-	34.8	4.8	58.3	80.4	99.2
			Growth rate (μ)			
A	0.070	0.061	0.063	0.055	0.045	0.015
B	0.067	0.059	0.068	0.055	0.045	0[2]
C	0.067	0.061	0.067	0.056	0.042	0.009
D	0.065					
E	0.065					
F	0.064					
average growth rate (μ)	0.066	0.061	0.066	0.055	0.044	0.008
inhibition (%)	-	9	1	17	34	88

[1] = Biomass did not increase in time, therefore the (increase) in biomass after 72 hours of testing compared to the control value is zero.

[2] = Growth rate did not increase in time, therefore the (increase) in growth rate after 72 hours of testing compared to the control value is zero

Table IV

Results of the chemical analysis. Concentrations are presented as mean values of duplicate analysis.

Nominal concentration (mg/l)	measured concentration (mg/l) with 95% confidence limits				Geometric mean (mg/l)	% of initial concentration			
time (h)	0	24	48	72	-	0	24	48	72
0	< 0.039[1]	< 0.039[1]	< 0.039[1]	< 0.039[1]	-	-	-	-	-
0.022	< 0.039[1]	< 0.039[1]	< 0.039[1]	< 0.039[1]	-	-	-	-	-
0.044	n.d.	n.d.	n.d.	n.d.	-				
0.089	0.059 ± 0.032	< 0.039[1]	< 0.039[1]	< 0.039[1]	0.043	100	-	-	-
0.178	n.d.	n.d.	n.d.	n.d.	0.051				
0.356	0.232 ± 0.032	0.151 ± 0.032	0.075 ± 0.032	0.051 ± 0.032	0.108	100	65	32	22
Stock	0.741 ± 0.031	n.d.	n.d.	n.d.					

[1] = Values were < detection limit (= 0.039 mg/l)

Table V

Summary of the test results

Parameter	Dimension	Value	95% confidence limits
N_0 ,inoculum	cells/ml	$1 \cdot 10^4$	-
N_{72} , cell density at the end of the test	cells/ml	$1.2 \cdot 10^6$	-
growth	no dimension	120	-
μ, growth rate	h^{-1}	0.066	-
Nominal concentrations			
Effect concentrations (EC_n)			
E_bC_{20}	mg/l	n.d.	-
E_bC_{50}	mg/l	0.081	0.069 - 0.095
E_bC_{80}	mg/l	n.d.	-
NOEC	mg/l	< 0.022	-
E_rC_{20}	mg/l	n.d.	-
E_rC_{50}	mg/l	0.200	0.179 - 0.221
E_rC_{80}	mg/l	n.d.	-
NOEC	mg/l	0.044	-
Measured concentrations			
Effect concentrations (EC_n)			
E_bC_{20}	mg/l	0.027	0.018 - 0.033
E_bC_{50}	mg/l	0.038	0.032 - 0.042
E_bC_{80}	mg/l	0.053	0.049 - 0.060
NOEC	mg/l	< 0.043	-
E_rC_{20}	mg/l	0.044	0.039 - 0.047
E_rC_{50}	mg/l	0.063	0.059 - 0.069
E_rC_{80}	mg/l	0.092	0.083 - 0.106
NOEC	mg/l	0.039	-

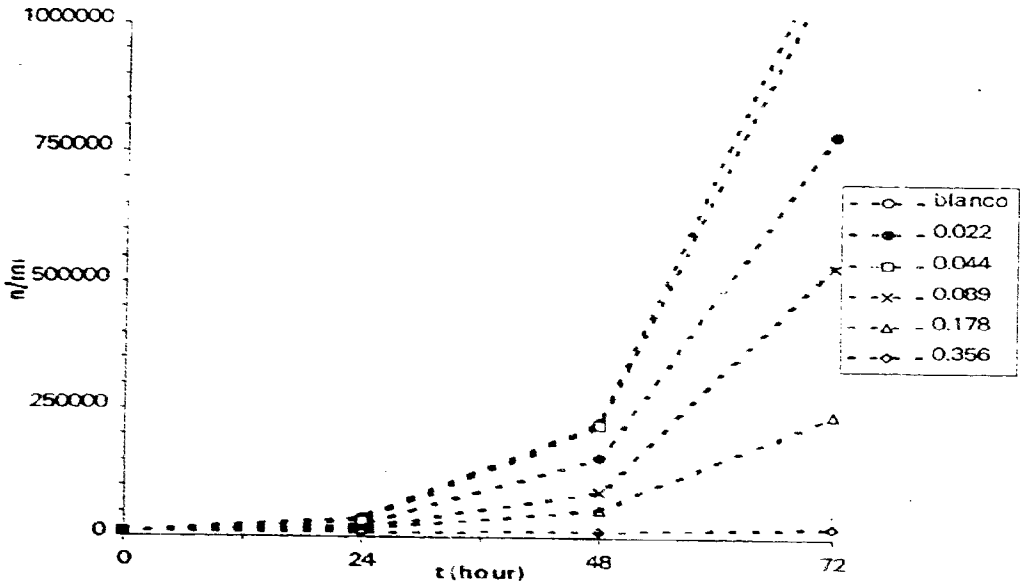


Figure 1

Growth curves of *Selenastrum capricornutum* over 72 hours of incubation at various test concentrations (nominal concentrations in mg/l) of Phantolid®.

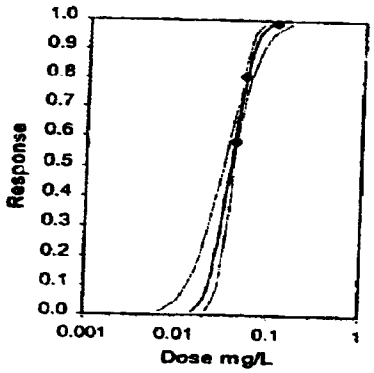


Figure 2
Concentration/effect relationship of Phantolid® calculated from the percentage of inhibition of the growth (biomass) at various test concentrations (measured).

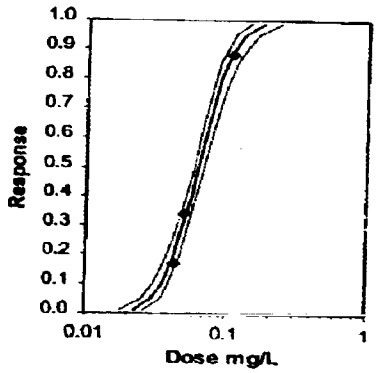
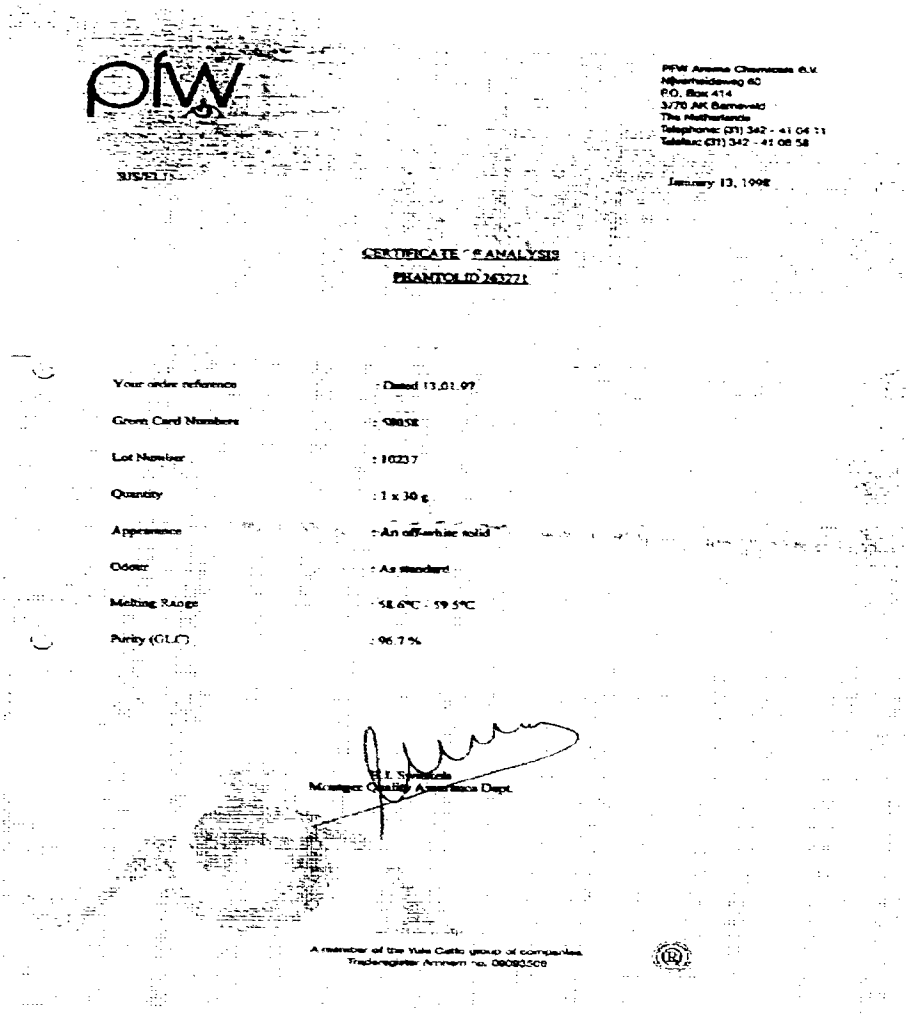


Figure 3
Concentration/effect relationship of Phantolid® calculated from the percentage of inhibition of the specific growth rate (μ) at various test concentrations (measured).

Annex I

PFW



PFW Aroma Chemicals B.V.
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The Netherlands
Telephone: (31) 342 - 41 04 11
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SUS/ELTS

January 13, 1998

CERTIFICATE OF ANALYSIS

PHANTOLID 263771

Your order reference	: Dated 13.01.97
Green Card Numbers	: 58058
Lot Number	: 10237
Quantity	: 1 x 30 g
Appearance	: An off-white solid
Odour	: As standard
Melting Range	: 58.6°C - 59.5°C
Purity (GLC)	: 96.7 %

H.I. Swinkels
Manager Quality Assurance Dept.

A member of the Yule Catto group of companies
Traderegister Arnhem no. 09093508

Annex II

04

AKZO NOBEL

Central Research
Location Arnhem

Final Research Report

RGL F98046 T 98005 A May 29, 1998

G. Stegeman, H. Henderiks

ANALYSIS OF PHANTOLID IN TEST MEDIA FROM TOXICITY TESTS

ICS-103 **CONFIDENTIAL**

Project identification

Client	Aquasense, Amsterdam	Client No	Project No
Subclient		Subclient No	Task No 9133

Drafted by department General Analytical and Environmental Chemistry

Keywords GC, ecotox, fish toxicity, Daphnia toxicity

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AKZO NOBEL

ABSTRACT

To determine toxicity of Phantolid a series of tox tests with fish and daphnids and an algal growth inhibition test were performed by Aquasense Amsterdam. The concentration of Phantolid in the used test media was determined by the General Analytical and Environmental Chemistry Department of Akzo Nobel Central Research. Samples to be analysed were test media with high, medium, low and zero concentration of Phantolid. Samples were taken at the beginning of the test (t=0 hour), at the end of the test and between times after every 24 hours. In this report procedures and results of the chemical analyses are described.

Analyses were carried out in compliance with the OECD Principles of Good Laboratory Practice (GLP). Test media samples were extracted with dibutylether using a so-called in-vial extraction procedure. The ether layer was subsequently analysed by means of gas chromatography (GC) with flame ionisation detection.

The concentration of Phantolid in the test media from the Daphnia magna tests at t=0 hours were between 0 and 1.14 mg/l. In the course of the test the concentrations decreased gradually. At the end of the test, after 48 hours, concentrations were roughly 50% of the initial concentration.

The initial concentration of Phantolid in the algal media ranged between 0 and 0.24 mg/l. The low concentration level was already below the detection limit of 0.04 mg/l. During the 72 hour test the Phantolid concentrations were found to decrease steadily. At the end of the test concentration levels in the test media were all below the detection limit except for the highest dose concentration, which had declined to 0.05 mg/l.

The concentration of Phantolid in test media from the 96 hour fish toxicity study were between 0 and 0.95 at the beginning of the test. Already after 48 hours the Phantolid concentration in all test media had decreased below the detection limit of 0.04 mg/l. This decrease in Phantolid concentration is more pronounced than in the algal and Daphnia test.

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AKZO NOBEL

ANALYSIS OF PHANTOLID IN TEST MEDIA FROM TOXICITY TESTS

Sponsor	Aquasense P.O. Box 95125 HC Amsterdam The Netherlands
Study monitor	Drs. Ing. A.G.M. Kroon

STUDY ORGANISATION

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Quality Assurance Unit	E.H.V. Derks
Management, Head of Department RGL	Dr.Ir. C.J. Groenenboom
Initiation date of the study	1998-05-15
Completion date of the study	1998-05-25

ARCHIVING AND STORAGE

The project file including the final report, amendments to the final report, the study plan, amendments to the study plan, records of quality assurance inspections, all letters, memos and notes and raw data pertaining to the study will be retained in the archives of Akzo Nobel Central Research Arnhem for a period of ten years. Other records including master schedule sheet, laboratory notebooks, logbooks, records of the maintenance and calibration of equipment, summary of training, curricula vitae and job descriptions of the personnel involved in the study, records related to location and storage of the test substance will also be kept in the Akzo Nobel Central Research Arnhem archives for a period of ten years. Test material will be stored deepfrozen under the sample code T 98005 for ten years or only as long as the quality of the test substance permits evaluation.

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GLP COMPLIANCE STATEMENT

The study reported here was carried out according to the study plan in compliance with the OECD Principles of Good Laboratory Practice (5.1). The report contains an accurate description of the results.

Study director
Dr. G. Stegeman

date 1998.05.29

Management, Head of Department RGL
Dr.Ir. C.J. Groenenboom

date 1998-06-02

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QUALITY ASSURANCE STATEMENT

This report was audited by the Quality Assurance Unit of Akzo Nobel Central Research Arnhem. It is considered to be an accurate presentation of the methods and procedures applied in the course of the study and an accurate reproduction of the data recorded.
Listed below are the dates of inspection of this study by the Quality Assurance Unit and the dates on which its findings were reported to Study Director and Management.

Dates of inspection	Dates of reporting
1998-05-20	1998-05-20
1998-05-29	1998-05-29

Quality Assurance Unit
E.H.V. Derks

date 1998.05.29

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AKZO NOBEL

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AKZO NOBEL

1. INTRODUCTION

To determine toxicity of Phantolid, a substance produced by PFW Aroma Chemicals, a series of tox tests with fish and daphnids and an algal growth inhibition test were performed by Aquasense Amsterdam (project No 1202B). The concentration of Phantolid in the used test media was determined by the General Analytical and Environmental Chemistry Department of Akzo Nobel Central Research. In this report the procedures and results of the chemical analyses are described.

The samples to be analysed were taken from test media at the beginning, at the end and during the toxicity tests at regular time intervals. The samples were extracted "in-vial" with dibutylether. The concentration of Phantolid in the dibutylether layer was determined by means of gas chromatography (GC) with flame ionisation detection. The analyses were carried out in compliance with the OECD Principles of Good Laboratory Practice (5.1) and according to Standard Operation Procedure N 1 (5.2).

2. MATERIALS

2.1 Test substance

The test substance Phantolid (sample code: T98005) was received from Aquasense on 1998.05.08. The following test substance data were submitted by the sponsor, who accepted full responsibility for the validity of these data. Information concerning the test material:

-	name of test substance	Phantolid
-	chemical name	5-acetyl-1,1,2,3,3,6-hexamethylindan
-	Cas reg.No.	15323-35-0
-	batch/lot No.	10237
-	Aquasense sample No	302900
-	purity	96.7 %
-	appearance	an off-white solid (powder)
-	storage until required	in a cool place in the dark
-	stability	stable under storage conditions

2.2 Samples

The samples to be analysed were received from Aquasense on 1998.05.15. On receipt all samples were still refrigerated, which was checked by taking some samples in the palm of the hand. After checking all the samples, they were immediately stored in a refrigerator until analysis. Sample codes and other relevant data supplied by Aquasense were assumed to be correct and were used without further check. Samples were analysed within one week after receipt.

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AKZO NOBEL

-	sample description	aqueous solutions of test substance
-	storage until required	in a refrigerator

2.3 Chemicals

Dibutylether, $[CH_3(CH_2)_3]_2$ O	99% GC-grade

2.4 Deionized water

Deionized water was produced from tap water in a water purification system (Spectrum Elgastat, Breukelen, The Netherlands). It had a conductivity of less than 5 µ $S.cm^{-1}$, a TOC content of less than 2 mg/l and it contained no more than 0.01 mg Cu per litre (Standard Operation Procedure K 10 (5.3)).

2.5 Apparatus

Description of the apparatus used and some relevant settings:

Gas chromatography system	Carlo Erba GC 8160
Autosampler	Carlo Erba AS 800
	sampling depth: -22 mm
Injection	cold on-column injection
	cool time: 12 seconds
	injection volume: 1 µl
	injection speed: maximum
	syringe : Hamilton microliter, 10 µl AS800
	syringe rinse procedure: standard AS800 procedure (bubble elimination = 5)
GC-column	J&W DB-1 with retention gap
	length: 30 meter
	internal diameter: 0.32 mm
	film thickness: 0.5 µm
Detection	Carlo Erba FID-80 flame ionization detector
	temperature: 300°C
	flame gases: 50 kPa H_2 and 100 kPa air
Carrier gas	Helium 5.0; 100 kPa head pressure
Temperature program	Initial temperature: 150°C (2 minutes)
	temperature rise: 4°C/min
	final temperature 225°C (5 minutes)
Data collection/integration	Fisons Multichrom rev.2 chromatography software
Recorder	Kipp & Zn BD-8, 1 mV

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2.6 Materials

Chromacol 2-ml glass GC autosampler vials with Teflon lined crimp caps.

3. METHODS

3.1 Preparations for GC analysis

The performance of the installed GC column was checked by a blank run (i.e. a chromatographic run without sample injection) and by analysis of a Phantolid standard in dibutylether. After checking column contamination, column bleed and peak shapes, the column was approved for chemical analyses.

3.2 Calibration of chromatographic system

A stock solution of Phantolid was prepared in dibutylether. From this stock solution a series of 6 calibration standards was prepared in dibutylether covering the expected concentration range of the samples.
The GC standards were prepared by adding 0.8 ml of deionized water and 0.8 ml of the standard solution to a GC sample vial. For each concentration level a duplicate GC standard was prepared. The capped vials were shaken vigorously for 2 minutes and were then left standing for at least 5 minutes to settle. The series of duplicate calibration standards were analyzed in single fold starting with the lowest concentration.

3.3 Control samples

To check on errors in the preparation of calibration standards, a second independent stock solution of Phantolid was prepared in dibutylether. From this stock solution a control sample of known Phantolid concentration was prepared within the concentration range of the calibration line. The control sample was prepared in duplicate according to the procedure described in 3.2. The duplicate control samples were analyzed in single fold.

3.4 Analysis of Phantolid samples

The refrigerated samples were brought to room temperature. The samples were shaken vigorously to homogenize and then sonicated for 10 minutes. If aggregates or particles were visible (e.g. algae of fish scales), the samples were centrifuged at 8000 rpm for 10 minutes. When necessary, samples were diluted in deionized water to obtain a nominal concentration within the calibration range.
From each test sample a duplicate sample for GC analysis was prepared. GC samples were prepared by adding 0.8 ml of the aqueous sample and 0.8 ml of dibutylether to a GC sample vial. The capped vials were shaken vigorously for 2 minutes to extract the aqueous layer and were then left standing for at least 5 minutes to settle.

Each GC sample vial was assayed in single fold (which means that each received test media sample was analyzed in duplicate since GC samples were prepared in duplicate). After a series of ten duplicate samples a calibration standard (except for the highest or lowest standard) was analyzed in single fold. It was checked if results satisfied quality criteria (see 3.6.3, 3.6.4).

3.5 Data processing

The peak area of the Phantolid peak (only one single Phantolid peak was detected) was determined using the Multichrom integration software. Key integration parameters were kept the same for calibration runs and sample analysis. Calibration line(s) were constructed by plotting peak area (in counts) against the concentration of test substance in the calibration standards using Statcal 6.50 software package [5.4]. The optimum polynomial fit of the calibration curve, coefficients of the polynomial (A, B, etc.) and standard deviation of residuals (S_R) were calculated.

For each sample the concentration of Phantolid in the dibutylether layer and the 95% confidence interval were calculated from the measured peak area and the calibration line using Statcal 6.50. To obtain the true Phantolid concentration a correction was made for impurity of the test substance. The concentration of Phantolid in the aqueous test samples were calculated assuming that the distribution coefficient of Phantolid, describing equilibrium distribution of Phantolid between the aqueous and ether phase, is the same for the calibration standards and the samples.

3.6 Data evaluation and quality control

3.6.1 Calibration standards

It was checked if the calibration standards were within the 99% confidence limits of the corresponding calibration line. If one data point was outside the 99% confidence interval this data point was rejected and the regression line was recalculated. If the calibration series contained more than one data point outside the 99% confidence interval the whole calibration series was rejected and a new calibration line was constructed with freshly prepared standards.

3.6.2 Control samples

If the known concentration of the control samples was outside the 99% confidence interval of the measured concentration another control sample, prepared from a fresh stock solution, was analyzed. If the measured concentration of this control sample was still deviating significantly (99% confidence) from the true concentration, the whole calibration line was rejected, a series of freshly prepared standards was analyzed and a new calibration line was constructed.

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3.6.3 Samples

Using Statcal 6.50 the width of the 99% confidence interval of the calibration line (= 2 * $t_{99\%}$*S_R, where the t-value is from the statistical two-tailed t-distribution) was calculated. If the difference between measured peak areas (in area counts) of duplicate analyses was larger than the width of the 99% confidence interval of the calibration line, the results for this sample were rejected and the sample was reanalyzed.

3.6.4 Control standards

If the measured concentration of the control standards, injected after each series of ten samples, deviated significantly from the true concentration (i.e. if the true concentration was not within the 99% confidence interval of the measured value), the control standard was reanalyzed in duplicate. If the true concentration remained outside the 99% confidence interval, the results for the previous 10 samples were rejected, the system was recalibrated using the original calibration standards and the 10 samples were reanalyzed.

4. RESULTS AND DISCUSSION

4.1 General

GC analysis of pure extraction solvent (dibutylether) and a Phantolid standard solution showed that Phantolid appears as a single peak which is free from interferences from the solvent and solvent impurities.
Analysed samples were test media samples containing high, medium, low and zero Phantolid dose concentrations. The samples were taken at the beginning of the test (t=0 hr), at the end of the test and between times after every 24 hours.
After shaking and sonication, none of the samples contained visible particles or aggregates so none of the samples was centrifuged.
Results of the analyses of the test media are summarized in table 6.1-6.3. The first three columns of each table ("Aquasense sample code", "Sampling date" and "Nominal sampling time") contain data provided by Aquasense. These data were taken from the sample dispatch form which was sent along with the samples. Reported Phantolid concentrations are corrected for impurity of the test substance and thus represent the concentration of pure Phantolid.

4.2 Samples from toxicity test with Daphnia magna.

The concentration of Phantolid in the test media from the Daphnia magna tests at t=0 hours were between 0 and 1.14 mg/l. In the course of the test the concentrations decreased gradually. At the end of the test, after 48 hours, concentrations were roughly 50% of the initial concentration. The current chemical analyses do not give a clue for the cause of the Phantolid losses.

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4.3 Samples from algal growth inhibition test

The initial concentration of Phantolid in the algal media was between 0 and 0.24 mg/l. The low dose concentration was already below the detection limit of 0.04 mg/l. During the 72 hour test the Phantolid concentrations were found to decrease steadily. At the end of the test concentration levels in the test media were all below the detection limit except for the highest dose concentration, which had declined to 0.05 mg/l.

4.4 Samples from toxicity tests with fish

The concentration of Phantolid in test media from the 96 hour fish toxicity study were between 0 and 0.95 at the beginning of the test. Already after 48 hours the Phantolid concentration in all test media had decreased below the detection limit of 0.04 mg/l. The observed decrease in Phantolid concentration is more pronounced than in the algal and Daphnia test.

5. REFERENCES

5.1 OECD, 1981. Principles of Good Laboratory Practice, C(81)30(final)

5.2 Standard Operation Procedure (SOP) N 1: Procedures and quality assurance of chemical analyses, procedure.

5.3 Standard Operation Procedure (SOP) K 10: Deionisator, method.

5.4 Statistics software package: Statcal 6.50, J. Kragten, University of Amsterdam, Amsterdam, 1995

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6. TABLES

Table 6.1 Analysis of Phantolid in test media from Daphnia magna toxicity test Aquasense project 1202B

Aquasense sample code	Sampling date	Nominal sampling time	Phantolid concentration and 95% confidence interval (mg/l)
DM stock, wit	1998-05-11	t= 0 hr	1.127 ± 0.031
DM [0], wit	1998-05-11	t= 0 hr	< 0.039 a)
DM [1], wit	1998-05-11	t= 0 hr	0.267 ± 0.032
DM [3], wit	1998-05-11	t= 0 hr	0.640 ± 0.031
DM [5], wit	1998-05-11	t= 0 hr	1.143 ± 0.031
DM [0], groen	1998-05-12	t= 24 hr	< 0.039 a)
DM [1], groen	1998-05-12	t= 24 hr	0.144 + 0.032
DM [3], groen	1998-05-12	t= 24 hr	0.362 ± 0.032
DM [5], groen	1998-05-12	t= 24 hr	0.840 ± 0.031
DM [0], rood	1998-05-13	t= 48 hr	< 0.039 a)
DM [1], rood	1998-05-13	t= 48 hr	0.123 ± 0.032
DM [3], rood	1998-05-13	t= 48 hr	0.337 ± 0.032
DM [5], rood	1998-05-13	t= 48 hr	0.659 ± 0.031

a) concentration below limit of detection

Table 6.2 Analysis of Phantolid in test media from the algal growth inhibition test Aquasense project 1202B

Aquasense sample code	Sampling date	Nominal sampling time	Phantolid concentration and 95% confidence interval (mg/l)
A stock, wit	1998-05-12	t= 0 hr	0.741 ± 0.031
A [0], wit	1998-05-12	t= 0 hr	< 0.039 a)
A [1], wit	1998-05-12	t= 0 hr	< 0.039 a)
A [3], wit	1998-05-12	t= 0 hr	0.059 ± 0.032
A [5], wit	1998-05-12	t= 0 hr	0.232 ± 0.032
A [0], groen	1998-05-13	t= 24 hr	< 0.039 a)
A [1], groen	1998-05-13	t= 24 hr	< 0.039 a)
A [3], groen	1998-05-13	t= 24 hr	< 0.039 a)
A [5], groen	1998-05-13	t= 24 hr	0.151 + 0.032
A [0], rood	1998-05-14	t= 48 hr	< 0.039 a)
A [1], rood	1998-05-14	t= 48 hr	< 0.039 a)
A [3], rood	1998-05-14	t= 48 hr	< 0.039 a)
A [5], rood	1998-05-14	t= 48 hr	0.075 ± 0.032
A [0], blauw	1998-05-15	t= 72 hr	< 0.039 a)
A [1], blauw	1998-05-15	t= 72 hr	< 0.039 a)
A [3], blauw	1998-05-15	t= 72 hr	< 0.039 a)
A [5], blauw	1998-05-15	t= 72 hr	0.051 ± 0.032

a) concentration below limit of detection

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Table 6.3 Analysis of Phantolid in test media from fish toxicity test Aquasense project 1202B

Aquasense sample code	Sampling date	Nominal sampling time	Phantolid concentration and 95% confidence interval (mg/l)
V stock, wit [b]	1998-05-11	t= 0 hr	1.127 ± 0.031
V [0], wit	1998-05-11	t= 0 hr	< 0.039 [a]
V [1], wit	1998-05-11	t= 0 hr	0.362 ± 0.032
V [3], wit	1998-05-11	t= 0 hr	0.650 ± 0.031
V [5], wit	1998-05-11	t= 0 hr	0.950 ± 0.031
V [0], groen	1998-05-12	t= 24 hr	< 0.039 [a]
V [1], groen	1998-05-12	t= 24 hr	< 0.039 [a]
V [3], groen	1998-05-12	t= 24 hr	< 0.039 [a]
V [5], groen	1998-05-12	t= 24 hr	0.063 ± 0.032
V [0], rood	1998-05-13	t= 48 hr	< 0.039 [a]
V [1], rood	1998-05-13	t= 48 hr	< 0.039 [a]
V [3], rood	1998-05-13	t= 48 hr	< 0.039 [a]
V [5], rood	1998-05-13	t= 48 hr	< 0.039 [a]
V [0], blauw	1998-05-14	t= 72 hr	< 0.039 [a]
V [1], blauw	1998-05-14	t= 72 hr	< 0.039 [a]
V [3], blauw	1998-05-14	t= 72 hr	< 0.039 [a]
V [5], blauw	1998-05-14	t= 72 hr	< 0.039 [a]
V [0], geel	1998-05-15	t= 96 hr	< 0.039 [a]
V [1], geel	1998-05-15	t= 96 hr	< 0.039 [a]
V [3], geel	1998-05-15	t= 96 hr	< 0.039 [a]
V [5], geel	1998-05-15	t= 96 hr	< 0.039 [a]

[a] concentration below limit of detection
[b] V stock.,wit = DM stock, wit (see table 6.1)

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