Committee for Risk Assessment
RAC

Annex 1

Background document

to the Opinion proposing harmonised classification and labelling at EU level of

(*R*)-p-mentha-1,8-diene; d-limonene

EC Number: 227-813-5
CAS Number: 5989-27-5

CLH-O-0000001412-86-275/F

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to public consultation. RAC has not changed the text of this CLH report but inserted text which is specifically marked as 'RAC evaluation'. Only the RAC text reflects the view of RAC.

Adopted
15 March 2019

CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2

Substance Name: (R)-p-mentha-1,8-diene; d-limonene

EC Number: 227-813-5
CAS Number: 5989-27-5
Index Number: 601-RST-VW-Y

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Version number: 2 **Date: March 2018**

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON (*R*)-P-MENTHA-1,8-DIENE; D-LIMONENE

CONTENTS

Part A.

In addition to the contents of this CLH report, a confidential annex has been made containing the full references of studies using vertebrate animals or human studies that are not publicly accessible via the open literature.

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON (*R*)-P-MENTHA-1,8-DIENE; D-LIMONENE

Part A.

1 PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Substance

Table 1: Substance identity

Substance name:	***(R)-p-mentha-1,8-diene***
EC number:	***227-813-5***
CAS number:	***5989-27-5***
Annex VI Index number:	***601-RST-VW-Y***
Degree of purity:	***confidential***
Impurities:	***None***

1.2 Harmonised classification and labelling proposal

Table 2: The current Annex VI entry and the proposed harmonised classification

	CLP Regulation
Current entry in Annex VI, CLP Regulation	Flam. Liq. 3 (H226) Skin Irrit. 2 (H315) Skin Sens. 1 (H317) Aquatic Acute 1 (H400) Aquatic Chronic 1 (H410) Note c
Current proposal for consideration by RAC	Asp. Tox 1 (H304) Skin Sens. 1B (H317) Aquatic Acute 1 (H400), M=1 Aquatic Chronic 3 (H412)
Resulting harmonised classification (future entry in Annex VI, CLP Regulation)	Asp. Tox 1 (H304) Flam. Liq. 3 (H226) Skin Irrit. 2 (H315) Skin Sens. 1B (H317) Aquatic Acute 1 (H400), M=1 Aquatic Chronic 3 (H412)

1.3 Proposed harmonised classification and labelling based on CLP Regulation

Table 3: Proposed classification according to the CLP Regulation

CLP Annex I ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification [1)]	Reason for no classification [2)]
2.1.	Explosives				Conclusive but not sufficient for classification
2.2.	Flammable gases				N/A
2.3.	Flammable aerosols				Data lacking
2.4.	Oxidising gases				N/A
2.5.	Gases under pressure				N/A
2.6.	Flammable liquids			Flam. Liq. 3 H226	Not considered in this report
2.7.	Flammable solids				N/A
2.8.	Self-reactive substances and mixtures				Not considered in this report
2.9.	Pyrophoric liquids				Not considered in this report
2.10.	Pyrophoric solids				Data lacking
2.11.	Self-heating substances and mixtures				Not considered in this report
2.12.	Substances and mixtures which in contact with water emit flammable gases				Not considered in this report
2.13.	Oxidising liquids				Conclusive but not sufficient for classification
2.14.	Oxidising solids				Data lacking
2.15.	Organic peroxides				Data lacking
2.16.	Substance and mixtures corrosive to metals				Not considered in this report
3.1.	Acute toxicity - oral				Not considered in this report
	Acute toxicity - dermal				Not considered in this report
	Acute toxicity - inhalation				Data lacking
3.2.	Skin corrosion / irritation			Skin Irrit. 2 H315	Not considered in this report
3.3.	Serious eye damage / eye irritation				Not considered in this report
3.4.	Respiratory sensitisation				Data lacking
3.4.	Skin sensitisation	Skin Sens. 1 H317		Skin Sens. 1B H317	

3.5.	Germ cell mutagenicity				Not considered in this report
3.6.	Carcinogenicity				Not considered in this report
3.7.	Reproductive toxicity				Not considered in this report
3.8.	Specific target organ toxicity –single exposure				Not considered in this report
3.9.	Specific target organ toxicity – repeated exposure				Not considered in this report
3.10.	Aspiration hazard	Asp. Tox. 1 H304			
4.1.	Hazardous to the aquatic environment	Acute 1 H400 Chronic 3 H412	M = 1	Acute 1 H400 Chronic 1 H410	
5.1.	Hazardous to the ozone layer				Not considered in this report

[1)] Including specific concentration limits (SCLs) and M-factors
[2)] Data lacking, inconclusive, conclusive but not sufficient for classification, not applicable (N/A) or not considered in this report.

Labelling:

GHS Pictograms:

Signal word: Danger
Hazard statements:
H226: Flammable liquid and vapour.
H315: Causes skin irritation.
H317: Can cause an allergic skin reaction.
H304: May be fatal if swallowed and enters airways.
H410: Very toxic to aquatic life with long lasting effects.

Precautionary statements: No precautionary statements are proposed since precautionary statements are not included in Annex VI of Regulation EC no. 1272/2008.

Notes assigned to an entry:

2 BACKGROUND TO THE CLH PROPOSAL

2.1 History of the previous classification and labelling

d-Limonene has a REACH registration with a tonnage band of 1000 - 10,000 tonnes per annum with Annex VI classification (entry 601-029-00-7) as part of a group entry of Flam. Liq. 3 (H226), Skin Irrit. 2 (H315), Skin Sens. 1 (H317), Aquatic Acute 1 (H400) and Aquatic Chronic 1 (H410). The classification was included in the 24th ATP based on the advice of the Commission Working Group on the Classification and Labelling of Dangerous Substances (April 1997).

2.2 Short summary of the scientific justification for the CLH proposal

d-Limonene is one of the ingredients of the active substance terpenoid blend QRD460. The terpenoid blend, consisting of p-cymene, d-limonene and alpha-terpinene, is accepted as an active substance for plant protection products. However, as it is a mixture and not a substance harmonised classification of terpenoid blend is not possible. Therefore, CLH proposals for the three ingredients are submitted.

Data on d-limonene were collected from the DAR of terpenoid blend, the registration dossier of d-limonene and other publically available data through a search using several databases including e-chemportal, PubMed, ToxNet and publications such as the US Environmental Protection Agency report on screening hazard characterization of monoterpene hydrocarbons (EPA 2009), a report by the European Food Safety Authority on the pesticide risk assessment of orange oil obtained from *Citrus aurantium* from Florida (USA) with a d-limonene content of 94.5 – 96.5 % (EFSA 2013) and the World Health Organisation (WHO) International Programme on Chemical Safety (IPCS) report on d-limonene (WHO 1998) Even though d-limonene has a harmonized Annex VI classification based on Regulation (EC) No. 1272/2008 (CLP Regulation) as part of a group entry, reassessment of the data suggests a change in classification to include Skin Sens. 1B (H317) and Asp. Tox 1 (H304) and modification of the current classification for environmental hazards.

Skin sensitisation

Studies on the local lymph node assay (LLNA) reported the estimated concentration giving rise to a 3 fold increase in lymphocyte proliferation (EC3) to 22% ; indicative of a moderate sensitizer and fulfilling the criteria for a harmonized classification of Skin Sens. 1B (H317).

Aspiration hazard

d-Limonene has a Kinematic viscosity at 25°C of 0.9 - 1.1 mm^2/s and is a hydrocarbon which results in classification of Asp. Tox 1 (H304).

For this reason, the dossier submitter considers adaptation of the harmonized classification of d-limonene to include Skin Sens. 1B (H317) and Asp. Tox 1 (H304).

Hazardous to the aquatic environment

Experimental endpoints on acute aquatic toxicity of d-limonene to algae, daphnids and fish range from 0.25 to 0.73 mg/L, and the relevant experimental chronic endpoints range from 0.14 to 0.32 mg/L. The substance is considered rapidly biodegradable. Therefore classifications as Aquatic Acute 1 (H400) and Aquatic Chronic 3 (H412) according to Annex I, Table 4.1.0 (Classification categories for hazardous to the aquatic environment) in Regulation (EC) 1272/2008 is warranted. For the acute classification, according to Annex I, Table 4.1.3 (Classification categories for hazardous to the aquatic environment) in Regulation (EC) 1272/2008 an M-factor of 1 is justified.

Currently, the substance is classified aquatic acute 1 and aquatic chronic 1 and no M-factors are given. Therefore, the dossier submitter considers adaptation of the harmonized classification to

change the aquatic chronic classification from 1 to 3 and to include the M-factor of 1 for the aquatic acute classification.

2.3 Current harmonised classification and labelling

2.3.1 Current classification and labelling in Annex VI, Table 3.1 in the CLP Regulation

d-Limonene has an Annex VI classification of Flam. Liq. 3 (H226), Skin Irrit. 2 (H315), Skin Sens. 1 (H317), Aquatic Acute 1 (H400) and Aquatic Chronic 1 (H410) as part of a group entry.

2.3.2 Current classification and labelling in Annex VI, Table 3.2 in the CLP Regulation

This paragraph is considered irrelevant seen the repeal of Directive 67/548/EEC with effect from 1 June 2015.

2.4 Current self-classification and labelling

2.4.1 Current self-classification and labelling based on the CLP Regulation criteria

Classification		Labelling			# of Notifiers	Total number of notifiers	Percent (%)	Notes
Hazard Class and Category Code	Hazard Statement Code	Hazard Statement Code	Pictograms, Signal Word Code(s)	Specific Concentration limits, M-factors				
Acute Tox. 4 dermal	H312	H312	GHS07 GHS02 GHS09 Wng		1	1981	0.1	Note C 1018/1981 51%
Acute Tox. 4 inhalation	H332	H332			1	1981	0.1	
Aquatic Acute 1	H400	H400			1884	1981	95.1	
Aquatic Chronic 1	H410	H410			1979	1981	99.9	
Aquatic Chronic 2	H411	H411			1	1981	0.1	
Asp. Tox. 1	H304	H304			395	1981	19.9	
Eye Irrit. 2	H319	H319			2	1981	0.1	
Flam. Liq. 3	H226	H226			1980	1981	99.9	
Skin Irrit. 2	H315	H315			1979	1981	99.9	
Skin Sens. 1	H317	H317			1965	1981	99.2	
Skin Sens. 1B	H317	H317			15	1981	0.8	

RAC general comment

d-Limonene is one of the ingredients of the active substance Terpenoid Blend QRD 460. The terpenoid blend, consisting of p-cymene, d-limonene and alpha-terpinene, was approved as an active substance (insecticide) for plant protection products under Regulation (EC) 1109/2009. Besides its use as a pesticide, it is widely used and can be found in foods, medicines, consumer products (e.g. use in cleaning agents and as a solvent), personal care products (as a fragrance) and cosmetics. It is registered under REACH.

3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

d-Limonene is found naturally in Orange oil, an essential oil present in oil glands located in the exocarp of citrus fruits. d-Limonene belongs to the family of terpenes and is an ingredient in insecticides. The terpenes can cause disruption of respiration causing insect death with insects that are more active or have larger spiracles likely to be more affected by the substances. Terpenes, including d-limonene are strong insecticides repelling insects such as thrips and whitefly. d-Limonene is one of the ingredients of the active substance terpenoid blend QRD460. The terpenoid blend, consisting of p-cymene, d-limonene and alpha-terpinene, is accepted as an active substance for plant protection products. However, as it is a mixture and not a substance harmonised classification of terpenoid blend is not possible. Therefore, CLH proposals for the three ingredients are submitted. Given that d-limonene is part of an active substance under Regulation (EC) No 1107/2009 (plant protection products), classification at Community Level is necessary. The formal justification is therefore a requirement for harmonised classification by another legislation or process.

Part B.

SCIENTIFIC EVALUATION OF THE DATA

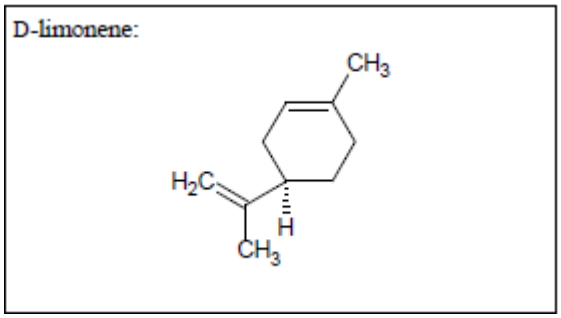
1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 4: Substance identity

EC number:	227-813-5
EC name:	(R)-p-mentha-1,8-diene
CAS number (EC inventory):	
CAS number:	5989-27-5
CAS name:	Cyclohexene, 1-methyl-4-(1-methylethenyl)-, (4R)-
IUPAC name:	(R)-4-isopropenyl-1-methylcyclohexene
CLP Annex VI Index number:	601-RST-VW-Y
Molecular formula:	$C_{10}H_{16}$
Molecular weight range:	136.24 g/mol
Other name	d-limonene

Structural formula:



1.2 Composition of the substance

Table 5: Constituents (non-confidential information)

Constituent	Typical concentration	Concentration range	Remarks
d-Limonene		confidential	See confidential Annex

Current Annex VI entry: Flam. Liq. 3 (H226), Skin Irrit. 2 (H315), Skin Sens. 1 (H317), Aquatic Acute 1 (H400) and Aquatic Chronic 1 (H410).

Table 6: Impurities (non-confidential information)

Impurity	Typical concentration	Concentration range	Remarks
Confidential			Not relevant for classification and labelling

Current Annex VI entry: n/a

Table 7: Additives (non-confidential information)

Additive	Function	Typical concentration	Concentration range	Remarks
None				

Current Annex VI entry: n/a

1.2.1 Composition of test material

The composition of the test material is included in the summary where relevant.

1.3 Physico-chemical properties

Table 8: Summary of physico - chemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
State of the substance at 20°C and 101,3 kPa	Colourless to slightly yellow liquid with citrus odour	EFSA (2013)	
Melting/freezing point	- 73.65 °C (199.5 K)	Gallis et al. (1996); Gallis et al. (2000)	Measured: Thermal analysis Purity: 99%
Boiling point	175 and 178 °C Orange oil : 176 °C	EFSA (2013); Farelo et al. (1991)	Measured: method not known Purity: 99.4%
Density	0.8384 g/cm^3 at 25°C (298.15 K)	Tamura and Li (2005a)	Measured: Anton Paar (DMA58) densimeter. Purity: 98%
Relative density	D_4^{20} = 0.844	Jobard (2010b)	Measured: oscillating densitimeter Purity: 95.2%
Vapour pressure	200 Pa.	Espinosa Diaz et al. (1999)	Measured: static method Purity: 99%
	133 Pa	(DAR 2013)	At 20°C
Surface tension	28.5 mN/m and 27.3 mN at 25 °C D-Limonene is not a surface active substance	EFSA (2013)	Measured: method not known Purity not given
Water solubility	The solubility of R(+)-limonene in water at 298.15 K was measured to be 12.3 mg/L (0.00000163 in mole fraction).	Tamura and Li (2005b)	Measured: flask method Purity: 98%
Partition coefficient n-octanol/water	23988 (log = 4.38) (S.E. = 0.05) at 37 °C. The calculated log Kow values of (+)-limonene were 4.36 (fragment method), 4.83 (atom /fragment contribution method) and 2.94 (atomistic method).	Griffin et al. (1999)	Measured: HPLC method Purity: 95% Nine compounds (including p-Cymene) of known log Kow (ranging from 1.1 to 4.1) and of similar chemical structure to that of terpenoids were used as standards in the determination of log Kow values. HPLC analysis of samples and standards was carried out with a C18 column and diode array detector. The HPLC method is generally not preferred over experimental

			determination of log Kow values. However the standards chosen were especially selected for terpenoids and p-Cymene which has a comparable structure to d-limonene was also included in the set of standards. Thus, this study is considered reduced reliable since it is an estimation method. The data are assigned a Klimisch score of 2. Since the reference compounds are similar to terpenoids, this value is preferred over the value used in the DAR.
	70795 (log = 4.85)	DAR (2013)	Measured: HPLC method OPPTS 830.7570, OECD 117 Purity not provided Comment DAR: Acceptable. Despite the GLP claim, it is unclear if the testing site has been GLP inspected. Study complies with GLP standards therefore no new data required. Dependency on pH is not expected. The Dossier submitter reassessed the original study report: Method used is not OPPTS 830.7570 (= estimation by HPLC). The study was conducted in triplicate by dispersing pure d-limonene (purity not reported) in water. Equal volume of *n*-octanol was added, followed by vigorous shaking. The *n*-octanol and water phases were then allowed to separate and were assayed by GC/MS. Therefore, this is a shake-flask study (OPPTS 830.7550; OECD 107). Shortcomings are: temperature, pH and test concentration were not reported. One ratio (1:1 v/v) was tested instead of required three ratios (2:1, 1:1 and 1:2 v/v). Water and *n*-octanol were not pre-saturated. Recovery was not reported. Above all, the shake-flask method can only be used to determine log P_{ow} values in the range -2 to 4. Thus, this study is considered unreliable. The data are

			assigned a Klimisch score of 3, and will not be used for classification.
	37154 (log = 4.57)	Li et al. (1998)	Shake flask method octanol solution of d-limonene was equilibrated with water by shaking gently for 20 min. Subsequently, the sample was centrifuged and both phases were analysed. Shortcomings are: temperature, pH and test concentration were not reported. One ratio (1:1 v/v) was tested instead of required three ratios. Water and *n*-octanol were not pre-saturated. Recovery was not reported. Above all, the shake-flask method can only be used to determine log P_{ow} values in the range -2 to 4. Thus, this study is considered unreliable. The data are assigned a Klimisch score of 3, and will not be used for classification.
Flash point	51°C	Jobard (2010a)	Measured: equilibrium method closed cup Purity: 95.2%
Flammability	d-Limonene: Highly flammable (flash point : 51 °C) Orange oil: Highly flammable (flash point : 43 °C)	EFSA (2013); Jobard (2010a)	Measured: equilibrium method closed cup d-Limonene: 95.2% Orange oil (Purity not given)
Explosive properties	Examination of the structure indicates that there are no chemical groups associated with explosive properties.	EFSA (2013)	
Self-ignition temperature	245 °C	Paulus (2010)	Measured: Auto-ignition test Purity: 95.2%
Oxidising properties	d-Limonene does not contain any functional group associated with oxidizing properties listed in the Guidance for the implementation of REACH R.7a table R.7.1-29.	ECHA (2014)	
Granulometry	Not applicable		
Stability in organic solvents and identity of relevant degradation products	In accordance with column 1 of REACH Annex IX, the stability	ECHA (2014)	

	in organic solvents study does not need to be conducted as the stability of the substance is not considered to be critical.		
Dissociation constant	In accordance with section 1 of REACH Annex XI, the dissociation constant study does not need to be conducted as the substance does not contain any functional groups that dissociate and therefore testing does not appear scientifically necessary.	ECHA (2014)	
Dynamic Viscosity	0.8462 mPa.s at 25°C (298.15 K)	Francesconi et al. (2001)	Measured: capillary method Purity: >97%

2 MANUFACTURE AND USES

2.1 Manufacture

Not applicable as d-limonene is imported.

2.2 Identified uses

d-Limonene is an ingredient of the plant protection product terpenoid blend. It is accepted as an active substance for plant protection products. d-Limonene is a very versatile chemical which can be used in a wide variety of applications. It is used for making paint solids, to impart an orange fragrance to products, and as a secondary cooling fluid. The largest growth segment has been the use of d-limonene in cleaning products. Therefore consumer exposure is through the use of fragrance products, coatings and inks, solvents or adhesives.

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

3.1 Physical and Chemical Properties

None considered in this report.

4 HUMAN HEALTH HAZARD ASSESSMENT

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

4.1.1 Non-human information

In shaved mice, the dermal absorption of [^{3}H]d/l-limonene from bathing water was rapid, reaching the maximum level in 10 minutes (von Schäfer and Schäfer 1982). In rats, the tissue distribution of radioactivity was initially high in the liver, kidneys, and blood after the oral administration of [^{14}C]d-limonene (Anonymous 1974); however, negligible amounts of radioactivity were found after 48 hours. Differences between species regarding the renal disposition and protein binding of d-limonene have been observed. For rats, there is also a sex-related variation (Anonymous 1989b, 1989a). The concentration of d-limonene equivalents was about 3 times higher in male rats than in females, and about 40% was reversibly bound to the male rat specific protein, α2:-globulin (Anonymous 1989a, 1992a). The biotransformation of d-limonene has been studied in many species, with several possible pathways of metabolism. Metabolic differences between species have been observed with respect to the metabolites present in both plasma and urine. Other reported pathways of limonene metabolism involve ring hydroxylation and oxidation of the methyl group (Anonymous 1976). In another study, perillic acid was reported to be the principal metabolite in plasma in rats (Anonymous 1992b).

4.1.2 Human information

d-Limonene has a high partition coefficient between blood and air and is easily taken up in the blood at the alveolus (Falk et al. 1990). d-Limonene is rapidly distributed to different tissues in the body and is readily metabolized. Clearance from the blood was 1.1 litre/kg body weight per hour in males exposed for 2 hours to d-limonene at 450 mg/m^{3} (Anonymous 1993). The net uptake of d-limonene in volunteers exposed to the substance at concentrations of 450, 225, and 10 mg/m^{3} for 2 hours during light physical exercise averaged 65% (Anonymous 1993). A high oil/blood partition coefficient and a long half-life during the slow elimination phase suggest high affinity to adipose tissues (Anonymous 1993; Falk et al. 1990). Orally administered d-limonene is rapidly and almost completely taken up from the gastrointestinal tract in humans as well as in animals (Anonymous 1974, 1976). Infusion of labelled d-limonene into the common bile duct of volunteers revealed that the chemical was very poorly absorbed from the biliary system (Anonymous 1991a). In one study (one hand exposed to 98% d-limonene for 2 hours), the dermal uptake of d-limonene in humans was reported to be low compared with that by inhalation (Anonymous 1991b); however, quantitative data were not provided. About 25–30% of an oral dose of d-limonene in humans was found in urine as d-limonene-8,9-diol and its glucuronide; about 7–11% was eliminated as perillic acid (4-(1-methylethenyl)-1-cyclohexene-1-carboxylic acid) and its metabolites (Anonymous 1969, 1976). d-Limonene-8,9-diol is probably formed via d-limonene- 8,9-epoxide (Anonymous 1976; Watabe et al. 1981). In another study, perillic acid was reported to be the principal metabolite in plasma in both rats and humans (Anonymous 1992b). Following the inhalation exposure of volunteers to d-limonene at 450 mg/m3 for 2 hours, three phases of elimination were observed in the blood, with half-lives of about 3, 33, and 750 minutes, respectively (Anonymous 1993). About 1% of the amount taken up was eliminated unchanged in exhaled air, whereas about 0.003% was eliminated unchanged in the urine. When male volunteers were administered (per os) 1.6 g [^{14}C]d-limonene, 50–80% of the radioactivity was eliminated in the urine within 2 days (Anonymous 1976). Limonene has been detected, but not quantified, in breast milk of non-occupationally exposed mothers (Anonymous 1982).

4.1.3 Summary and discussion on toxicokinetics

See section 4.1.1 and section 4.1.2. Generally, the amount of d-limonene absorbed via the oral route is similar in different species; reported values range from 50-96% in rats, guinea-pigs, hamsters and dogs whilst those in human volunteers are reported as 50-80% (Anonymous 1974, 1976). Absorption via de inhalation route is also rapid; the percentage absorbed is reported to be on average about 65% (Falk et al. 1990).

4.2 Acute toxicity

Not considered in this report.

4.2.1 Non-human information

4.2.1.1 Acute toxicity: oral

Not considered in this report.

4.2.1.2 Acute toxicity: inhalation

Not considered in this report.

4.2.1.3 Acute toxicity: dermal

Not considered in this report.

4.2.1.4 Acute toxicity: other routes

Not considered in this report.

4.3 Irritation

4.3.1 Skin irritation

Not considered in this report.

4.3.1.1

4.3.2 Eye irritation

Not considered in this report.

4.3.3 Respiratory tract irritation

This paragraph is considered irrelevant seen the repeal of Directive 67/548/EEC with effect from 1 June 2015.

4.4 Corrosivity

Not considered in this report.

4.5 Sensitisation

4.5.1 Skin sensitisation

Table 9: Summary table of relevant skin sensitisation studies

Method	Results	Remarks	Reference
In vivo Mouse local lymph node assay (LLNA) Female CBA/Ca mice (8-12 weeks old) Purity: 99.7% 0, 10, 25, 50, 75 or 100% v/v d-limonene in ethanol/diethyl phthalate (3: 1 v/v)	Stimulation Index d-Limonene 10% v/v = 1.3 25% v/v = 3.4 50% v/v = 4.0 75% v/v = 8.8 100% v/v = 6.5 EC3 = 22% v/v (5500 µg/cm^2)	Main Study OECD429 with minor deviations: no certificate of analysis of the test substance	Anonymous (2004)
In vivo Mouse local lymph node assay (LLNA) Female CBA/Ca mice (8-12 weeks old) Purity: 99% 0, 25, 50, 75 or 100% v/v d-limonene in e acetone/olive oil (4:1 v/v)	Stimulation Index d-Limonene 25% v/v = 1.84 50% v/v = 2.44 100% v/v = 3.95 EC3 = 68.5% vv	Supporting Study OECD429 with deviations: no data on physicochemical properties of test substance; no data on individual weights, housing conditions of test animals; no information on time course of onset and signs of toxicity	Anonymous (2001)

4.5.1.1 Non-human information

Betts (2004)
In a local lymph node assay performed in CBA/Ca strain mice according to OECD guideline 429 and in compliance with GLP, groups of mice (4 females/dose) were applied with 25 µL of d-limonene at concentrations of 0 (vehicle control), 10, 25, 50, 75 or 100% v/v in ethanol/diethyl phthalate (3: 1 v/v) to the dorsal surface of each ear for three consecutive days. On Day 6, all animals were injected with ^{3}H-methyl thymidine and after five hours the draining (auricular) lymph nodes were excised and measured for radioactivity expressed as number of disintegrations per minute (DPM). Historic data of hexylcinnamaldehyde (5, 10 and 25 % w/v) in acetone/olive oil (4:1 v/v) was used as the data for positive control group (Anonymous 2004).

Mean DPM for 0, 10, 25, 50, 75 or 100% d-limonene were observed to be 2511, 3319, 8554, 9916, 22063 or 16259 dpm, respectively (Table 10). Stimulation index for 10, 25, 50, 75 or 100% d-limonene were calculated to be 1.3, 3.4, 4.0, 8.8 or 6.5, respectively (Table 10). The estimated concentration giving rise to a 3 fold increase in lymphocyte proliferation (EC3) was 22% v/v (5500 µg/cm^2). No increase in visual levels of irritancy to the ear skin was observed during the study (Anonymous 2004).

Warbrick et al. (2001)

In a skin sensitization study conducted following a method similar to OECD guidelines 429, four groups of CBA/Ca strain female mice (4/concentration) were exposed topically on the dorsum of both ears to 25 µL of vehicle (acetone/olive oil, 4:1 v/v), 25, 50 and 100% v/v of limonene daily for 3 consecutive days. Five days following initiation of exposure all mice were injected 250 µL of phosphate buffered saline (PBS) containing 20 µCi of [^{3}H] methyl thymidine (^{3}H-TdR). The animals were then sacrificed after 5 hours and suspension of lymph node cells was prepared from auricular lymph nodes. The incorporation of ^{3}H-TdR was measured by β-scintillation counting as disintegrations per minute (dpm) per node for each experimental group. A Stimulation index of 3 or greater was considered to be indicative of a potential to cause contact sensitization.
Limonene at exposure concentration of 0 (vehicle), 25, 50 and 100% v/v resulted in 476, 877, 1164 and 1882 dpm/node and stimulation index of 1, 1.84, 2.44 and 3.95, respectively (Table 11).
The calculated EC3-value for limonene was found to be 68.5% (Table 11).

Table 10: Skin sensitisation potential of d-limonene in ethanol/diethyl phthalate (3:1 v/v) (Anonymous 2004)

Concentration of test substance (% v/v)	Number of lymph nodes assayed	Disintegrations per min (dpm)	dpm per lymph node	Test:control ratio
0 (vehicle only)	8	2511	314	Not applicable
10	8	3319	415	1.3
25	8	8554	1069	3.4
50	8	9916	1240	4.0
75	8	22063	2758	8.8
100	8	16259	2032	6.5
EC3	Estimated to be 22% (5500 µg/cm2)			

Table 11: Local lymph node assay responses to limonene (Anonymous 2001)

Exposure Concentration (% v/v)	dpm/node	Stimulation index (SI)	EC3 (%)
0	476	1	68.5
25	877	1.84	
50	1164	2.44	
100	1882	3.95	

Effects of air-oxidation in the sensitising potency of d-limonene
Experimental studies on the sensitizing potential of d-limonene show diverging results and some studies have shown that the sensitizing potential of d-limonene increases with prolonged air exposure. d-Limonene was found to be a sensitizer after prolonged exposure to air according to 2 Freund's complete adjuvant test (FCAT) experiments and 1 guinea pig maximization test (GPMT) study. No significant response was obtained to d-limonene not air exposed, even if the animals were sensitized to oxidized d-limonene. 5 main oxidation products of d-limonene were identified. (R)-(-)-carvone and a mixture of cis and trans isomers of (+)-limonene oxide were found to be potent sensitizers, while no significant reactions were obtained in the animals induced with a mixture of cis and trans isomers of (-)-carveol. Therefore, air oxidation of d-limonene is essential for its sensitizing potential (Karlberg et al. 1992).

4.5.1.2 Human information

Limited information is available on skin sensitisation in humans. In a Kligman Maximization test, human volunteers (25) were exposed to d-limonene (unknown amount and concentration) on a skin site pre-treated for 24 hours with 5% aqueous sodium laurel sulfate (SLS). The test-substance exposure lasted 48 hours under occlusion. The induction phase with five exposures was 15 days. Following a 10-day rest period, a new skin site was washed with 10% SLS for 1 hour after which there was a challenge with an 8% solution of the test substance. A sensitization reaction with d-limonene was not observed in this assay (Grief (1967) summarized in EPA (2009)).

Because terpenes such as d-limonene oxidize upon exposure to air, studies have shown that the generated oxidized products of d-limonene are responsible for the induction of allergic contact dermatitis (skin sensitisation) in humans (Matura et al. 2005). Limonene-1-hydroperoxide and limonene-2-hydroperoxide are two oxidation products of d-limonene that showed a strong sensitising response in the LLNA. Limonene-1-hydroperoxide and limonene-2-hydroperoxide at 0.5% in petrolatum, and 10 week air-oxidized (4h/day) limonene 3.0% in petrolatum were tested in 763 consecutive dermatitis patients. Results showed that limonene-1-hydroperoxide gave most reactions, with 2.4% of the patients showing positive patch test reactions. Limonene-2-hydroperoxide and oxidized d-limonene (0.5%) gave 1.7% and 1.2% positive patch test reactions, respectively (Christensson et al. 2014). Oxidized d-limonene 3.0% (containing limonene hydro peroxides at 0.33%) in petrolatum was tested in 2900 consecutive dermatitis patients in Australia, Denmark, the United Kingdom, Singapore, Spain, and Sweden. Results showed that 5.2% (range 2.3-12.1%) of 2900 patients had a positive patch test reaction to oxidized d-limonene (Brared Christensson et al. 2014).

Karlberg and Dooms-Goossens (1997) exposed d-limonene to air for 4h/day for 10-20 weeks and applied the resulting product at various concentrations (2-5%) in patches to dermatitis patients from two locations (Leuven and Stockholm). Additionally they applied 0.5-1% of purified d-limonene hydroperoxides. The air exposed product contained up to 12.5% of oxidised d-limonene and sensitisation reactions were observed in 0.9-1.6% of the patients from Leuven, but 1.9-5.1% in patients from Stockholm. Some doubtful cases were observed as well in Stockholm (2-6% of patients). 0.4% of the patients in Leuven reacted to 0.5% d-limonene hydroperoxide while this was 2.4% in Stockholm and 3.2% when applied at a 1% concentration (Stockholm only).

These results support the specificity of the allergenic activity of d-limonene hydroperoxide analogues and the importance of oxidized limonene as a cause of contact allergy in humans.

4.5.1.3 Summary and discussion of skin sensitisation

Two LLNA studies investigating the sensitizing potential of d-limonene in mice reported an EC3 of 22% and 65%, respectively; indicative of a clear positive response (Stimulation index of 3 or greater). As these tests were performed with a highly purified form of d-limonene (99% and 99.7%), the results are considered relevant for all purities. However, a higher potency for skin sensitisation may be present in d-limonene with a higher level of oxidation products. Some human data in volunteers are available but these are of limited value because of the small number of subjects and lack of information on exposure. The relevance of sensitisation potential of products containing pure or high concentrations of oxidised d-limonene is debatable. In 2015, the RAC concluded for Linalool, that exposure to its oxidised form was not relevant considering its current use and classification was therefore based on the compound linalool only. In the studies mentioned in section 4.5.1.2, D-limonene was air-oxidised for at least 10 weeks (4h/day), a situation that is unlikely to occur for products containing d-limonene, similar to the case for linalool. Additionally, according to the manual screening by Greece in 2016, commercial products contain between 0.007 - 0.5% d-limonene although the Dutch National Institute for Health and the Environment (RIVM)

reported d-limonene concentrations of up to 2.1% in commercial products (RIVM 2009). Karlberg and Dooms-Goossens (1997) measured fewer than 12.5% of oxidised d-limonene of which <5% d-limonene hydroperoxide in its air-exposed products. If reported, the other studies mentioned in sections 4.5.1 of this report found lower concentrations of oxidised d-limonene compounds in the air-oxidised products. The concentrations of air-oxidised d-limonene tested in the studies, including the purified hydroperoxide, were generally tested at higher concentrations in comparison to the prevalence of d-limonene in commercial products. In a worst case scenario, where 2% of d-limonene is present in a product, at most 0.25% of the product would be oxidised d-limonene. But again this is unlikely as no commercial product containing d-limonene (mostly fragrances) are exposed to air 4h/day for 10 weeks while being well stirred. In summary, it is more logic to classify for the sensitisation of d-limonene and use studies that contain d-limonene with a possible naturally occurring fraction of oxidised d-limonene because the product is being used.

4.5.1.4 Comparison with criteria

According to the decision logic for classification of substances in section 3.4.2.2.6 of the CLP, a substance is categorized in Skin Sens. 1 (H317) if there is no sufficient information for sub-categorization. Following Table 3.4.4 of the CLP Regulation, skin sensitisation potency in the LLNA is described where an EC3 (%w/v) greater than 2 is indicative of a moderate sensitizer with a classification of Skin Sens. 1B (H317). Therefore, the sub-categorization from Skin Sens. 1 (H317) to Skin Sens. 1B (H317) for d-limonene is warranted. Skin sensitisation of the oxidised form of d-limonene is not considered being relevant for its current use, therefore there is no reason d-limonene may be classifiable as skin sens 1A.

4.5.1.5 Conclusions on classification and labelling

Based on data on the mouse LLNA, there is sufficient data available for sub-categorization and therefore classification for Skin Sens. 1B (H317: Can cause an allergic skin reaction) is warranted.

RAC evaluation of skin sensitisation

Summary of the Dossier Submitter's proposal

Human information

In the Kligman Maximization test on human volunteers (25) exposed to d-limonene under occlusion for 48 hours (unknown amount and concentration) with five induction exposures during 15 days, the substance did not induce skin sensitisation reactions (Grief, 1967; summarised in EPA, 2009) suggesting lack of, or low skin sensitising potential of d-limonene in humans.

Some studies have shown that the skin sensitising potential of d-limonene increases with prolonged air exposure (Matura *et al*., 2005). Karlberg and Dooms-Goossens (1997) have demonstrated that 0.9-1.6 % of patients with dermatitis in Leuven and 1.9-5.1 % of patients with dermatitis in Stockholm responded with skin sensitisation reactions when exposed in the patch test to a product of air exposed d-limonene containing up to 12.5 % of oxidised d-limonene.

Experimental studies

For evaluation of the skin sensitising potential of d-limonene the DS presented results of two local lymph node assays (LLNAs) in mice (Betts, 2004; Warbrick *et al*., 2001). In the first assay (Betts, 2004) conducted according to OECD TG 429 and in compliance with GLP, d-limonene induced a stimulation index above 3, with an effective concentration (EC3) equal to 22 % v/v (5 500 µg/cm^2).

In a second skin sensitisation assay (Warbrick *et al*., 2001), conducted according to a method similar to OECD TG 429, the stimulation index was also above 3 and the calculated EC3-value for d-limonene was found to be 68.5 % (Warbrick *et al*., 2001).

The DS also noted that d-limonene was found to be a sensitiser after prolonged exposure to air according to two Freund's complete adjuvant tests (FCAT) and one guinea pig maximization test (GPMT) study (Karlberg *et al*., 1992).

In the opinion of the DS, there is sufficient data available for sub-categorisation based on the mouse LLNA results, and therefore classification for Skin Sens. 1B (H317: Can cause an allergic skin reaction) is warranted.

Comments received during public consultation

Two MSCA agreed that classification as Skin Sens. 1B; H317 is warranted.

One MSCA informed that positive reactions to oxidised limonene, air exposed limonene or limonene hydroperoxides were reported in three studies using human patch test data from dermatitis patients (Christensson, 2014; Brared Christensson, 2014; Karlberg and Dooms-Goossens, 1997) and asked for a more thorough evaluation of the human data and an indication whether these data fulfil the criteria for Skin Sens. 1A or 1B classification. Additionally, to complement the human database, the MSCA listed additional studies, which should be considered for inclusion in the human data section.

In their response, the DS summarised the existing human data, including those raised by the MSCA (please see the DS response to comment number 8 in the "response to comments" document for details). The DS also compared the existing human data with the CLP criteria for sub-category 1A, and concluded that the weight of evidence from several human studies indicates that classification for oxidised d-limonene products as Skin Sens. 1A is warranted. However, d-limonene itself could not be considered as allergenic in humans because in the human patch tests only products of d-limonene air oxidation were used: limonene-1-hydroperoxide (Christensson *et al*., 2014), oxidized d-limonene (Brared Christensson *et al*. 2014; Karlberg and Dooms-Goossens, 1997), oxidation mixture of both the *R*- and *S*-enantiomers of limonene (Matura *et al.*, 2006), oxidized R-(+)-limonene mixture and R-(+)-limonene hydroperoxide (Matura *et al*., 2002, 2003). No reactions to pure R-(+)-limonene were observed in 30 subjects sensitised to the oxidised limonene patch test materials of Matura *et al*. (2002, 2003).

The DS agreed that the oxidized products formed when d-limonene is exposed to air may be classifiable as Skin Sens. 1A. However, the harmonised classification should deal with the substance itself rather than any impurities or substances that result from chemical reactions by incidental contact with e.g., air or water. The DS also emphasised that the animal data with d-limonene produced reactions that fall within the criteria for Skin Sens. 1B and that these reactions were not close to meeting the criteria of Skin Sens. 1A (which is with an EC3 value

≤ 2 %), while the EC3 found in animal studies were above 22 %. There is no indication the oxidised products will be formed to a significant extend in practice that can produce reactions severe enough for Skin Sens. 1A. Most human studies were performed with air-oxidised d-limonene after at least 10 weeks of air exposure (4 h/day stirred). This is considered unrealistic for most situations. Overall, the DS was of the opinion Skin Sens. 1B is warranted for d-limonene as it likely represents the practical situation most.

Assessment and comparison with the classification criteria

In two LLNA studies, d-limonene (purity 99.7 % and 99 % respectively) stimulated proliferation of cells with EC3 values equal to 22 % v/v and 68.5 %, respectively, both above the EC3 value > 2 %, thus meeting the criteria for classification of a substance in the subcategory Skin Sens. 1B. In both assays, a clear dose-response relationship was observed. It can be excluded that the criteria for Category 1A can be met, as it is not possible that d-limonene at concentration below 2 % would induce a stimulation index of 3 (to meet the criterion for a skin sensitising response at a given concentration), because in two LLNA tests at much higher concentrations of 10 % and 25 %, d-limonene produced the stimulation index values of, 1.3 and 1.84, respectively, thus well below 3.

The existing data indicate that when exposed to air, d-limonene undergoes oxidation, and some oxidised products of d-limonene can produce allergic contact dermatitis in humans and produce a high stimulation index in the LLNA. Several oxidation products of d-limonene were identified. Some of them, such as limonene-1-hydroperoxide, limonene-2-hydroperoxide, oxidized d-limonene and (*R*)-(-)-carvone and a mixture of cis and trans isomers of (+)-limonene oxide were found to be potent sensitisers, while with others no significant reactions were obtained in the animals. No information is available on the concentrations of these products of d-limonene oxidation in closed containers of d-limonene, but it is assumed that it is rather very low. The existing data for individual oxidation products of d-limonene seem to be insufficient for the proposal of harmonised classification, however they indicate that they are more potent skin sensitisers than d-limonene.

The Scientific Committee on Consumer Safety (SCCS) opinion on fragrance allergens in cosmetic products (SCCS/1459/11, 2012) noted that pure d-limonene, d-limonene containing some (low) level of oxidation products or d-limonene oxidised by air exposure (conditions of exposure not specified) did induce in the LLNAs a stimulation index (SI) above 3, but all of them produced EC3 values above 2 %, thus none of them met the classification criteria for category 1A (EC3 value ≤ 2 %). Still, the oxidised d-limonene with an EC3 of 3 % was more potent than pure d-limonene with EC3 of 30 % (Christensson *et al.,* 2008; see additional references below).

Based on the results of LLNA tests, RAC agrees with the DS proposal, and is of the opinion that **d-limonene warrants classification as Skin Sens. 1B; H317 – May cause an allergic skin reaction**.

RAC considers that it is not appropriate to add Note D to Annex VI entry for d-limonene since no data are available on the effectiveness of potential stabilisers in preventing oxidation of d-limonene.

4.5.2 Respiratory sensitisation

Not considered in this report.

4.6 Repeated dose toxicity

Not considered in this report

4.7 Germ cell mutagenicity (Mutagenicity)

Not considered in this report.

4.8 Carcinogenicity

Not considered in this report.

4.9 Toxicity for reproduction

Not considered in this report.

4.10 Other effects

4.10.1 Aspiration and toxicity hazard

4.10.1.1 Viscosity of d/limonene

Table 12: Summary table of Kinematic viscosity

Method	Results	Remarks	Reference
Kinematic viscosity at 25°C of d-limonene	1.1 mm^2/s	None	COM (2014); (1988)
Kinematic viscosity at 25°C by read across of (S)-(-)-limonene	1.002 mm^2/s	None	Francesconi et al. (2001)
Kinematic viscosity at 25°C of d-limonene	0.897 mm^2/s	None	Clará et al. (2009)

Read-across data
Data for (S)-(-)-limonene (CAS 5989-54-8) can be used for d-limonene since enantiomers share the same chemical properties. A study was conducted similarly to OECD Guideline 114 to determine the dynamic viscosity of (S)-(-)-limonene using Schott measuring system equipped with capillary viscometers. The kinematic viscosity was calculated from the average of six flow times using equation: $\nu = k\ (t - \theta)$. The dynamic viscosity was calculated by the equation: $\eta = \rho\nu$ (ρ is density). Under the test conditions, the dynamic viscosity of (S)-(-)-limonene (97% purity) at 25°C (298.15 K) was determined to be 0.8462 mPa.s (Francesconi et al. 2001). This study was performed similarly to OECD Guideline 114 with minor deviations: test was conducted at one temperature (25 °C) only and individual analytical determinations were not reported. The density of S-limonene is

0.844 g/mL at 25°C and the kinematic viscosity (dynamic viscosity divided by the density of the liquid) is 1.002 mm^2/s.

Both the kinematic viscosity of d-limonene (0.9 - 1.1 mm^2/s) and S-limonene (1.002 mm^2/s) at 25°C are much lower than 20.5 mm^2/s and higher values are not expected at 40°C.

4.10.1.2 Comparison with criteria

Aspiration toxicity hazard category 1 (Asp. Tox. 1) (H304: May be fatal if swallowed and enters airways) is warranted for liquid substances and preparations because of their low viscosity. Low viscosity leads to flow and low surface tension leads to spread of a liquid through the respiratory tract. Aspiration toxicity hazard category 1 (Asp. Tox. 1) is warranted, if the substance is a hydrocarbon and has a kinematic viscosity *v* of 20.5 mm^2/s or less, measured at 40°C (Regulation (EC) No 1272/2008, section 3.10.2).

4.10.1.3 Conclusions on classification and labelling

Based on the kinematic viscosity, a classification for Asp. Tox. 1 (H304: May be fatal if swallowed and enters airways) is warranted.

RAC evaluation of aspiration toxicity

Summary of the Dossier Submitter's proposal

A summary of the kinematic viscosity data submitted by DS is provided below:

Method	Results	Reference
Kinematic viscosity at 25 °C of d-limonene	1.1 mm^2/s	COM, 2014, 1988
Capillary method performed similarly to OECD TG 114. Kinematic viscosity at 25 °C of (S)-(-)-limonene (purity: > 97 %) was used by read across for d-limonene since enantiomers share the same chemical properties	1.002 mm^2/s	Francesconi *et al.,* 2001
Kinematic viscosity at 25 °C of d-limonene	0.897 mm^2/s	Clará *et al.,* 2009

Both the kinematic viscosity of d-limonene (0.9-1.1 mm^2/s) and l-limonene (1.002 mm^2/s) at 25 °C are much lower than 20.5 mm^2/s and higher values are not expected at 40 °C, which might indicate the potential for aspiration toxicity.

The DS has proposed to classify d-limonene for Aspiration toxicity as Asp. Tox. 1; H304 – May be fatal if swallowed and enters airways.

Comments received during public consultation

Three MSCAs agreed with the classification proposed by DS as Asp. Tox. 1; H304 for d-limonene.

Assessment and comparison with the classification criteria

The criteria for classification for aspiration toxicity are given in Table 3.10.1, Annex I of the CLP Regulation.

Given that d-limonene is a hydrocarbon and has a kinematic viscosity between 0.9-1.1 mm^2/s at 25 °C and its expected kinematic viscosity at 40 °C would be lower than its viscosity at 25 °C, thus lower than 20.5 mm^2/s. Therefore, RAC agrees with the DS proposal, and considers that d-limonene should be classified as **Asp. Tox 1; H304 – May be fatal if swallowed and enters airways**.

4.10.2 Neurotoxicity

Not considered in this report.

5 ENVIRONMENTAL HAZARD ASSESSMENT

The environmental hazards of Terpenoid blend which contains d-limonene were assessed in the Draft Assessment Report, addenda and Proposed Decision of the Netherlands prepared in the context of the possible approval Terpenoid blend QRD 460 under Reg. (EC) 1107/2009. The DAR is publicly available via the EFSA web site (http://dar.efsa.europa.eu/dar-web/provision).
Where available endpoints for d-limonene are taken over from the DAR, however since the DAR is for the Terpenoid blend containing more substances, only little data on d-limonene as single compound is available in the DAR. Additional data is searched for in public literature and databases. Endpoints from databases were only used for classification purposes when original test reports could be assessed for their reliability. When available, QSARs have been used to complement the dataset. When reliable experimental endpoints are available, QSAR endpoints are only used for informational purposes.

5.1 Degradation

Table 13: Summary of relevant information on degradation

Method	Results	Remarks	Reference
Hydrolysis	d-limonene does not contain any functional groups that are susceptible to hydrolysis under environmental conditions.	Statement in the DAR	DAR (2013)
Half-life in air	QSAR estimations: hydroxyl radicals: 53 min. ozone: 37.3 min. nitrate radicals: 0.9-9 min.	AOPWIN in EPI Suite 4.11	US-EPA (2012)
Ready biodegradability	readily biodegradable	71.4% over 28 days	King (1992)

5.1.1 Stability

No experimental data is available. d-Limonene is not expected to undergo hydrolysis since it lacks functional groups that hydrolyse under environmental conditions (DAR 2013; CICAD 1998) However, the Henry's law constant is determined to be 1.30×10^{-3} Pa m^3/mol and from this and level III fugacity modelling, d-limonene is expected to partition from water and soil to air. In air it will be degraded rapidly (the DT100 was determined to be 33.6 hours) by interaction with hydroxyl and nitrate radicals (see section 5.2.3) (DAR 2013). D-limonene is not expected to be affected by photolytic degradation (DAR 2013).

5.1.2 Biodegradation

5.1.2.1 Biodegradation estimation

The BIOWIN v4.10 QSAR contained within EPI Suite™ version 4.11 (US-EPA 2012) consists of six models. D-limonene is predicted to biodegrade fast using linear (BIOWIN 1) and non-linear (BIOWIN 2) biodegradation models. Ultimate biodegradation, i.e., conversion of d-limonene to carbon dioxide (BIOWIN 3), is predicted to occur within weeks while initial steps of biodegradation (BIOWIN 4) are predicted to occur within days to weeks. In two of the models, BIOWIN 5 and 6, representing MITI testing, d-limonene was not considered to be readily biodegradable based on microbial oxygen uptake in the OECD 301C test. D-limonene is not predicted to biodegrade quickly under anaerobic conditions (BIOWIN 7) (DAR 2013). Thus, even though BIOWIN 3 estimates ultimate biodegradation within "weeks", as BIOWIN 5 indicates that d-limonene will not be readily biodegradable, the overall conclusion is that d-limonene is estimated to be not readily biodegradable.

5.1.2.2 Screening tests

The following studies provide information on the biodegradation of d-limonene. Screening studies for d-limonene were not available in the DAR.

Ready biodegradation study

Reference	:	King (1992)	study type		OECD 310
year of execution	:	1992	incubation time	:	28 days
GLP statement	:	No	nominal concentration	:	10 mg/L
Guideline	:	OECD 301B with adaptations	Temperature	:	20-23°C
test substance	:	*d-limonene*	Degradability	:	71.4% based on CO_2
Purity	:	95%	Metabolites		not reported
test system	:	sealed vessel	Acceptability	:	**acceptable with restrictions**

The study was performed according to OECD guideline 301B with adaptations for volatile substances (sealed vessel). The test method as adapted is in line with the latest adopted OECD test guideline 310 (Ready Biodegradability - CO_2 in sealed vessels (Headspace Test)). The study tested the degradation of d-limonene with a purity of 95% by an inoculum from an unacclimated sludge plant in a sealed vessel. The test was performed at 20-23°C and the pH of the medium was adjusted to 6.5. Samples were taken at day 3, 7, 10, 14, 16, 21, 24 and 28 when the concentration of inorganic CO_2 is determined in the headspace and medium with an inorganic carbon analyser. The amount of inorganic carbon was related to that produced in a control to determine the extent of degradation. Details on the control are not reported, neither are details on a reference substance given. The percentage of degradation based on the CO_2 development is given in the table below.

Table 14: Degradation of d-limonene

Day	Percentage biodegradation of d-limonene
3	25.5
7	29.8
10	60.6
14	58.8
16	64.7
21	71.1
24	62.6
28	71.4

After 28 days the biodegradation was 71.4% (95% confidence interval 68.3 - 74.5%). On this basis, it is concluded that d-limonene is readily biodegradable fulfilling the 10d window criterion because after 10 days, 60.6% degradation was achieved. The study can be considered reliable with restriction as for example details on the controls are not provided. The data are assigned a Klimisch score of 2, and are used for classification purposes.

In addition to the study above, data were obtained from the public registration information on the ECHA dissemination website (https://echa.europa.eu/web/guest/information-on-chemicals/ registered-substances; date of access 27-9-2016). Four studies were available on this website, these are discussed below.

ECHA dissemination site key study (Author not disseminated 2010)
The key study in the REACH dossier is an OECD guideline 301D Closed Bottle test performed with non-adapted activated sludge from a domestic sewage treatment plant from 2010. The biodegradation was assessed by the determination of the oxygen consumption. After 28 days the O_2 consumption was 5.3 mg/L related to a reduction in concentration of 80%. For the reference compound the O_2 consumption related to 82% was 4.5 mg/L and this was achieved at day 14. Details on the biodegradation are given in Table 15.

Table 15: Oxygen consumption (mg/L) and the percentages biodegradation of the test substance, dipentene (BOD/ThOD) and sodium acetate (BOD/ThOD) in the Closed Bottle test.

Time (days)	Oxygen consumption (mg/L)		Biodegradation (%)	
	Test substance	Acetate	Test substance	Acetate
0	0.0	0.0	0	0
7	2.7	4.1	41	76
14	4.7	4.5	71	83
21	5.0		76	
28	5.3		80	

Although the test seems reliable, the details on the ECHA website give insufficient details on the substance actually tested. Under the heading test material no information is given, only in the heading of a result table and in the applicant summary was it mentioned that actually dipentene was tested. According to the database on the ECHA website, three different reaction masses are registered under the name dipentene (EC numbers: 205-341-0; 907-808-0 and 939-009-8). Although all three different mixtures contain d-limonene the actual content of the d-limonene is not specified . The dossier submitter was informed by the registrant that the dipentene tested consisted of 48.4% d-limonene; 20.6% β-phellandrene; 9.8% α-terpinene; 5.8% γ-terpinene and 4.5% terpinolene (personal communication, September 2016). A rationale could be given that these structures have a structural resemblance and will be similarly biodegradable but taken the complexity of the mixture

consisting of five different components, the actual extent of the biodegradation of d-limonene is not known. Therefore this study is only used as supporting information on the biodegradability of d-limonene.

ECHA dissemination site supporting study "002" (Author not disseminated 1980)
In the REACH dossier, the reliability of this study from 1980 was reported as "not assignable". Indeed the information in the REACH dossier was too limited to assess this study for the purpose of the current report and the results will not be used for classification purposes.

ECHA dissemination site supporting study "003" (Author not disseminated 1997)
In the REACH dossier this study is described as a ready biodegradation study soil-slurry biodegradation assay with an inoculum originating from a forest soil. The inoculum was described as 20% (w/v) soil-slurry. Volumetric biodegradation rate and soil-normalised biodegradation rate were determined to be 0.38 mg/L/h and 1.9 µg/g/h. The details in the REACH dossier are too limited to actually assess the reliability of the study. For example, the test protocol is poorly described and a test guideline is not mentioned. Additionally, the registrant has indicated that this study is not in line with the standard test methods for ready biodegradability (personal communication, September 2016). Given the above mentioned, the results of this study are not used in this report for classification purposes.

ECHA dissemination site supporting study "004" (Author not disseminated 1996)
In the REACH dossier this study is described as a ready biodegradation study with enriched cultures from a forest soil. A degradation rate for cultures unadapted to the test material was reported of 0.044 mg/L/h with a lag period of 180 hours. The details in the REACH dossier are too limited to actually assess the reliability of the study. For example, the test protocol is poorly described and a test guideline is not mentioned. Additionally, the registrant has indicated that this study is not in line with the standard test methods for ready biodegradability (personal communication, September 2016). Given the above mentioned, the results of this study are not used in this report for classification purposes.

5.1.2.3 Simulation tests

In the DAR two studies have been assessed that have addressed the fate and behaviour of Terpenoid Blend QRD 460 by testing the three terpene constituents, i.e. alpha-terpinene, *p*-cymene and d-limonene, individually in separate test vessels. The relevant sections of the DAR summaries that report on **d-limonene** as a single compound are provided below.

Aquatic simulation study *DAR reference STUDY IIA, 7.8.3/001*

reference	:	Moser (2011)	study type	:	non-standard study with natural lake water similar to OECD 309
year of execution	:	2011	incubation time	:	48 hours
GLP statement	:	yes	nominal concentration	:	1 mg/L
guideline	:	none	Temperature	:	18.1-21°C
test substance	:	d-limonene, *p*-cymene, alpha-terpinene	DT50	:	3.0 hours (for d-limonene)
purity	:	95% (d-limonene; lot #810763)	Metabolites		not detected
test system	:	Filtered (0.45µ) lake water	Acceptability	:	**acceptable**

This study is not a water sediment study, rather a study in natural waters that is similar to OECD 309. Degradation of alpha-terpinene, *p*-cymene and d-limonene, QRD 460, was studied in natural lake water (Lake Constance, Horn, CH, see details below). **The test substances were tested**

individually to provide information on the degradability and the formation of degradation products of each compound, if possible. Test vessels (20mL borosilicate glass tubes with Teflon-lined screw cap) were covered with aluminium foil to exclude light and incubated at 20 ± 2 ºC. The test was performed in a flow-through system with air slowly passing. Stock solutions of the three test items were filled into test vessels equipped with traps containing iso-octane to collect volatile test item or possible degradation products. Samples for analysis were taken at application and after 1, 3, 6, 24 and 48 hours and analysed immediately. Their respective trapping solutions were also analysed.
Application solutions were prepared with a concentration of 0.946 mg a.i./L for d-limonene (1.01 mg a.i./L for alpha-terpinene and 0.993 mg a.i./L for *p*-cymene). The test substances were tested individually by adding 20 ml of test solution to a test vessel.
Duplicate samples were analysed at each test interval. The entire water sample was extracted with n-hexane containing an internal standard. The n-hexane phase was then analysed by GC-FID. The trapping solution was analysed by GC-FID without any further treatment. Method validation revealed mean recoveries for d-limonene of 72.4% (low concentration) and 55.9% (10x concentration), respectively. Recovery of the three terpenes was low which is attributed to the high volatility. The repeatability of the test was good and high accuracy and precision were achieved.
The purity of the supplied test items was also tested using analytical standards.
A GC-MS method was applied for further characterisation to identify possible degradation products.
The disappearance time DT_{50} and DT_{90} was calculated using the GC-FID results and are based on the percentage a.i. found at t=0 h. Calculation were performed using SFO kinetics using FOCUS kinetics spreadsheet for 2 replicates. The RSS was minimized by adjusting M0 and k values.
Only the results for d-limonene are shown and discussed below.
The purity of d-limonene was determined to be 93.7%, which is slightly higher from the value reported with the test item.
Water Quality*:* Different batches of lake water were analysed. Characterisation of the lake water at the time of sampling yielded the following: pH of 7.86-8.28; dissolved oxygen of 6.73-9.13 mg O_2/L; TOC of 2.25-9.17 mg C/L; conductivity of 275-300 µS/cm; hardness of 142-164 mg $CaCO_3$/L; and alkalinity of 105-128 mg $CaCO_3$/L.
Test results: For d-limonene the extracted concentration at t=0 was 0.295 and 0.314 mg a.i./L resp., which correspond to a recovery of 31.1 and 33.2% of the initial concentration. This is much lower than the recovery in method validation. Further continuous decrease in concentration was measured until below LOQ after 48 hours. (R)-(+) Limonene was found in the trapping solution at 48h, but only in one replicate. All other results were below the LOQ of 0.0197 mg a.i./L. The results for (R)-(+) Limonene are given in Table 16.
GC-MS measurements of representative samples did not result in detection of degradation products of the test items.

Table 16: Concentration of d-limonene in extracts and trapping solutions (DAR 2013)

Time hour	Concentration in the extract [mg a.i./L]	Mean recovery [%]	Concentration test item used for DT_{50} [mg/L][b]	Concentration in trapping solutions [mg a.i./L]
0	0.295		0.310	-
0	0.314	32.2	0.331	
0[a]	0.501			-
0[a]	0.466	51		
1	0.248		0.261	<LOQ
1	0.231	25.3	0.243	<LOQ
3	<LOQ		<LOQ	<LOQ
3	0.227	12	0.238	<LOQ

6	<LOQ		<LOQ	<LOQ
6	0.219	11.6	0.321	<LOQ
24[a]	<LOQ			<LOQ
24[a]	0.332	17.5		<LOQ
48	<LOQ		<LOQ	<LOQ
48	<LOQ		<LOQ	0.252

[a)] Repeated measurements – not used for calculation of half-life

[b)] The concentration was calculated using the mg a.i./L divided by the purity of the test item, which was 95% for (R)-(+) Limonene

Note: <LOQ was defined to be 0 for further calculations

LOQ Limit of Quantification. Determined as 0.0197 mg a.i./L in extract and 0.2 mg a.i./L in trapping solution

Degradation rate: In Figure 1 the results of the kinetic fit using the FOCUS Kinetics spreadsheet are presented.

Figure 1: SFO degradation plot and error level Chi² test of d-limonene (DAR 2013)

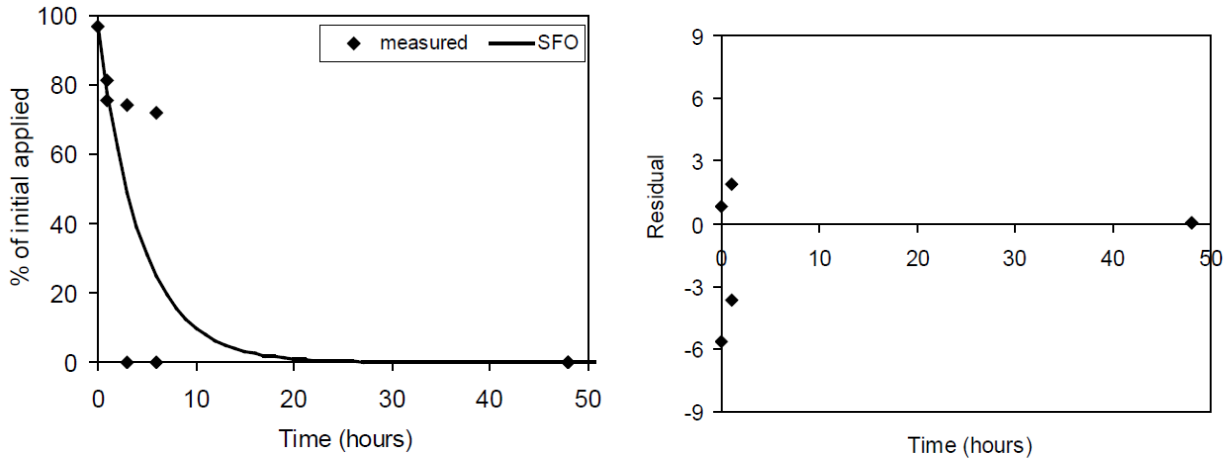


Table 17: Summary of DT50 and DT90 values, SFO parameters and chi2 test (DAR 2013)

	DT_{50} [hours]	**DT_{90} [hours]**	**M0 (fitted)**	**K (fitted)**	**Error level Chi^2 test**
d-limonene	3.0	10.0	97.61	0.229	11.8

Conclusion: **d-limonene volatilized from the natural water test systems rapidly with a DT50 of 3.0 and DT90s of 10.0 hours**. The trapping solution did show the presence of the test substance in one case but not of any degradates. Degradates in the water were also not detected. Thus, **rapid escape (fugacity via volatility) appears to be the predominant pathway for d-limonene in natural water**.

The study was performed with non-radio labelled test material and therefore, no mass balance can be given. No metabolisation products were detected by GC-MS analyses, neither in the extracts of the aquatic systems not in the trapping solutions. The author arguments the test items volatilised from the water, however, only the test with *p*-cymene showed an increase in concentration of the a.i. in the trapping solution. The distribution of residuals is acceptable. No t-test was performed. The DT_{50} value derived for d-limonene of 3.0 hours, can be used for the risk assessment.

Degradation in soil *DAR reference STUDY IIA, 7.2.1/01*

Reference	:	Moser (2010)	study type	:	aerobic soil degradation according to OECD 307
year of execution	:	2010	incubation time	:	up to 4 d
GLP statement	:	Yes	nominal concentration	:	-
guideline	:	OECD 307 (2002)	temperature	:	20°C
test substance	:	d-limonene, *p*-cymene, alpha-terpinene	DT50	:	<24 h
purity	:	95.0% (d-limonene; lot # 810763).	metabolites	:	not applicable
soils	:	Sandy loam	acceptability	:	**acceptable**

The **aerobic soil degradation of** alpha-terpinene, *p*-cymene and **d-limonene** was studied in one representative sandy loam soil. The test soil was field collected in Sevelen (Switzerland), sieved (2 mm) and stored refrigerated until 5 days before use and then acclimatised to test temperature. Test vessels (500 ml) containing 100 g (dry weight) soil were pre-incubated under aerobic conditions for four days prior to application. **The three test substances were applied individually** to achieve final nominal concentrations of approximately 1.82 mg/kg alpha-terpinene, 0.68 mg/kg *p*-cymene and 0.55 mg/kg d-limonene, this reflects the relative proportion of each terpene in the active substance QRD 460. A continuous flow-through test system was used at a temperature of 20 ± 2ºC in the dark at 50% of MWHC. Aerobic conditions were maintained by continuously bubbling moistened air through the water layer. Each replicate was equipped with a trap containing iso-octane as trapping solution to collect volatile test item or possible degradation products. Samples were analysed after 0 and 7 hours, and 1, 2 and 3 days after application. The trap of the respective sample was analysed too.

Duplicate samples for each test item were analysed at each sampling interval. The soil was extracted with acetonitrile. The acetonitrile fraction was further extracted by liquid/liquid extraction with hexane. The hexane was concentrated and then analysed by GC. The trapping solution was analysed by GC without any further treatment. The analytical method was subject to validation as part of the study. The LOQ was 0.038 mg a.i./kg soil for d-limonene.

Only the results for d-limonene are shown and discussed below.

Table 18: Concentration of d-limonene, in soil extracts and trapping solutions (DAR 2013)

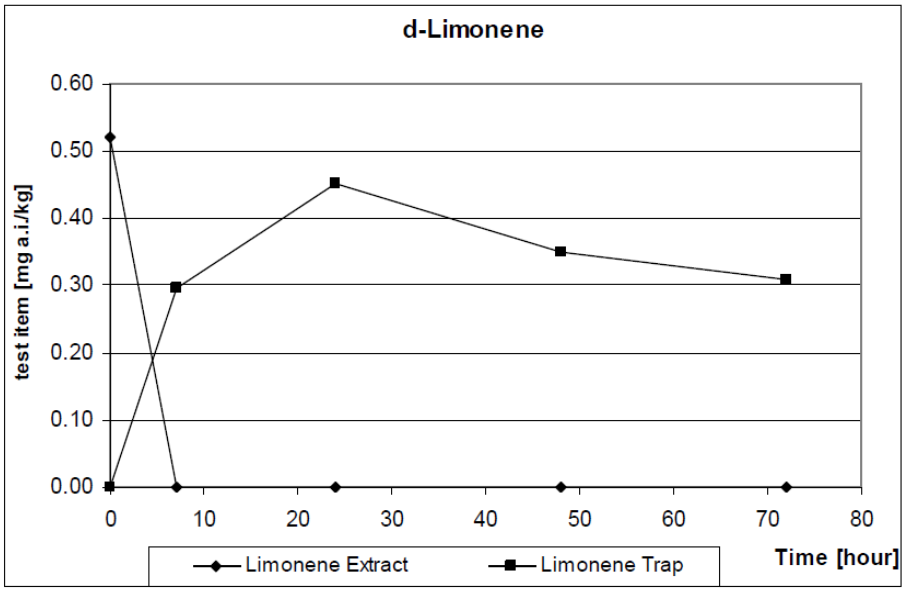
Sample	**Sample time [hours]**	**Concentration [mg a.i./kg]**
Soil extract	0	0.53 0.51
	7	<LOQ <LOQ
	12	<LOQ <LOQ
	24	n.d. n.d.
	36	n.d. <LOQ
		Concentration [mg a.i./L]
Trap	7	0.19 0.40
	12	0.45 0.45
	24	0.33 0.37
	36	0.39 0.23

n.d. not detectable
LOQ = 0.038 mg a.i./kg

LOQ trapping solution = 0.196 mg a.i./L. (concentration of lowest analytical standard)

In the soil extract of d-limonene of T0, 0.51 and 0.53 mg a.i./kg were found. These calculated values were slightly higher than the applied 0.46 mg a.i./kg of d-limonene. No degradation products were detected. By 7 hours after application, both replicates showed a concentration <LOQ (0.038 mg a.i./kg). From day 2 onwards there was no detectable residue.
For all three test items levels of volatile test item and/or degradation products increased from 7 hours to one day after application. Thereafter amounts decreased. **The test item and their degradation products disappeared from the soil into the trapping solution. Due to the continuous aeration, the test items were pushed out of the trapping solution with ongoing time**. The study was performed with **non-radio labelled test material**. Therefore, no mass balance can be given.

Figure 2: Distribution of d-limonene in soil extract and trapping solution (DAR 2013)



It was concluded that d-limonene disappears rapidly from the soil into the trapping solution by evaporation. The DT_{50} was calculated to be <24 hours. The DT_{90} which was actually also the DT_{100} was <48 hours.
This study confirms the assumptions made based on the physical chemical properties of the terpenoid blend QRD 460 and the fugacity models conclusions that the fate of the terpenoid blend (alpha-terpinene, p-cymene and d-limonene) QRD 460 in soil is of limited relevance as it volatilises and evaporates rapidly into the air compartment. **No kinetics of degradation could be calculated as the substances dissipated within 24 hours**. The result that alpha-terpinene, *p*-cymene and d-limonene disappear rapidly from the soil with a **DT_{50} of <24 hours** can be used for risk assessment.

5.1.3 Summary and discussion of degradation

No experimental stability data is available for d-limonene.
Biodegradation estimates with BIOWIN predict that d-limonene is not readily biodegradable.
The available water and soil degradation studies in the DAR with d-limonene show rapid DT50 values. The aquatic simulation study was not a water sediment study, rather a study in natural water. The water was continuously aerated, and the non-radiolabelled d-limonene was only detected in one of the trapping solutions. No degradation products were detected. The DT50 was calculated to be 3.0 hours but the disappearance was considered to be caused by evaporation rather than

biodegradation. The aerobic soil simulation study also used non-radiolabelled d-limonene, and evaporation to the trapping solution was shown as the predominant disappearance route. Therefore, these studies cannot be used to assess the biodegradability of d-limonene.
The study of King (King 1992) provided by the registrant has shown that d-limonene is readily biodegradable, after 28 days biodegradation was 71.4%. This is supported by the results from the key study in the REACH dossier. In this case, the experimental data are preferred over the calculated QSAR data. Therefore, d-limonene is considered rapidly degradable for classification purposes.

5.2 Environmental distribution

5.2.1 Adsorption/Desorption

No experimental studies on the sorption behaviour of d-limonene in soil are available. In the DAR a Koc value of 6324 L/kg, calculated with KOCWIN in EPIsuite, is used in PEC calculations. The height of this value indicates that it should sorb relatively strong to soil and sediment. It should be however noted that considering the high volatility of the substance, it is expected that it will remain shortly in the terrestrial environment.

5.2.2 Volatilisation

According to the DAR (2013), D-limonene has a vapour pressure of 1.33 x 10^2 Pa at 20 °C and the Henry's law constant was estimated to be 1.3 x 10^3 Pa x m^3/ mol. The substance is considered to be highly volatile and will dissipate from water rapidly.

5.2.3 Distribution modelling

In the DAR, it is stated that the main environmental compartment receiving d-limonene was air which also degraded d-limonene much faster than the soil, sediment and water compartments. It was also remarked that the environmental compartment distribution in Level III is based on reaching steady state conditions and not equilibrium in a closed system. Therefore, d-limonene entering the air will quickly degrade. Full degradation in the total system was predicted to be 33.6 hours.

5.3 Aquatic Bioaccumulation

Table 19: Summary of relevant information on aquatic bioaccumulation for d-limonene

Method	Results	Remarks	Reference
QSAR BCFBAF v3.01	361 L/kg	regression based method, log Kow = 4.38	US-EPA (2012)
QSAR BCFBAF v3.01	728 L/kg	Arnot-Gobas method, log Kow = 4.38	US-EPA (2012)

5.3.1 Aquatic bioaccumulation

In the DAR, an experimentally determined log K_{ow} of 4.85 is reported but this value is considered unreliable by the dossier submitter. Therefore preference is given to the value of 4.38 (see Table 8). The values of 4.38 is also presented as experimental value by the estimation programs Bioloom (Biobyte 2006) and EPIsuite (US-EPA 2012). It is also stated in the DAR, that in general for terpenes (including d-limonene) because of their high volatility and low water solubility, the residence time of terpenes in water is too low for accumulation by fish or other aquatic organisms. Also no accumulation in soil is expected since the dissipation time in soil is lower than 24 hours. Furthermore, it is stated that naturally occurring substances like terpenes will not have a propensity to bioaccumulate or bioconcentrate in aquatic organisms. These arguments could be used as supporting information but according to the guidance, in absence of experimentally determined bioconcentration data, conclusions on bioaccumulation should be based on the experimentally determined log Kow.

5.3.1.1 Bioaccumulation estimation

QSAR calculations can be performed with BCFBAF v3.01 in EPI Suite (US-EPA 2012). On the basis of the log Kow of 4.38, BCFs of 361 L/kg and 728 L/kg are estimated with the regression based method and Arnot-Gobas method respectively.

5.3.1.2 Measured bioaccumulation data

No information is available on test for bioaccumulation.
However, there is ADME data available for d-limonene in the DAR (see section 4.1 for details). It was reported that absorption is rapid for d-limonene in rats with recovery within 48 hours. Several possible pathways for metabolism are reported. Thus, in mammals d-limonene appears to be readily metabolized to substances that are rapidly excreted within 48 hours.

5.3.2 Summary and discussion of aquatic bioaccumulation

According to the guidance (section 4.1.3.2.3.3), the log Kow of 4.38 being higher than 4, indicates that the substance has a high potential for bioaccumulation.

5.4 Aquatic toxicity

Table 20: Summary of information on aquatic toxicity

Method	Results (mg/L)	Remarks	Reference
Experimental endpoints			
Fish			
Short-term fish toxicity according to ASTM E729 method; GLP not reported.	96 h LC50 = 0.702 (test 1) 96 h EC50 = 0.702 (test 1) 96 h LC50 = 0.720 (test 2) 96 h EC50 = 0.688 (test 2)	*Pimephales promelas* purity 99%; flow-through Ri=2	(Anonymous 1990b)

Chronic toxicity to fish according to OECD test guideline 212 GLP reported	NOEC growth = 0.059 (EC10 between 0.37 and 0.67 mg/L) NOEC hatching = 0.37 NOEC behaviour = 0.19 EC10 survival = 0.32 NOEC survival = 0.37	*Pimephales promelas* endpoints based on mean measured concentration; EC10 for growth could not be statistically determined. Ri=2	(Anonymous 2015)
<u>Invertebrates</u>			
Short-term invertebrate toxicity according to OECD test guidance 202 GLP reported	48 h EC50 = 0.307 (mobility)	*Daphnia magna* endpoint based on mean measured concentration Ri=1	Betat (2013b)
Short-term invertebrate toxicity according to OECD test guidance 202	48 h EC50 = 0.456 (mobility)	*Daphnia magna* endpoint based on mean measured concentration Ri=1	Delpit (2014)
Short-term invertebrate toxicity according to OECD test guidance 202	48 h EC50 = 0.51 (mobility)	*Daphnia magna* endpoint based on mean measured concentration Ri=1	Bjørnestad (2013)
Short-term invertebrate toxicity method and GLP not reported.	EC50 = 7.85	*Daphnia magna* exposure concentrations exceed water solubility; based on nominal concentrations Ri=3	Park et al. (2011)
Short-term invertebrate toxicity according to ASTM E729 method; GLP not reported.	48 h LC50 = 0.924 (mortality) 48 h LC50 = 0.577 (mortality) 48 h EC50 = 0.42 (mobility)	*Daphnia magna* purity 87%; flow-through based on mean measured concentrations Ri=2	(Anonymous 1990b)

Short-term invertebrate toxicity method and GLP not reported	48 h EC50 = 69.6	*Daphnia magna* endpoint based on nominal concentrations, endpoint considered unreliable	May Passino and Smith (1987)
Short-term invertebrate toxicity according to OECD test guidance 202, GLP reported	48 h EC50 = 0.36	*Daphnia magna* endpoint based on nominal concentrations, endpoint considered unreliable	Author not disseminated (2007)
Chronic invertebrate toxicity according to OECD test guideline 211	21 day EC10 = 0.153	*Daphnia magna* renewal test, endpoint based on mean measured concentration Ri=1	Kamper (2016b, 2016a)
<u>Algae/Aquatic Plants</u>			
Aquatic toxicity to algae according to OECD guideline 201	72 h ErC50 = 0.32 72 h ErC10 = 0.174	*P. subcapitata* endpoint based on mean measured concentration Ri=2	Betat (2013a)
Aquatic toxicity to algae according to OECD guideline 201	48 h ErC50 = 0.25 48 h ErC10 = 0.14 72 h ErC50 = 0.15 72 h ErC10 = 0.09	*P. subcapitata* endpoint based on mean measured concentration Endpoints for 48 h: Ri=2 Endpoints for 72 hours: Ri=3	Seierø (2015)
Aquatic toxicity study to algae Method and GLP not reported.	24 h NOEC <0.05 24 h NOEC <1.5	*Pseudokichneriella subcapitata* Ri =3	(Anonymous 1990b; LMC ASIS 2014)
QSAR calculated endpoints			
QSAR - fish toxicity	96 h LC50 = 0.459 28 day NOEC = 0.080	iSafeRat® Holistic HA-QSAR and iSafeRat® HA-QSAR for chronic aquatic toxicity	KREATiS (2015d) KREATiS (2015e)

	96 h LC50 = 0.845 30 day NOEC = 0.073	ECOSAR v1.11 neutral organics based on log Kow of 4.38	US-EPA (2012)
QSAR - invertebrate toxicity	48 h EC50 = 0.62 21 day NOEC = 0.05	iSafeRat® Holistic HA-QSAR and iSafeRat® HA-QSAR for chronic aquatic toxicity	KREATiS (2015c) KREATiS (2015b)
	96 h LC50 = 0.154 (mysid) 48 h LC50 = 0.577 (daphnid) 16 d NOEC = 0.074 (daphnid)	ECOSAR v1.11 neutral organics based on log Kow of 4.38	US-EPA (2012)
QSAR - algae toxicity	72 h EC50 = 0.50	iSafeRat® Holistic HA-QSAR	KREATiS (2015a)
	LC50 = 1.07 NOEC = 0.32	ECOSAR v1.11 neutral organics based on log Kow of 4.38	US-EPA (2012)

5.4.1 Fish

5.4.1.1 Short-term toxicity to fish

Experimental EC50 values for *Pimephales promelas* are available in the OECD toolbox (LMC ASIS 2014) and PAN database (Kegley et al. 2014). These values range from 0.2 to 35 mg/L. Considering the high volatility of the substance the original test reports should be assessed in order to ensure that the toxicity endpoints are based on the actual exposure concentrations. Most of these test reports cited in these databases could be retrieved (Anonymous 1990a, 1990b, 1997).

The study, from Anonymous (1990b), is a very thorough study where care is taken that the actual exposure concentrations were determined. It is a 96 h flow-through study with 30-34 days old juvenile *Pimephales promelas* with a wet weight of 49 to 177 mg and length of 15.4 to 21.8 mm. The test volume was replaced 50.4 times a day and the fresh test medium was generated directly before addition from a continuously generated near saturated solution. The test was performed in two tests with d-limonene from two different sources. The test concentrations were analysed every 24 hours and the toxicity endpoints are based on the average test concentrations of d-limonene ranging from 0.18 to 1.11 mg/L for test 1 and 0.25 to 1.89 for test two. The reported LC50 and EC50 for 96 hours of exposure are both 0.702 mg/L for test 1 and respectively 0.720 and 0.688 mg/L for test 2. These endpoints can be considered as reliable and will be used for classification purposes.

The analysis of test media showed the presence of additional substances (8–11%), not being the parent compound. The authors of the study concluded these substances to be hydrolysis products. We do not agree with hydrolysis conclusion, as d-limonene does not hydrolyse (see Table 14 in

section 5.1). For the hydrolysis of an alkene to occur, strong acidic conditions are required which is not the case here (Chemgapedia 2016). The additional substances may be either oxidation or hydration products. The molar mass of formed compounds was indicating an incorporation of a water molecule in the compound (the weight of the product is 18 mass units higher). This reaction only occurs directly after addition to water since the ratios between the parent and products is the same between stock and test solutions. In either case, formed metabolites are expected to be more polar than the parent compound, having lower toxicity (See Annex, 7.1). The test concentrations of d-limonene and hydration products are expressed as d-limonene. This is the only aquatic study that reports the presence of additional components other than the parent substance after exposure to water.

The study of Anonymous (1990a) is the same as the first test from Anonymous (1990b) and in the publication of Anonymous (1997) no data on d-limonene could be found. Therefore these references are not further discussed in this report. One more acute study with fish is mentioned in the OECD toolbox, the original reference of this could not be retrieved but in the toolbox was also mentioned that the exposure concentrations were not measured and therefore could already be concluded that the endpoint would not be reliable for the purpose of classification. In the public literature, other references (e.g. Anonymous (2003)) are available where d-limonene is tested as component in a commercial product. Since in the tests with these products the effects of other components cannot be excluded, these study are also not taken into account.

QSAR generated information
In addition to the studies above, the registrant has submitted a QSAR generated endpoint (KREATiS 2015d). This endpoint was calculated with the iSafeRat® Holistic HA-QSAR and was supplemented with a QMRF document. This QSAR resulted in a 96 h LC50 of 0.459 mg/L with confidence limit of 0.40 - 0.52 mg/L. In addition, the dossier submitter calculated QSAR based (neutral organics) LC50 values for fish with ECOSAR v1.11 available in EPIsuite. Based on the log Kow value of 4.38, LC50 values of 0.845 and 1.041 mg/L were estimated for fresh and saltwater fish respectively. These estimations are in the same order of magnitude as the experimental values.

5.4.1.2 Long-term toxicity to fish

The registrant has submitted an early life stage study on *Pimephales promelas*, this study is summarised below.

Reference	:	Anonymous (2015)	water solubility	:	4.0-5.7
type of study	:	Early life stages	species	:	Pimephales promelas, embryos
year of execution	:	2013	exposure duration	:	8 days (4 days post hatch)
GLP statement	:	Yes	nominal concn.	:	0, 2.5*%, 5.3%,11%, 32% and 48.6% of saturation
			Time weighted mean measured concn.		0, *, 0.059, 0.19, 0.37 and 0.67 (mg/L)
Guideline	:	OECD 212	dosing method	:	Renewal
test substance	:	d-limonene	acceptability	:	Reliable with restriction (Klimisch score of 2)
Purity	:	minimum p>99%	NOEC	:	0.059 mg/L (growth, measured) (EC10 between 0.37 and 0.67 mg/L)
			NOEC		0.19 mg/L (appearance)
			EC10		0.32 (survival)
			NOEC		0.37 (survival)

*The 2.5% solutions were not analysed as they were considered not relevant for the determination of the EC or NOEC values.

Embryos were used in an early life stages test to evaluate the sub-lethal effects of d-limonene. The substance was tested at the following nominal concentrations: 0 , 2.5%, 5.3%, 11.0%, 23.2% and 48.6% of a saturated solution of the test item in test medium. Time weighted average test concentrations were 0.059, 0.19, 0.37 and 0.67 mg/L for the nominal concentrations of 5.3%,

11.0%, 23.2% and 48.6%. The 2.5% solutions were not analysed, as they were not relevant for the determination of the EC or NOEC values. Thirty eggs (3 replicates of 10 eggs each) were exposed to each concentration of the test item and the control. The test vessels were 100 ml flasks sealed with PTFE coated screw caps. No aeration was used. The test medium was prepared by dilution of a saturated solution, renewal was performed at day 3 and 6 and test concentrations were analysed at start and termination of the experiment and before and after each renewal. Duration of the test was 8 days (4 days post hatch). The test was carried out at 23.5-25.3°C, a light: dark regime of 16:8 was maintained and each test concentration was tested in triplicate. Dissolved oxygen, temperature and pH were measured at the beginning, renewal and end of the test. The validity criteria specified in the test guidelines were met.

At termination of the test, the growth of the hatched larvae was determined and during the test, hatching, survival, abnormal appearance, and behaviour was observed and recorded daily. Actual measured test concentrations were used in the data analysis of NOEC, LOEC, LC10 and LC50 values. LC10 and LC50 values for the endpoint survival was calculated on the basis of the analytical results, by use of the standard procedure for Probit analysis and NOEC and LOEC values were estimated by use of Students T-test. Special considerations were taken considering the volatility of the test substance and endpoints are based on time weighted mean measured concentrations. The analyses of the test concentrations seem to be prone to uncertainty as test concentrations appear to increase between renewals. It is noted that due to the volatility and lipophilicity of the compound, d-limonene is a difficult substance to determine in the water phase and to assure a constant concentration during the exposure period. Therefore, the reported fluctuations in the test concentrations are considered acceptable but it reduces the reliability of the results and a Klimisch score of 2 (=reliable with restriction) is assigned.

The results show slight to moderate effect on the appearance and behaviour of fish at 0.37 mg/L and increased hatching rate at 0.67 mg/L (highest concentration). Statistically significant chronic effects were observed for survival rate at 0.67 mg/L (100% at mortality) and on the growth rate at the end of the test at, 0.19 and 0.37 mg/L. The NOEC and LOEC for growth rate are determined to be 0.059 and 0.19 mg/L, respectively. The effects on growth rate observed at 0.19 and 0.37 mg/L was less than 10% and more than 10% for 0.67 mg/L (the data did not allow the calculation of EC10 and EC50). For survival, an EC10 value of 0.32 mg/L was determined. A NOEC for survival was not given in the report but up to 0.37 mg/L the mortalities were not significantly different from the control, therefore the NOEC for survival is considered to be 0.37 mg/L. An overview of the observed mortalities at test termination is given in the table below.

Saturated solution of *d*-Limonene (%)	Total number of eggs			Day 8 - 2013.10.23											
				Malformed larvae			Total number of dead eggs and larvae			Total number of hatched larvae			Total alive		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Control	10	10	10	-	-	-	3	3	2	7	7	8	7	7	8
2.5	10	10	10	-	-	-	1	0	7	9	10	3	9	10	3
5.3	10	10	10	-	-	-	2	2	0	8	9	10	8	8	10
11	10	10	10	-	-	-	1	2	0	10	9	10	9	8	10
23.2	10	10	10	-	1	-	2	4	5	10	9	9	8	6	5
48.6	10	10	10	-	-	-	-	-	-	-	-	-	0	0	0

Tests performed according to OECD test guideline 210 are preferred because they cover more sensitive life stages and as such are considered to be more sensitive. Nevertheless, a study according to OECD test guideline 212 is also considered a chronic study because the CLP guidance

(section I.2.1.2) indicates that chronic studies can vary from 7 days to over 200 days. Furthermore, in the REACH guidance (R.7.8.4.1), OECD test guideline 212 is listed as a chronic study.
Where EC10 values are available, they are preferred over NOEC values for the same endpoint (ECHA 2015; OECD 2006). The $NOEC_{growth}$ of 0.059 mg/L was concluded with a statistically significant effect on growth of 4%, observed at two consecutive concentrations of 0.19 and 0.37 mg/L. The use of the $NOEC_{growth}$ of 0.059 mg/L is considered inappropriate for classification purposes as EC10 values are preferred over NOECs and for this endpoint, it is certain that the effects for growth at the 0.1 mg/L treshold will be limited and the EC10 will be higher than 0.37 mg/L. The next lowest value is the NOEC of 0.19 mg/L for appearance and behaviour but this data is only recorded for support of mortality data and it is not used for classification purposes. Therefore, the endpoint to use for classification purposes will be the EC10 of 0.32 mg/L for survival.

QSAR generated information
In addition to the laboratory study, the registrant has also submitted a QSAR generated endpoint (KREATiS 2015e). This endpoint was calculated with the iSafeRat® HA-QSAR for chronic aquatic toxicity and was supplemented with a QMRF document. This QSAR resulted in a 28 day NOEC of 0.080 mg/L with confidence limit of 0.056 – 0.11 mg/L. In addition, the dossier submitter calculated QSAR based (neutral organics) NOEC values for fish with ECOSAR v1.11 available in EPIsuite (US-EPA 2012). Based on the log Kow value of 4.38, NOECs of 0.073 and 0.34 mg/L were estimated for fresh and saltwater fish respectively (ECOSAR generates ChV values, these are converted to a NOEC by: NOEC = ChV/√2). The log Kow value used was within the domain of the freshwater QSAR. The QSAR for the saltwater fish is based on only two endpoints and its endpoint is therefore considered unreliable.

5.4.2 Aquatic invertebrates

5.4.2.1 Short-term toxicity to aquatic invertebrates

The registrant has submitted three study reports with acute toxicity tests on Daphnia magna. These studies are summarised below:

Reference	:	Betat (2013b)	water solubility	:	4.0 mg/L
type of study	:	Acute toxicity study	Species	:	*Daphnia magna*
year of execution	:	2012	exposure duration	:	48 hours
GLP statement	:	yes	nominal conc.	:	0.2 - 1.2 mg/L
Guideline	:	OECD 202, EU C.2	dosing method	:	Renewal
test substance	:	d-limonene	Acceptability	:	Reliable (Klimisch score of 1)
Purity	:	96.3%	48-h EC50	:	0.307 mg a.s./L (0.257-0.354, 95% c.i.) (mean measured)

Juveniles of D. magna were exposed to six test concentrations of d-limonene (0.2, 0.3, 0.4, 0.6, 0.8 and 1.2 mg/L). The test concentrations were prepared from a saturated solution of the test substance in water and the medium was renewed after 24 hours. The test was performed in 20 ml flasks sealed with screw caps. Test temperature was 20°C, light:dark regime was 16:8h and no aeration was performed during the test but dissolved oxygen was >60 % of the air saturation value. pH ranged from 6.86 to 7.68. Four replicates were performed per test concentration and control containing five daphnids. The control consisted of the same dilution water, test conditions and test organisms, but no test substance. Samples for chemical analysis of the test item were taken at t=0, t= 24 h (before after renewal) and t=48 h. Observations were made at 24 and 48 hours.
A 48-h EC50 for mobililty of 0.307 mg/L (0.257-0.354, 95% conf.int.) based on mean measured concentrations was reported. Special considerations were taken considering the volatility of the test

substance. The results are assigned an Ri of 1 (=reliable). The 48-h EC50 value from this study is used for classification purposes

Reference	:	Delpit (2014)	water solubility	:	5.46 mg/L
type of study	:	Acute toxicity study	Species	:	*Daphnia magna*
year of execution	:	2013	exposure duration	:	48 hours
GLP statement	:	yes	nominal conc.	:	0.2 - 1.2 mg/L
Guideline	:	OECD 202, EU C.2	dosing method	:	Renewal
test substance	:	d-limonene	Acceptability	:	Reliable (Klimisch score of 1)
Purity	:	95.5%	48-h EC50	:	0.456 mg a.s./L (0.353-0.551, 95% c.i.) (mean measured)

Juveniles of *D. magna* were exposed to six test concentrations of d-limonene (nominal: 0.5, 0.7, 1.0, 1.5, 2.1 and 3.0 mg/L). The test concentrations were prepared from a saturated solution of the test substance in water and the medium was renewed after 24 hours. The test was performed in 20 ml flasks sealed with screw caps. Test temperature was 20°C, light:dark regime was 16:8h and no aeration was performed during the test but dissolved oxygen was >3 mg/L. pH ranged from 7.52 to 8.31. Four replicates were performed per test concentration and control containing five daphnids. The control consisted of the same dilution water, test conditions and test organisms, but no test substance. Samples for chemical analysis of the test item were taken at t=0, t= 24 h (before after renewal) and t=48 h. Observations were made at 24 and 48 hours.
A 48-h EC50 for mobility of 0.456 mg/L (0.353-0.551, 95% conf.int.) based on mean measured concentrations was reported. Special considerations were taken considering the volatility of the test substance. The results are assigned an Ri of 1 (=reliable). The 48-h EC50 value from this study is used for classification purposes

Reference	:	Bjørnestad (2013)	water solubility	:	"very low"
type of study	:	Acute toxicity study	Species	:	*Daphnia magna*
year of execution	:	2013	exposure duration	:	48 hours
GLP statement	:	yes	nominal conc.	:	19.8 - 100% of saturation
Guideline	:	OECD 202, ISO 6341	dosing method	:	Renewal
test substance	:	d-limonene	Acceptability	:	Reliable (Klimisch score of 1)
Purity	:	>99%	48-h EC50	:	0.51 mg a.s./L (0.46-0.59, 95% c.i.) (mean measured)

Juveniles of D. magna were exposed to six test concentrations of d-limonene derived by dilution of a saturated stock solution. The stock solution was prepared by siphoning off the mid fraction of a solution with excess of the test compound (1 g/L). The nominal test concentrations were 19.8, 29.6, 44.4, 66.7 and 100% of the stock solution. The test medium was renewed after 24 hours. The test was performed in 42 ml flasks sealed with PTFE coated screw caps and a minor headspace. Test temperature was 21°C ± 0.8, light:dark regime was 16:8h and no aeration was performed during the test but dissolved oxygen saturation was 100 % in all tested concentrations. pH of the test solution was 7.8 ± 0.5. Four replicates were performed per test concentration and control containing five daphnids. The control consisted of the same dilution water, test conditions and test organisms, but no test substance. Samples for chemical analysis of the test item were taken at t=0, t= 24 h (before after renewal) and t=48 h. Observations were made at 24 and 48 hours.
A 48-h EC50 for mobility of 0.51 mg/L (0.46-0.59, 95% conf.int.) based on mean measured concentrations was reported. Special considerations were taken considering the volatility of the test substance. The results are assigned an Ri of 1 (=reliable). The 48-h EC50 value from this study is used for classification purposes
In addition to the reported studies from the registrant, one peer reviewed publication is available that presents toxicity data for d-limonene to *Daphnia magna* (Park et al. 2011). In the test 24 h old daphnids were exposed to d-limonene in glass tanks. The actual test concentrations were not monitored and the reported endpoint of 7.85 mg/L is based on nominal concentrations. A separate residue test was performed with a solution of 100 mg/L which was measured after 2 and 7 days.

This test could be used to determine time weighted average concentration but since the concentration in the residue test exceed the water solubility with a factor of 8 it is considered not representative for the reported endpoint. Because of the low water solubility and high vapour pressure of d-limonene, the reported EC50 is likely to be an underestimation of the actual toxicity of the compound and the endpoint is considered unreliable (Ri=3). The OECD toolbox and the PAN database (Kegley et al. 2014) both contain EC50 values for *Daphnia magna* (ranging from 0.275 to 69.6 mg/L). Considering the high volatility of the substance, the original test reports should be assessed in order to ensure that the toxicity endpoints are based on the actual exposure concentration. Where available, the original references were retrieved (Anonymous 1990b; May Passino and Smith 1987; Park et al. 2011).

The study, from Anonymous (1990b), is a very thorough study where care is taken that the actual exposure concentrations were determined. It is a 48 h flow-through study with <24 hours old *Daphnia magna*. The test volume of 200 ml and a temperature of 20 °C was replaced 50.4 times a day and the fresh test medium was generated directly before addition from a continuously generated near saturated solution. The test was performed in to tests with d-limonene from two different sources. The test concentrations were analysed every 24 hours and the toxicity endpoints are based on the average test concentrations of d-limonene ranging from 0.24 to 1.35 mg/L for test 1 and 0.29 to 1.63 for test two. For test 1, the reported LC50 for 48 hours of exposure is 0.924 mg/L. For test 2 an LC50 of 0.577 mg/L and an EC50 for mobility of 0.421 mg/L is reported. For the LC50 of the first test and EC50 of the second test should be noted that they are calculated as the geometric mean of the NOEC and LOEC since at the LOEC 100% effect was observed. Nevertheless, these endpoints should be considered as reliable (Ri=2) and will be used for classification purposes.

The analysis of test media showed the presence of additional substances (8–11%), not being the parent compound. The authors of the study concluded these substances to be hydrolysis products. We do not agree with this conclusion, as d-limonene does not hydrolyse (see section 5.1, Table 14). For the hydrolysis of an alkene to occur, strong acidic conditions are required which is not the case here (Chemgapedia 2016). In our view, the additional substances may be either oxidation or hydration products. The molar mass of formed compounds was indicating an incorporation of a water molecule in the compound (the weight of the product is 18 mass units higher). This reaction only occurs directly after addition to water since the ratios between the parent and products is the same between stock and test solutions. In either case, formed metabolites will be more polar than the parent compound, having lower toxicity (See section, 7.1). The test concentrations of d-limonene and hydration products are expressed as d-limonene.

The study of May Passino and Smith (1987) tested toxicity of d-limonene to *Daphnia magna* in a static test system and reported an EC50 of 69.6 mg/L based on nominal concentrations. Because of the low water solubility and high vapour pressure of d-limonene, the reported EC50 is likely to be an underestimation of the actual toxicity of the compound. Therefore the endpoint is considered to be unreliable and will not be used for classification purposes.

On the ECHA dissemination site one study (Author not disseminated 2007) is presented that is not available in the dossier nor has its endpoints been discussed in this report. The study is performed according to OECD test guideline 202 in a static test set-up. The test concentrations are only confirmed by analysis at the start of the test and the endpoints are based on nominal concentrations. The EC50 reported for 48 hours is 0.36 mg/L. Because of the low water solubility and high vapour pressure of d-limonene, the reported EC50 is likely to be an underestimation of the actual toxicity of the compound since it is no based on time-weighted-average concentrations. Therefore the endpoint is considered to be unreliable and will not be used for classification purposes.

QSAR generated information

In addition to the laboratory study, the registrant has also submitted a QSAR generated endpoint (KREATiS 2015c). This endpoint was calculated with the iSafeRat® Holistic HA-QSAR and was supplemented with a QMRF document. This QSAR resulted in a 48 h EC50 of 0.62 mg/L with

confidence limit of 0.55 – 0.69 mg/L. In addition, the dossier submitter calculated QSAR based (neutral organics) LC50 values for daphnids and mysids (saltwater) with ECOSAR v1.11 available in EPIsuite. Based on the log Kow value of 4.38, LC50 values of 0.577 and 0.154 mg/L were estimated for daphnids and mysids respectively. The log Kow value is within the domain of the QSARs (max log Kow of 6.4). The QSAR and experimental endpoints are in the same order of magnitude.

5.4.2.1 Long-term toxicity to aquatic invertebrates

The registrant has submitted a chronic toxicity test on Daphnia magna. This study is summarised below:

Reference	:	Kamper (2016a)	water solubility	:	4.0-5.7 mg/L
type of study	:	Reproduction toxicity study	Species	:	*Daphnia magna*
year of execution	:	2016	exposure duration	:	21 days
GLP statement	:	yes	nominal conc.	:	2.5 - 16% of saturation
Guideline	:	OECD 211	dosing method	:	Renewal
test substance	:	d-limonene	acceptability	:	Reliable (Klimisch score of 1)
Purity	:	>99%	NOEC	:	0.08 mg a.s./L
			EC10		0.153 mg a.s./L (0.083-0.0.222, 95% c.i.) (mean measured)

Juveniles of D. magna were exposed to five test concentrations of d-limonene derived by dilution of a saturated stock solution. The stock solution was prepared by siphoning off the mid fraction of a solution with excess of the test compound. The nominal test concentrations were 2.5, 4.0, 6.5, 10 and 16% of the stock solution. Ten daphnids (female <24 hours) were exposed to each test concentration and each animal was placed in an individual test flask of 50 ml that was thereafter sealed with a PTFE-coated screw cap. Renewal was performed every Monday, Wednesday and Friday by transferring the test animal to a new flask containing fresh prepared test solution. The animals were fed (algae) at each renewal. The test temperature was 19.9°C ± 0.1, light:dark regime was 16:8h and no aeration was performed during the test but dissolved oxygen saturation was 100 % in all tested concentrations. pH of the test solution was 7.8 ± 0.5. The control consisted of the same dilution water, test conditions and test organisms, but no test substance. Samples for chemical analysis of the test item were taken at start and termination of the test and at each renewal (before after renewal) from an additional test flask containing no daphnids and algae (feed). Observations were made at each renewal and at termination. The parameters monitored were: number of offspring, mortality of parents, time for first offspring, dead offspring. After submission of the study report, additional analysis was performed on the highest tested concentration (Kamper 2016b). The results of this analysis are also included here. Time weighted average test concentrations were 23, 50, 80, 173 and 288 µg/L. Mortality of the parents in the control was at most 10%. An EC10 of 157 µg/L and a NOEC of 80 µg/L were determined based on the number of life offspring. Special considerations were taken considering the volatility of the test substance and endpoints. The results are assigned an Ri of 1 (=reliable). The EC10 value from this study is used for classification purposes. It is scientifically preferred since it is based on interpolation of the concentration effect data while the NOEC is dependent of the test design.

QSAR generated information

In addition to the laboratory study, the registrant has also submitted a QSAR generated endpoint for *D. magna*(KREATiS 2015b). This endpoint was calculated with the iSafeRat® HA-QSAR for chronic aquatic toxicity and was supplemented with a QMRF document. This QSAR resulted in a 21 day NOEC of 0.050 mg/L with confidence limit of 0.035 – 0.070 mg/L. In addition, the dossier submitter calculated QSAR based (neutral organics) NOEC values for daphnids and mysids with

ECOSAR v1.11 available in EPIsuite (US-EPA 2012). Based on the log Kow value of 4.38, NOECs of 0.074 and 0.005 mg/L were estimated for daphnids and mysids respectively (ECOSAR generates ChV values, these are converted to a NOEC by: NOEC = ChV/√2). The log Kow value is within the domain of the QSARs (max log Kow of 8) but it should be noted that the QSAR for the mysids is based on only two endpoints and its endpoint is therefore considered unreliable. The QSAR and experimental endpoints for *D. magna* are in the same order of magnitude.

5.4.3 Algae and aquatic plants

The registrant has submitted two algal toxicity tests. These studies are summarised below:

Reference	:	Betat (2013a)	water solubility	:	3.4-5.7 mg/L
type of study	:	Growth inhibition study	Species	:	*Pseudokirchneriella subcapitata*
year of execution	:	2012	exposure duration	:	72 hours
GLP statement	:	yes	nominal conc.	:	0.2 - 2.0 mg/L
Guideline	:	OECD 201, EU C.3	dosing method	:	Static
test substance	:	d-limonene	acceptability	:	Reliable with restrictions (Klimisch score of 2)
Purity	:	96.3%	72 h EC50	:	0.32 mg a.s./L (0.291-0.355, 95% c.i.)
			72 h EC10		0.174 mg a.s./L (0.137-0.202, 95% c.i.) (growth rate, mean measured)

Algal cells of *Pseudokirchneriella subcapitata* were exposed to an aqueous solution of d-limonene at nominal concentrations of 0.2, 0.3, 0.5, 0.8, 1.3 and 2.0 mg/L. The stock solution was prepared by sampling the bottom and mid fraction of a solution with excess of the test compound which was thereafter directly diluted to obtain the test solutions. Inoculation occurred with such an amount of algae that the initial concentration in the test vessels was 5 x 10^3 cells/ml. The test flasks were sealed with a fritted glass stopper. Incubation occurred under continuous shaking. The test temperature was 23.0 - 23.2°C, mean light intensity was 5474 lux and did not vary more than 15%. pH of the test solution ranged from 7.68 - 10.21, variation was observed most at the end of the test. The control consisted of the same dilution water, test conditions and test organisms, but no test substance. Samples for chemical analysis of the test item were taken at start and termination of the test from all concentrations and biotic and abiotic control. Cell density was counted daily and increased 114 times within 72 hours. Geometric mean measured test concentrations were 0.134, 0.189, 0.306, 0.536 and 0.938 mg/L for the nominal concentrations of 0.3, 0.5, 0.8, 1.3 and 2.0 mg/L. For the nominal concentration of 0.2 mg/L, at start, the concentrations was already below the detection limit (LOD) and an actual concentration could not be determined. For the nominal concentration of 0.3 and 0.5 mg/L the concentration was also below the LOD in the biotic systems. Because of this, it is unclear if the mean concentrations are a good representative for the actual exposure concentration since it is unclear how the actual decline in the exposure concentrations develops. This lowers the reliability of the derived endpoints especially because the endpoints are at the level of these test concentration. Endpoints are based on the mean measured concentrations and results for the lowest test concentrations were not included. For growth rate an EC50 of 0.320 mg/L and an EC10 of 0.174 mg/L was derived. For yield the EC50 and EC10 were 0.214 and 0.149 mg/L respectively. The results are assigned an Ri of 2 (=reliable with restrictions) because of the high variation in the pH at the end of the test and the uncertainty in the lower test concentrations. The EC50 and EC10 value for growth rate from this study are used for classification purposes.

Reference	:	Seierø (2015)	water solubility	:	4.0-5.7 mg/L
type of study	:	Growth inhibition study	Species	:	*Pseudokirchneriella subcapitata*
year of execution	:	2014	exposure duration	:	48 and 72 hours
GLP statement	:	yes	nominal conc.	:	7, 10, 16, 24, 35, 53 and 80% of saturation
			Time weighted mean measured conc. (48 h)		0.09, 0.14, 0.23 and 0.30 mg/L for 7 - 24%*

			Time weighted mean measured conc. (72 h)		0.05, 0.08, 0.12 and 0.17 mg/L for 7 - 24%*
Guideline	:	OECD 201, ISO 8692	dosing method	:	Static
test substance	:	d-limonene	acceptability	:	48 h: Reliable with restrictions (Klimisch score of 2) 72 h: Unreliable (Klimisch score of 3)
Purity	:	>99%	48 h EC50	:	0.25 mg a.s./L (0.24-0.27, 95% c.i.)
			48 h EC10		0.14 mg a.s./L (0.13-0.16, 95% c.i.)
			72 h EC50		0.15 mg a.s./L (0.15-0.16, 95% c.i.)
			72 h EC10		0.09 mg a.s./L (0.08-0.09, 95% c.i.)
					(for all: growth rate, mean measured)

*The 35, 53 and 80% solutions were not analysed because at 24% already 100% effect was observed.

Algal cells of *Pseudokirchneriella subcapitata* were exposed to an aqueous solution of d-limonene at nominal concentrations of 7, 10, 16, 24, 35, 53 and 80% of a saturated solution of the test item in test medium. The stock solution was prepared by sampling the mid fraction of a solution with excess of the test compound which was thereafter directly diluted to obtain the test solutions. The initial concentration of algae in the test vessels was 2.5 x 10^3 cells/ml, this amount was chosen to enable exponential growth throughout the incubation period. The test was carried out with minor headspace in 42 mL glass vials sealed with PTFE coated caps. Incubation occurred under continuous shaking. The test temperature was 22.3 ± 0.1°C, mean light intensity was 60-120 µmol/m^2/sec. pH of the test solution ranged from 7.9 - 9.4, variation was observed most at the end of the test. The control consisted of the same dilution water, test conditions and test organisms, but no test substance. Samples for chemical analysis of the test item were taken every 24 hours from all concentrations and control, frozen (-20°C) and sent frozen to an external laboratory for analysis where they were kept refrigerated until analysis. Analysis was performed via headspace analysis and detection with GC-MS. The storage conditions were checked for difference between frozen storage or refrigerated storage, no significant differences in analytical results were found between the two methods of storage. Cell density was counted daily and control growth rate was 1.7 per day over 72 hours. Geometric mean measured test concentrations were determined only for the nominal concentrations of 7, 10, 16 and 24%, because at 24% already 100% effect was observed, higher test concentrations were not analysed. These geometric mean concentrations were 0.9, 0.14, 0.23 and 0.30 mg/L over 0-48 hours and 0.05, 0.08, 0.12 and 0.17 mg/L over 0-72 hours respectively. For all concentration apart from 24%, the concentration at 72 hours was below the LOD, these were included in the calculations of the geometric mean as 0.005 mg/L. An overview of all measured concentrations is given in Table 21 below.

Table 21: Results of the chemical analysis (mg/L) of subsamples from the test solutions without algae and the calculated geometric mean concentrations (mg/L).

nominal test concentration (% of saturated stock solution)	t = 0 h	t = 24 h	t = 48 h	t = 72 h	Geometric mean 0-48 hours	Geometric mean 0-72 hours
Control	< 0.010	-	-	<0.010	-	<0.010
7%	0.08	0.12	0.06	<0.010	0.09	0.05
10%	0.13	0.15	0.12	<0.010	0.14	0.08
16%	0.20	0.29	0.17	<0.010	0.23	0.12
24%	0.36	0.31	0.24	0.010	0.30	0.17

Reported endpoints are based on the mean measured concentrations. Growth inhibition and growth were calculated for each test concentration relative for the control without addition of test item. ECx values for growth and yield were determined using the computer program TOXEDO and NOEC and LOEC values were estimated by the computer program Dunnett's procedure as the highest tested concentration at which no significant inhibition was observed. For growth rate an 72 hour EC50 of 0.15 mg/L and an EC10 of 0.09 mg/L was derived. For yield the EC50 and EC10 were

0.09 and 0.05 mg/L respectively. As all tested concentrations caused significant inhibition on the yield, no NOEC could be determined.
Because the concentrations in the 72 hour samples were below the limit of detection, it was recommended in the report to use the 48 hour endpoints rather than the 72 hour endpoints. It is however not explained what would have caused this decrease in detectability. The test concentrations seems not to decrease over the first 48 hours, with even increases (up to 50%) for most test concentrations between t=0 and t=24. A rapid decline was then observed over the last 24 hours. These fluctuations in concentration are inconsistent and it is strange that no evaporation seems to occur over the first period followed by a massive evaporation over the last period. This is also not explained in the report and deviations from the test protocol that could explain this observation are also not given in the report. The decline over the last 24 hours indicate significant changes in the test conditions that does indeed indicate that endpoints derived over 72 hours of exposure are not reliable. Where it concerns the proposed use of endpoints derived over 48 hours, the analytical result of the test concentrations show a high fluctuation in the test concentrations over this period, for example and increase for the 16% test solution from 0.20 mg/L to 0.29 mg/L. This suggests a high uncertainty in the analysis of the samples taken at different time points. This shows that the analysis of the test concentrations is prone to uncertainty but due to the volatility and lipophilicity of the compound, it is a difficult substance to determine in the water phase and the results are considered best achievable. Therefore, the reported fluctuations in the test concentrations are considered acceptable but it reduces the reliability to Ri 2 (= Reliable with restrictions). In this view together with the fact that the endpoints from the study of Seierø (2015) are in the same order of magnitude, the use of results of 48 hours exposure for classification purposes is supported.
For additional endpoints, the OECD toolbox was checked for additional toxicological data, experimental NOECs for *Pseudokirchneriella subcapitata* are given of <0.05 and <1.5 mg/L. The original test report (Anonymous 1990b) of these values has been assessed. These endpoints are based on a static test where after 24 hours all of the test compound had dissipated form the test solution but a dissipation curve is not available. Although no significant effects were observed at any test concentration, time weighted average test concentrations cannot be determined. It is also unclear how the endpoints in the OECD toolbox were derived from the test results. Therefore these endpoints are considered unreliable (Ri=3) and they will not be used for classification purposes.

QSAR generated information
In addition to the laboratory study, the registrant has also submitted a QSAR generated endpoint for algae (KREATiS 2015a). This endpoint was calculated with the iSafeRat® Holistic HA-QSAR and was supplemented with a QMRF document. This QSAR resulted in a 72 hour EC50 for growth rate of 0.50 mg/L with confidence limit of 0.42 – 0.60 mg/L. In addition, the dossier submitter calculated QSAR based (neutral organics) LC50 values for algae with ECOSAR v1.11 available in EPIsuite (US-EPA 2012). Based on the log Kow value of 4.38, an LC50 values of 1.07 mg/L and a NOEC of 0.32 mg/L were estimated for algae (ECOSAR generates ChV values, these are converted to a NOEC by: NOEC = ChV/√2). The log Kow value is within the domain of the QSARs (max log Kow of 8.0). The QSAR and experimental endpoints are in the same order of magnitude.

5.4.4 Other aquatic organisms (including sediment)

A few studies are available where d-limonene has been tested on *Aedes aegypti* and/or *Aedes albopictus* (Cheng et al. 2009; Giatropoulos et al. 2012; Liu et al. 2013; Park et al. 2011; Santos et al. 2011; Silva et al. 2008). In these studies that were water-only tests, the exposure concentrations exceed the water solubility of d-limonene and the endpoints are based on nominal concentrations. Furthermore several of these tests were performed in paper or polypropylene cups which could have caused sorption of the test substance making it even less available in the water phase. The reported

endpoints are in the range of 19.8 to 50 mg/L but considering the shortcomings of the studies, they are likely to be an underestimation of the actual toxicity of d-limonene to these mosquito's. The endpoints will therefore not be used for classification purposes and are not included in the summary table.

5.5 Comparison with criteria for environmental hazards (sections 5.1 – 5.4)

CLP - Acute aquatic hazards

For d-limonene there are reliable acute data for all three trophic levels. The lowest endpoint for fish is 0.695 mg/L (this is the geometric mean of 0.702 and 0.688 mg/L which are values based on the same endpoint determined in tests performed at comparable exposure scenarios), for invertebrates this is 0.42 mg/L for *Daphnia magna* (this is the geometric mean of 0.307, 0.456, 0.51 and 0.42 mg/L which are values based on the same endpoint determined in studies performed at comparable exposure scenarios) and for algae this is 0.25 mg/L for *P. subcapitata* (the lowest available endpoint for 48 hours of exposure). These values are considered preferable over the QSAR generated endpoints. The lowest value of 0.25 mg/|L is lower than 1.0 mg/L and d-limonene does fulfil the criteria for classification as Aquatic Acute Cat. 1. An M factor of 1 is warranted based on the EC50 of 0.25 mg/L.

CLP - Chronic aquatic hazards

The substance has a high potential for bioaccumulation and is considered rapidly degradable.

Experimental chronic toxicity endpoints are available for all three trophic levels. The relevant chronic endpoints for fish is 0.32 mg/L for *Pimephales promelas*, for invertebrtates this is 0.153 mg/L for *Daphnia magna* and for algae this is 0.14 mg/L for *P. subcapitata* (the lowest available endpoint for 48 hours of exposure). The lowest values of 0.14 mg/L is between 0.1 and 1 mg/L and the substance is considered rapidly biodegradable. Based on the criteria set out in CLP, Annex I, section 4.1, Table 4.1.0(b) (ii), d-limonene fulfils the criteria for classification as Aquatic Chronic 3.

5.6 Conclusions on classification and labelling for environmental hazards (sections 5.1 – 5.4)

Conclusions on classification and labelling for environmental hazards of d-limonene.

	CLP regulation	
	Classification	M-factor
Resulting harmonised classification.	Aquatic Acute category 1. H400: Very toxic to aquatic life. Aquatic Chronic category 3 H412: Harmful to aquatic life with long lasting effects	M = 1

RAC evaluation of aquatic hazards (acute and chronic)

Summary of the Dossier Submitter's proposal

d-Limonene is currently listed in Annex VI of the CLP Regulation with Aquatic Acute 1 and Aquatic Chronic 1 classifications without any M-factors. The DS proposed to classify d-limonene as Aquatic Acute 1 with an M-factor of 1 and Aquatic Chronic 3. There were reliable acute data for all three trophic levels. The lowest endpoint for fish was 0.695 mg/L (geometric mean of 0.702 and 0.688 mg/L), for invertebrates 0.42 mg/L for *Daphnia magna* (geometric mean of 0.307, 0.456, 0.51 and 0.42 mg/L) and for algae 0.25 mg/L for *Pseudokirchneriella subcapitata.* The acute toxicity values were in range 0.1 < LC_{50} ≤ 1 mg/L leading to an M-factor of 1. The substance had a high potential for bioaccumulation and was considered rapidly degradable. Experimental chronic toxicity endpoints were available for all three trophic levels. The lowest value of 0.14 mg/L for algae *Pseudokirchneriella subcapitata* was between 0.1 and 1 mg/L. Thus, d-limonene fulfilled the criteria for classification as Aquatic Chronic 3.

Degradation

No experimental data was available on the stability of the substance. d-Limonene was not expected to undergo hydrolysis since it lacks functional groups that hydrolyse under environmental conditions. The Henry's law constant was determined to be 1.30 × 10^3 Pa m^3/mol and d-limonene is expected to partition from water and soil to air. In air, it will be degraded rapidly (the DT_{100} was determined to be 33.6 hours) by interaction with hydroxyl and nitrate radicals. d-Limonene is not expected to undergo photolytic degradation.

There was a biodegradation study available performed according to OECD TG 301B following GLP with adaptations for volatile substances (sealed vessel). The test method adaptation was in line with the latest adopted OECD TG 310 (Ready Biodegradability – CO_2 in sealed vessels (Headspace Test)). The nominal test concentration was 10 mg/L. After 28 days the biodegradation was 71.4 %. On this basis, it was concluded that d-limonene was readily biodegradable, fulfilling the 10-day window criterion as after 10 days 60.6 % degradation was achieved. In addition, one of the ready biodegradability studies available at the ECHA dissemination site was used as supportive evidence. The key study in the REACH dossier was an OECD TG 301D Closed Bottle test. After 28 days the biodegradation was 80 %. The dossier submitter was informed by the registrant that the substance tested was dipentene consisting of 48.4 % d-limonene; 20.6 % β-phellandrene; 9.8 % alpha-terpinene; 5.8 % γ-terpinene and 4.5 % terpinolene. According to the DS, these substances have a structural resemblance and will be similarly biodegradable. However, given the complexity of the mixture consisting of five different components, the actual extent of the biodegradation of d-limonene is not known. Therefore, this study is only used as supportive evidence.

In a simulation study in natural waters, similar to OECD TG 309, degradation of alpha-terpinene, p-cymene and d-limonene (components of QRD 460) was studied in natural lake water. The test substances were tested individually. d-Limonene volatilized from the natural water test systems rapidly with a DT_{50} of 3.0 and DT_{90} of 10.0 hours. The trapping solution did show the presence of the test substance in one case but no degradants were detected. Furthermore, no degradants were detected in the water. Thus, rapid escape (fugacity via volatility) appeared to be the predominant pathway for d-limonene in natural waters. The DS

also presented QSAR calculations done with the BIOWIN v4.10 QSAR contained within EPI Suite™ version 4.11 (US-EPA 2012). The overall conclusion of the six models used was that d-limonene was not readily biodegradable.

The DS concluded that d-limonene was considered rapidly degradable for classification purposes based on the ready biodegradation test result of 71.4 % degradation in 28 days. This was supported by the results from the key study in the REACH registration dossier.

Bioaccumulation

An experimentally determined log K_{ow} of 4.85 was reported in the DAR but this value was considered unreliable by the DS. Preference was given to the value of 4.38 at 37 °C and at pH 7.2 from a study equivalent or similar to OECD TG 117 using nine compounds of known log K_{ow} (ranging from 1.1 to 4.1) and of similar chemical structure to that of terpenoids as standards in the determination of log K_{ow} values. The HPLC method is generally not preferred over experimental determination of log K_{ow} values. However, the standards chosen were especially selected for terpenoids and p-cymene, which has a comparable structure to d-limonene, was also included in the set of standards. Since the reference compounds are similar to terpenoids, this value was preferred over the value used in the DAR. The log K_{ow} of 4.38 being higher than the classification criteria cut-off 4, indicates that the substance has a high potential for bioaccumulation. There is no fish bioconcentration study available.

Aquatic toxicity

Table. *Reliable information on aquatic toxicity of d-limonene*

Test method, reference	Test species	Result (mg/L)	QSARs for d-limonene
Fish			
99 % d-limonene Short-term fish toxicity ASTM E729 method, flow-through[3] Anonymous (1990b)	*Pimephales promelas*	Test 1[1]: 96-h LC_{50}: 0.702 96-h EC_{50}: 0.702 (mobility) Test 2[1]: 96-h LC_{50}: 0.720 96-h EC_{50}: 0.688 (mobility) based on measured average concentrations	96-h LC_{50}: 0.459 mg/L (iSafeRAT® Holistic HA-QSAR) LC_{50}*: 0.845 mg/L (freshwater fish); 1.041 mg/L (saltwater fish) (ECOSAR v.1.11)
> 99 % d-limonene Chronic toxicity to fish OECD TG 212, GLP, semi-static, renewal every third day [3,6] exposure duration: 8 days (4 days post hatch) Anonymous (2015)	*Pimephales promelas*	8-d NOEC growth: 0.059 (EC_{10} between 0.37 and 0.67 mg/L, could not be statistically determined) 8-d NOEC hatching: 0.37 8-d NOEC behavior: 0.19 8-d EC_{10} survival: 0.32 8-d NOEC survival: 0.37 8-d LC_{50} for survival: 0.41 based on time weighted mean measured concentrations	28-day NOEC: 0.080 mg/L (iSafeRAT® Holistic HA-QSAR) NOEC*: 0.073 (freshwater fish) (ECOSAR v.1.11)
Invertebrates			
96.3% d-limonene Short-term invertebrate toxicity, OECD TG 202,	*Daphnia magna*	48-h EC_{50}: 0.307 (mobility) mean measured 82-110 %	48-h EC_{50}: 0.62 mg/L (iSafeRAT® Holistic HA-QSAR)

GLP, semi-static, renewal after 24 hours.[3,5] Betat (2013b)		of nom.[2]	LC_{50}*: 0.577 mg/L, daphnids; LC_{50}*: 0.154 mg/L, saltwater mysids (ECOSAR v.1.11)
99.5 % d-limonene Short-term invertebrate toxicity, OECD TG 202, GLP, semistatic, renewal after 24 hours.[3,5] Delpit (2014)	*Daphnia magna*	48-h EC_{50}: 0.456 (mobility) based on mean measured concentration	
> 99 % d-limonene Short-term invertebrate toxicity, OECD TG 202, GLP, semi-static, renewal after 24 hours.[3,6] Bjørnestad (2013)	*Daphnia magna*	48-h EC_{50}: 0.51 (mobility) based on mean measured concentrations	
87 % d-limonene Short-term invertebrate toxicity according to ASTM E729 method, GLP not reported, flow-through[3] Anonymous (1990b)	*Daphnia magna*	Test 1: 48-h LC_{50}: 0.924[7] (mortality) Test 2: 48-h LC_{50}: 0.577 (mortality) 48-h EC_{50}: 0.421[7] (mobility) based on mean measured concentrations	
> 99 % d-limonene Chronic invertebrate toxicity, OECD TG 211, semi-static, renewal three times a week, GLP.[3,6] Kamper (2016b, 2016a)	*Daphnia magna*	21-day EC_{10}: 0.153 21-day NOEC: 0.080[2] based on mean measured concentrations	21-day NOEC: 0.050 mg/L (iSafeRAT® Holistic HA-QSAR) NOEC*: 0.074 mg/L, daphnids (ECOSAR v.1.11)
Algae/Aquatic plants			
96.3 % d-limonene Aquatic toxicity to algae according to OECD TG 201, GLP, static[3,8] Betat (2013a)	*Pseudokirchneriella subcapitata*	72-h ErC_{50}: 0.32 72-h ErC_{10}: 0.174 based on geometric mean measured concentrations[2]	72-h ErC_{50} 0.50 mg/L (iSafeRAT® Holistic HA-QSAR) LC_{50}*: 1.07 mg/L; NOEC*: 0.32 mg/L (ECOSAR v.1.11)
Aquatic toxicity to algae according to OECD TG 201, GLP, static[3,6] Seierø (2015)	*Pseudokirchneriella subcapitata*	**48-h ErC_{50}: 0.25** **48-h ErC_{10}: 0.14** 72-h results not reliable based on geometric mean measured concentrations	

[1] d-limonene from two different sources
[2] REACH Registration Dossier
[3] dilution of saturated solution of the test item added into the test medium
[5] flasks with screw caps

[6] PTFE coated screw caps

[7] geometric mean of the NOEC and LOEC since at the LOEC 100 % effect was observed

[8] fritted glass stopper
[*] neutral organics, based on log K_{ow} 4.38

Acute Aquatic toxicity

There was only one reliable fish study available for d-limonene. The study was performed in two tests with d-limonene from two different sources. The 96-hour LC_{50} and EC_{50} were both 0.702 mg/l for test 1 and 0.720 and 0.688 mg/L for test 2 based on average measured test concentrations. The analysis of test media showed a presence of additional substances (8-11 %). The DS thought that the additional substances may be either oxidation or hydration products which are expected to be more polar than the parent compound, having lower toxicity. The test concentrations of d-limonene and hydration products were expressed as d-limonene.

There were four reliable acute *Daphnia* studies available. In the three studies performed according to the OECD TG 202 and following GLP, the 48-hour EC_{50}s for mobility were 0.307 mg/L, 0.456 mg/L and 0.51 mg/L, respectively, based on mean measured concentrations. In addition, there was a *Daphnia* study performed according to the ASTM E729 Method. Data was derived from two tests with d-limonene from two different sources. For test 1, the reported LC_{50} for 48 hours of exposure is 0.924 mg/L. For test 2, an LC_{50} of 0.577 mg/L and an EC_{50} for mobility of 0.421 mg/L was reported. The LC_{50} of the first test and EC_{50} of the second test were calculated as the geometric mean of the NOEC and LOEC since at the LOEC 100 % effect was observed. The analysis of test media showed the presence of additional substances (8-11 %), not being the parent compound, similarly to the acute fish test.

There were two *Pseudokirchneriella subcapitata* algae tests available. Both tests were performed according to the OECD TG 201 following GLP. In the first test, an ErC_{50} of 0.320 mg/L was derived. Endpoints were based on the mean measured concentrations and results for the lowest test concentrations were not included. For the nominal concentration of 0.2 mg/L, at start, the concentrations were already below the detection limit (LOD) and an actual concentration could not be determined. For the nominal concentration of 0.3 and 0.5 mg/L the concentration was also below the LOD in the biotic systems. Due to this, it is unclear if the mean concentrations were a good representative for the actual exposure concentration since it is unclear how the actual decline in the exposure concentrations develops. To conclude, there were uncertainties because of the high variation in the pH at the end of the test and the uncertainty in the lower test concentrations. Despite the uncertainties the DS considered the results reliable for classification purposes.

In the second *Pseudokirchneriella subcapitata* test, a 72-hour ErC_{50} of 0.15 mg/L and an ErC_{10} of 0.09 mg/L was derived. Cell density was reduced from that required in the OECD TG 201 to achieve exponential growth. All validation criteria were fulfilled in the test. The nominal test concentrations were 0, 7 %, 10 %, 16 %, 24 %, 35 %, 53 % and 80 % of a saturated solution of the test item in test medium. As the test item was volatile, a closed test system with a minor headspace was used in the test. As the chemical analyses showed a major decrease in the test concentrations from the 48-hour sample to the 72-hour sample, the statistical calculation of the effect concentrations was calculated based on geometric mean concentrations covering analysed concentrations both from the 0-48-hour and 0-72-hour exposure period, and on the nominal test concentrations. The difference between the 48-hour and 72-hour endpoint values was expected to be due to the significant decrease in

detectability of the test item in the period 48-72 hours and not to an increased toxicity of the test substance with time. It was therefore recommended to use the 48-hour end-points. Due to the volatility and lipophilicity of the compound, it was a difficult substance to determine in the water phase and the results were considered as the best achievable. The geometric mean measured 48-hour E_rC_{50} was 0.25 mg/L.

The DS concluded that d-limonene warranted classification Aquatic Acute 1, M = 1, based on the E_rC_{50} 0.25 mg/L derived from OECD TG 201 using the algae *Pseudokirchneriella subcapitata*.

Chronic Aquatic toxicity

In a fish test performed according to the OECD TG 212, *Pimephales promelas* embryos were used in an early life stages test to evaluate the sub-lethal effects of d-limonene. The DS considered this test as chronic although it did not cover the sensitive life stages as does OECD TG 210. Special considerations were taken considering the volatility of the substance. The endpoints were based on time weighted mean measured concentrations. The 8-day NOEC for growth rate were determined to be 0.059 mg/L. The data did not allow the calculation of EC_{10} and EC_{50} for growth rate. For survival, an 8-day EC_{10} value of 0.32 mg/L was determined. A 8-day NOEC for survival was not given in the report but up to test concentrations of 0.37 mg/L the mortalities were not significantly different from the control. Therefore, the 8-day NOEC for survival was considered to be 0.37 mg/L. The DS chose the 8-day EC_{10} of 0.32 mg/L for chronic classification.

In a GLP OECD TG 211 *Daphnia* reproduction toxicity study, time weighted average test concentrations were 0.023, 0.050, 0.080, 0.173 and 0.288 mg/L. A 21-day mean measured EC_{10} of 0.153 mg/L and NOEC of 0.080 mg/L were determined based on the number of live offspring.

There were two algae studies available on *Pseudokirchneriella subcapitata*, both following GLP and OECD TG 201 (see Acute toxicity for details). In a 72-hour study, a mean measured EC_{10} of 0.174 mg/L for growth rate was determined. In the other study, the 72-hour results were not considered reliable. The mean measured 48-hour EC_{10} for growth rate was 0.14 mg/L.

The DS concluded that d-Limonene warranted classification Aquatic Chronic 3 based on the E_rC_{10} 0.14 mg/L derived from OECD TG 201 using the algae *Pseudokirchneriella subcapitata*.

Additional information – in-depth analysis by RAC

QSARs for acute and chronic toxicity

The REACH registrant had submitted QSAR generated endpoints for fish, *Daphnia* and algae. The endpoints were calculated (iSafeRAT® Holistic HA-QSAR) and were supplemented with the QSAR Model Reporting Format (QMRF) document. In addition, the DS calculated QSAR based (neutral organics) toxicity values for fish, daphnids and algae based on the log K_{ow} of 4.38 with ECOSAR v.1.11 available in EPISuite. The log K_{ow} is within the domain of the QSARs. The QSARs based only on two endpoints (chronic saltwater fish, chronic mysids) are considered unreliable and were not included in the summary table above. The DS concluded that the estimations on acute fish, *Daphnia* and algae toxicity and on chronic *Daphnia* and algae toxicity were in the same order of magnitude as the experimental values.

Comments received during public consultation

Two Member States (MS) supported the classification proposed by the Dossier Submitter. Two MSs supported the Aquatic Acute 1, M = 1 classification. They also supported the conclusion that d-limonene is rapidly degradable and potentially bioaccumulative but they were uncertain about the long-term hazard classification. The other MS proposed classification as Aquatic Chronic 2 based on the NOEC for growth of 0.059 mg/L from the *Pimephales promelas* test. One MS wanted more information to assess the reliability of the OECD TG 301B study. They also wanted more details on the QSAR predictions. Depending on the details presented d-limonene might not be considered rapidly degradable and the default position of not rapidly degradable might apply. They also questioned the use of geometric mean from 2 data points only. They also brought up that the OECD TG 212 test used as a basis for chronic classification was a short-term test and invited the DS to consider a surrogate approach which would result in classification as Aquatic Chronic 1, M = 1, which is also supported by the QSARs. They recommended to check the reliability of the QSARs. They also had questions concerning the validity of the algae tests.

In their response, the DS explained their choice to use the OECD TG 212 to evaluate chronic toxicity. They also gave details on the selection of endpoint from the chronic fish test. The observed effects for mortality and effects on growth at lower concentrations than the EC_{10} for survival were lower than a 10 % effect. They considered that the EC_{10} values for mortality and growth will be higher than the EC_{10} for survival and, therefore, the EC_{10} for survival was preferred.

The OECD TG 212 test also reported an 8-day LC_{50} for survival of 0.41 mg/L and the DS would keep that as a key endpoint for acute aquatic toxicity to fish. The DS agreed that the use of the surrogate method would lead to Aquatic Chronic 1, M=1 classification. In addition, more data on the OECD TG 301B study was presented in the RCOM although the original study report contains limited data on the validity criteria. The output of the BioWin 4.10 calculations for d-limonene were also given. More information provided for chronic aquatic toxicity in fish showed that the iSafeRat® Holistic HA-QSAR QSAR provided had a domain between log water solubility (in log (mol/L)) of -5.56 to -0.32 and covered the class of non-polar narcotic compounds. The training set consisted of data for six fish species and 26 chemicals. d-Limonene fell within the domain. Explanations concerning the validity of the algae tests were given.

Assessment and comparison with the classification criteria

Degradation

In a study performed according to OECD TG 301B with adaptations for volatile substances (sealed vessel), the biodegradation was 71.4 % after 28 days. The 10-day window criteria were fulfilled. Seven fragrance ingredients were tested showing degradation from 2.9 to 85.3 % after 28 days. The biodegradation for days 3, 7, 10, 14, 16, 21, 24 and 28 were 25.5 %, 29.8 %, 60.3 %, 58.8 %, 64.7 %, 71.1 %, 62.6 % and 71.4 %, respectively. The confidence limits were 68.3-74.5 %. Consequently, d-limonene was considered to be readily biodegradable. The study report lacks information needed for checking study validity *e.g.* information on replicates and CO_2 evolution in the inoculum blank at the end of the test. The study by King (1992) 'The Biodegradability of Perfume Ingredients in the Sealed Vessel Test' refers to a study report published in Chemosphere, Vol. 23, No.4, pp 507-524 (1991) by Birch,

R.R. and Fletcher, R.J for development and validation of the method used. The publication is titled 'The Application of Dissolved Inorganic Carbon Measurements to the Study of Aerobic Biodegradability'. The article is about developing a test that is essentially the same as the Sturm CO_2 production test (OECD TG 301B) but with greater simplicity of the technique and the high precision of the data. It does not include any validity criteria as such. This study has been referenced and used as the basis of the OECD TG 310. RAC is of the opinion that this adds to the reliability of the King study even if the validity information is not available. RAC considers the test reliable.

The BIOWIN v.4.0 QSAR estimation predicted that d-limonene was not readily biodegradable. The estimation used a combination of two models Biowin3 (ultimate survey model) and Biowin 5 (MITI linear model). The Biowin 3 model estimate was 'weeks' and the Biowin 5 model estimate was 'not readily degradable'. This method is based on the application of Bayesian analysis to ready biodegradation data for US Premanufacture Notification (PMN) chemicals, derived collectively from all six OECD301 test methods plus OECD TG 310. The linear and nonlinear MITI models (Biowin 5 and 6) also predict ready biodegradability, but for degradation in the OECD TG 301C test only, and based exclusively on data from the Chemicals Evaluation and Research Institute Japan (CERIJ) database (http://www.cerij.or.jp/ceri_en/otoiawase/otoiawase_menu.html). d-Limonene is not in the training set of either of the models.

RAC is of the opinion that there is no reason to doubt the reliability of QSAR estimates but experimental data is preferred when existing and reliable. RAC realises that the study report on the ready biodegradability test does not contain all information needed for validity checking but on the other hand the referred publication strengthens the reliability. Therefore, RAC considers d-limonene as rapidly degradable for classification purposes.

Bioaccumulation

There is no fish bioconcentration study available and therefore RAC agrees to use the log K_{ow} of 4.38 for assessing bioaccumulation potential. The surface tension of d-limonene is 28.5 mN/m and 27.3 mN at 25°C indicating that the substance might be surface active. However, there is neither a hydrophobic nor a hydrophilic group in the structure of the substance and hence it seems unlikely that d-limonene would display surface-active properties. In this case, the HPLC method used to derive the log K_{ow} 4.36 can be considered suitable. The Log K_{ow} value being higher than the classification criteria cut-off of 4, indicates that the substance has a high potential for bioaccumulation.

Acute Aquatic toxicity

There was one reliable acute fish study available. The 96-hour LC_{50} and EC_{50} (mobility) values ranged from 0.688 and 0.720 mg/L in the two tests included in the study. In the response to the PC comments, the DS informed about an LC_{50} for survival of 0.41 mg/L from the OECD TG 212 test. RAC notes that this was an 8 d study.

There were four reliable *Daphnia* studies available. The lowest 48-hour EC_{50} was 0.307 mg/L for mobility. The DS had proposed to use a geometric mean 0.42 mg/L of the four test results available. Three of the studies were semi-static with renewal alter 24 hours. One of the studies was a flow-through study. RAC is of the opinion that the conditions in these tests are different and consequently the geometric mean should not be used.

Regarding the Seierø (2015) algae test, RAC agrees to use the 48-hour mean measured

concentrations from the 72-hour test because the difference between endpoint values was expected to be due to significant decrease in detectability of the test item in the period 48-72 hours and not to an increased toxicity of the test substance with time. Consequently, the lowest ErC_{50} for algae was 0.25 mg/L.

Consequently, there were acute toxicity data on three trophic levels, the lowest value being an ErC_{50} value of 0.25 mg/L for algae that forms the basis for the aquatic acute classification proposal.

Chronic Aquatic toxicity

An OECD TG 212 (Fish, Short-term Toxicity Test on Embryo and Sac-fry Stages) test was available for fathead minnow. The test duration was 8 days, with exposure from 4 days post hatch and the 8-day EC_{10} for survival was 0.32 mg/L. The test guideline notes that only tests incorporating all stages of the life-cycle of fish are generally able to give an accurate estimate of the chronic toxicity of chemicals to fish and that any reduced exposure with respect to life stages may reduce the sensitivity and thus underestimate the chronic toxicity. It was therefore expected that the embryo and sac-fry test would be less sensitive than the Full Early Life Stage test (OECD TG 210), particularly with respect to chemicals with high lipophilicity (log P_{ow} > 4) and chemicals with a specific mode of action. However, smaller differences in sensitivity between the two tests would be expected for chemicals with a non-specific, narcotic mode of action. d-Limonene has a log K_{ow} of 4.38 and when comparing the experimental toxicity test results to QSAR estimates, it seems that d-limonene has a narcotic mode of action. RAC concludes that data from this test can be taken into account for assessing chronic toxicity in fish.

The 21-day EC_{10} of 0.153 mg/L for *Daphnia magna* is the only reliable chronic toxicity value for invertebrates.

Regarding algae test data, RAC agrees to use the 48-hour mean measured concentrations from the 72-hour test because the difference between endpoint values was expected to be due to significant decrease in detectability of the test item in the period 48-72 hours and not to an increased toxicity of the test substance with time. Consequently, the lowest ErC_{10} for algae was 0.14 mg/L.

Consequently, there are experimental data for three trophic levels and QSARs are used only as supportive evidence. In case any chronic test data on fish toxicity becomes available, this classification might have to be revisited.

RAC acknowledges that the use of EC_{10} results is preferable to the use of NOECs for determining chronic aquatic toxicity and that reliable EC_{10} results are available for all three trophic levels, the lowest of which is the EC_{10} of 0.14 mg/L in algae.

Conclusion

RAC concludes to classify d-limonene with Aquatic Acute 1; H400 (M = 1), based on the lowest acute toxicity value of 0.25 mg/L for algae (*Pseudokirchneriella subcapitata*) and with Aquatic Chronic 3; H412 based on the lowest chronic toxicity value for algae (*Pseudokirchneriella subcapitata*) of 0.14 mg/L for a rapidly degradable substance.
Overall, RAC agrees with the DS that d-limonene warrants classification as **Aquatic Acute 1; H400 (M = 1) and Aquatic Chronic 3; H412.**

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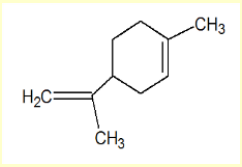
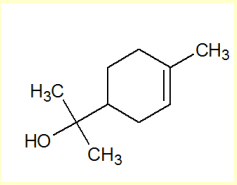
7 ANNEX

7.1 Comparison of aquatic acute toxicity data for d-limonene and identified substance in the Anonymous (1990b) study.

	d-limonene (purity 67% in test water)	**Possible hydrate product: p-menth-3-en-8-ol (alpha Terpineol) CAS number 98-55-5**
Structure		
Physical chemical properties	LogKow = 4.38 Water solubility = 12.3 mg/L Molecular weight = 136.23	LogKow = 3.28 Water solubility = 360.6 Molecular weight = 154.25
Acute Toxicity : Experimental lowest experimental value (mg/L)		
Fish	0.695	70* (geometric average)
Daphnia	0.307	73* (nm)
Algae	0.15	68* (TWA)
Acute Toxicity : Estimated toxicity data, ECOSAR (mg/L)		
Fish	0.845	8.068
Daphnia	0.577	5.180
Algae	1.07	6.416

*ECHA dissemination site: aquatic toxicity tests are carried out with Terpineol multi (a multi-constituent substance with alpha-Terpineol and gamma-Terpineol as constituents).

ECOSAR predications are provided when experimental data is not available.