Committee for Risk Assessment

RAC

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

Adopted

15 March 2019

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CLH-O-0000001412-86-275/F

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonised classification and labelling (CLH) of:

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

EC Number: **227-813-5**

CAS Number: **5989-27-5**

The proposal was submitted by **Netherlands** and received by RAC on **19 April 2018.**

In this opinion, all classification and labelling elements are given in accordance with the CLP Regulation.

PROCESS FOR ADOPTION OF THE OPINION

Netherlands has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **21 May 2018**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **20 July 2018**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Bogusław Barański**

Co-Rapporteur, appointed by RAC: **Riitta Leinonen**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The RAC opinion on the proposed harmonised classification and labelling was adopted on **15 March 2019** by **consensus**.

Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATE	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	601-029-00-7	(*R*)-p-mentha-1,8-diene; d-limonene	227-813-5	5989-27-5	Flam. Liq. 3 Skin Irrit. 2 Skin Sens. 1 Aquatic Acute 1 Aquatic Chronic 1	H226 H315 H317 H400 H410	GHS02 GHS07 GHS09 Wng	H226 H315 H317 H410			Note C
Dossier submitters proposal	TBD	(*R*)-p-mentha-1,8-diene; d-limonene	227-813-5	5989-27-5	**Retain** Aquatic Acute 1 **Add** Asp. Tox. 1 **Modify** Skin Sens. 1B Aquatic Chronic 3	**Retain** H400 **Add** H304 **Modify** H317 H412	**Retain** GHS07 GHS09 **Add** GHS08 **Modify** Dgr	**Retain** H410 **Add** H304 **Modify** H317		**Add** M=1	
RAC opinion	TBD	(*R*)-p-mentha-1,8-diene; d-limonene	227-813-5	5989-27-5	**Retain** Flam. Liq. 3 Aquatic Acute 1 **Add** Asp. Tox. 1 **Modify** Skin Sens. 1B Aquatic Chronic 3	**Retain** H226 H400 **Add** H304 **Modify** H317 H412	**Retain** GHS02 GHS07 GHS09 **Add** GHS08 **Modify** Dgr	**Retain** H226 H317 H410 **Add** H304		**Add** M=1	
Resulting Annex VI entry if agreed by COM	TBD	(*R*)-p-mentha-1,8-diene; d-limonene	227-813-5	5989-27-5	Flam. Liq. 3 Skin Irrit. 2 Skin Sens. 1B Asp. Tox. 1 Aquatic Acute 1 Aquatic Chronic 3	H226 H315 H317 H304 H400 H412	GHS02 GHS07 GHS08 GHS09 Dgr	H226 H315 H317 H304 H410		M=1	

GROUNDS FOR ADOPTION OF THE OPINION

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.

HUMAN HEALTH HAZARD EVALUATION

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.

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**.

ENVIRONMENTAL HAZARD EVALUATION

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 $0.1 < LC_{50} \leq 1$ 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)	*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 behaviour: 0.19 8-d EC_{10} survival: 0.32 8-d NOEC survival: 0.37 8-d LC_{50} for survival: 0.41	28-day NOEC: 0.080 mg/L (iSafeRAT® Holistic HA-QSAR) NOEC*: 0.073 (freshwater fish) (ECOSAR v.1.11)

Test method, reference	Test species	Result (mg/L)	QSARs for d-limonene
Anonymous (2015)		based on time weighted mean measured concentrations	
Invertebrates			
96.3 % d-limonene Short-term invertebrate toxicity, OECD TG 202, GLP, semi-static, renewal after 24 hours.[3],[5] Betat (2013b)	*Daphnia magna*	48-h EC_{50}: 0.307 (mobility) mean measured 82-110 % of nom.[2]	48-h EC_{50}: 0.62 mg/L (iSafeRAT® Holistic HA-QSAR) 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)

Test method, reference	Test species	Result (mg/L)	QSARs for d-limonene
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	LC_{50}*: 1.07 mg/L; NOEC*: 0.32 mg/L (ECOSAR v.1.11)

[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. An 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*.

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 (Biowin5 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.**

Additional references

Scientific Committee on Consumer Safety (SCCS) Opinion on fragrance allergens in cosmetic products (SCCS/1459/11; adopted at its 15th plenary meeting of 26-27 June 2012)

Christensson J B, Johansson S, Hagvall L, Jonsson C, Borje A, Karlberg A T. Limonene hydroperoxide analogues differ in allergenic activity. Contact Dermatitis 2008:59: 344-352.

ANNEXES:

Annex 1 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in 'RAC boxes'.

Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).