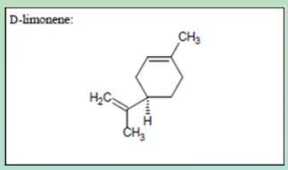
Long term ecotoxicity testing of limonene for hazard classification: not such a lemon after all

Thomas P [1)] ; Balk F [2)] ; van Bergen H [3)] ; Jenner K [4)] ; Kamper A [5)]; Gimeno S [6)]

1 CEHTRA, 38080 L'Isle d'Abeau, France
2 Royal Haskoning DHV, 6500 AD Nijmegen, The Netherlands
3 Para-Celsus Concept, 3984 CT Odijk, The Netherlands
4 Givaudan UK Limited, TN24 0LT Ashford, United Kingdom
5 DHI, DK-2970 Hørsholm, Denmark
6 Firmenich Belgium SA, 1348 Louvain-La-Neuve, Belgium

Introduction

Limonene is a stereoisomeric substance taking its name from lemon, which dominates the composition of the essential oils of citrus fruits. When synthesised as dipentene it is a racemate. However, in botanical sources it is present as the D- or L-enantiomer in Natural Complex Substances (NCSs) obtained at anything from traces in some plant extracts to over ninety percent in orange oil. These oils are commercially used as fragrance and flavour ingredients in a wide range of applications (cosmetic products, food manufacture, fragrance perfumery, botanical insecticide, household cleaning products, etc). Therefore, it is predominantly released back to the environment after use. **The racemate and both D- and L-limonene received a harmonised classification under Annex VI of the EU C&L legislation as Aquatic acute category 1: (Very toxic to aquatic life) and Aquatic chronic category 1: (Very toxic to aquatic life with long-lasting effects)**. Both classifications have a severe impact on storage, handling and transport requirements of limonene and the many (natural) complex substances and fragrance and flavour mixtures that contain even small amounts of it. The existing classification is a result of a limited data set notably for chronic endpoints. The chronic category 1 classification is extrapolated from the acute category 1 toxicity, log Kow >4 and erroneously assumed not-rapid biodegradability of the substance. As limonene is a narcotic substance its Chronic 1 assignment was expected to be conservative.
Due to doubts on the chronic classification and the consequences for labelling, storage, handling and transport, **long term aquatic ecotoxicity studies were conducted** to obtain a solid basis for the environmental classification.



Materials and Methods

Despite the existence of a guidance document, considered the ecotoxicologists' Bible for testing difficult substances (OECD, 2000), the chronic studies were fraught with difficulties. Limonene is a highly volatile, hydrophobic, low solubility (Table 1), rapidly biodegradable, non-polar narcotic substance and testing such materials under chronic conditions adds levels of complexity beyond those found when testing under acute conditions: **All studies were performed using closed conditions** (sealed with PTFE coated screw caps with minimum headspace) but as the substance is readily biodegradable, further contingencies had to be made to **prevent build up of biofilm on glassware** used in the study. Flow-through conditions would have been preferred but the low water solubility concentration and the **desire not to use solvents or dispersants to prevent physical effects meant that daphnids and fish were tested under semi-static conditions**. Rapid accumulation and metabolism of the test substance meant that regular replacement was necessary and thus **losses could occur during preparation** of new test solutions. Moreover, **low testing concentrations, adsorption on glassware in the test or during analysis together with the necessity to find a low LOQ** also posed a problem in the studies confounding measurement of No Effect Concentrations. The obligatorily static test design for algae and the closed semi-static regime for fish meant that more **limited test durations needed to be considered**.

Property	Value
Vapour pressure	200 Pa
Water solubility	The solubility of R(+)-limonene in water at 298.15 K is 5 to 12 mg/L
Partition coefficient n-octanol/water	log = 4.38-4.85

Table 1: Physicochemical properties of d-limonene

Results and Discussion

In the **algae study**, concentrations of limonene were followed regularly to monitor loss over time. As expected losses became significant when the algae population reached a concentration high enough to metabolise the substance rapidly. This occurred only at 72 hours. Prior to this the concentrations of test substance were maintained and therefore the result was based on measured concentrations over a **48 h period** as growth in the control already met the OECD validity criteria at this time.

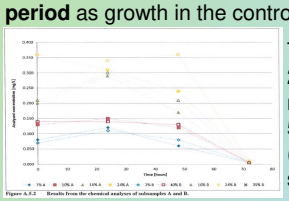


Fig 1: algae test concentrations. Note significant loss at 72h

The test concentrations in the **daphnid reprotoxicity test** following OECD 211 were even more complicated to maintain and the study needed to be repeated several times under increasingly rigorous test conditions including: 50 ml **PTFE screw capped flasks for testing with minimized headspace** (approx. 3 ml); **replacement with fresh perfectly clean glassware** at solution renewal; **sample freezing** and maintaining samples on dry ice; sampling into headspace vials allowing **direct analysis** in an autosampler and double sampling as a sanity checker for analytical results.

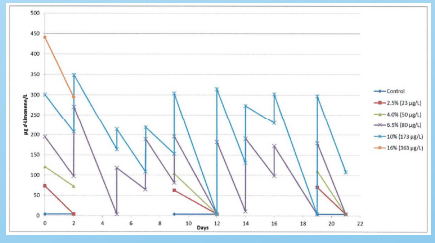


Fig 2: daphnid chronic test concentrations over the test

The **fish test** was performed according to OECD 212 (short term toxicity test on embryo and sac fry stages) on ***Pimephales promelas*** for **technical and ethical reasons**. A full Early Life Stage test following OECD 210 would have been technically challenging after swim up as the regular addition of food, maintenance of test concentrations of a highly volatile, biodegradable test substance in the presence of biofilm all contribute to technical difficulties that would make final results difficult to interpret. The test was **performed using a closed system under semi-static conditions with 95% solution replacement on two occasions during the 8 d test**. Validation criteria were met. The test concentrations fluctuated due to analytical issues but **valid EC10 results were obtained using geometric means of measured concentrations (considered scientifically more pertinent than NOECs).**

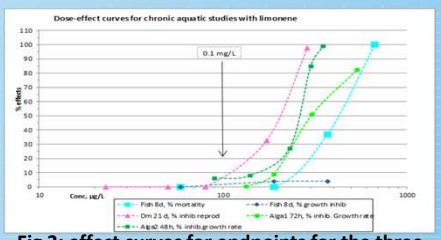


Fig 3: effect curves for endpoints for the three chronic tests

Aquatic toxicity to algae according to OECD guideline 201	72 h ErC50 = 0.32 72 h ErC10 = 0.174 48 h ErC10 = 0.150	*P. subcapitata*	Betat (2013b)
Chronic invertebrate toxicity according to OECD test guideline 211	21 day EC10 = 0.153	*Daphnia magna*	Kamper (2016a 2016b)
Chronic toxicity to fish according to OECD test guideline 212	EC10s 0.37 to 0.67 NOEC growth = 0.059 NOEC hatching = 0.37 NOEC behaviour = 0.19 EC10 survival = 0.32	*Pimephales promelas*	Petersen (2015)

Table 2: overview of chronic test effect concentrations

Conclusions

Despite the technical difficulties associated with testing a substance with multiple difficult properties, the final result was worth the many months of effort necessary from a Classification & Labelling perspective as **the substance was found to be considerably less chronically toxic than originally believed based on the base set data**. Taking into account the **ready biodegradability** of the substance which was affirmed during the course of this work and the conclusion of the chronic studies performed EC10s >0.1 and <=1 mg/L), a **chronic 3 classification has been proposed replacing the Chronic 1 currently in force** based on results from acute studies. The subsequent regulatory procedure to implement the classification in the EU regulations is currently ongoing.

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References

1. OECD (2000) Guidance Document No 23. Aquatic toxicity testing of difficult substances and mixtures

Poster No. WE388

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