

# *European Commission*



**Combined Draft Renewal Assessment Report prepared according to  
Regulation (EC) N° 1107/2009  
and  
Proposal for Harmonised Classification and Labelling (CLH Report)  
according to Regulation (EC) N° 1272/2008**

**EUGENOL**  
**2-methoxy-4-(prop-2-en-1-yl)phenol**  
**Volume 3 – B.9 (AS)**

Rapporteur Member State : SPAIN  
Co-Rapporteur Member State : GREECE

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## Version History

<b>When</b>	<b>What</b>
July 2022	Initial DAR-RMS Spain
November 2022	DRAR after CoRMS and Applicant comments
February 2023	RAR updated after EFSA CoCh received on 24/01/2023

The RMS is the author of the Assessment Report. The Assessment Report is based on the validation by the RMS, and the verification during the EFSA peer-review process, of the information submitted by the Applicant in the dossier, including the Applicant's assessments provided in the summary dossier. As a consequence, data and information including assessments and conclusions, validated and verified by the RMS experts, may be taken from the applicant's (summary) dossier and included as such or adapted/modified by the RMS in the Assessment Report. For reasons of efficiency, the Assessment Report should include the information validated/verified by the RMS, without detailing which elements have been taken or modified from the Applicant's assessment. As the Applicant's summary dossier is published, the experts, interested parties, and the public may compare both documents for getting details on which elements of the Applicant's dossier have been validated/verified and which ones have been modified by the RMS. Nevertheless, the views and conclusions of the RMS should always be clearly and transparently reported; the conclusions from the applicant should be included as an Applicant's statement for every single study reported at study level; and the RMS should justify the final assessment for each endpoint in all cases, indicating in a clear way the Applicant's assessment and the RMS reasons for supporting or not the view of the Applicant.

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## **B.9. ECOTOXICOLOGY DATA**

### **B.9.1. EFFECTS ON BIRDS AND OTHER TERRESTRIAL VERTEBRATES**

#### **B.9.1.1. Effects on birds**

##### ***B.9.1.1.1. Acute oral toxicity to Birds***

One acute oral avian toxicity study with Mevalone was previously evaluated as part of the EU review for the EU inclusion of eugenol (DAR, Volume 3, Annex B.9, 2011, B.9.1.1, ██████████ 2007). The data on the representative formulation, Mevalone, are sufficient to address the active substance data requirement. The full summary is provided in Vol. 3 CP Study B.9.1.1.1/01. A  $LC_{50} > 10000$  mg product/kg bw.(corresponding to  $>320$  mg eugenol/kg bw based on the nominal eugenol content of 3.2% w/w) was calculated.

##### ***B.9.1.1.2. Short-term dietary toxicity to birds***

This study type is no longer required for the risk assessment according to the current EFSA Guidance Document on Risk Assessment for Birds and Mammals (EFSA Journal 2009; 7(12): 1438). No further data are required. Nevertheless, one acute short-term avian dietary toxicity study was previously evaluated as part of the EU review for the EU inclusion of eugenol (DAR, Volume 3, Annex B.9, 2011, B.9.1.2, ██████████., 2007), giving an acute dietary  $LC_{50}$  value  $>5866$  mg product/kg bw/day (corresponding to  $>187.7$  mg eugenol/kg bw, based on the nominal eugenol content of 3.2% w/w). However, according with the EFSA Conclusion of the active substance Eugenol (EFSA Journal, 2012 ; 10(11) :2914) this study « was considered no valid within the peer review of thymol and therefore, for consistency was not used for the short-term risk assessment for eugenol ».

##### ***B.9.1.1.3. Sub-chronic toxicity and reproduction to birds***

It is noted that a data gap for further information to address the long-term risk to birds was identified during the first EU review of eugenol (EFSA Journal 2012; 10(11):2914). Further information on natural background levels of eugenol was provided as confirmatory data, but this was not considered sufficient by EFSA to enable a comparison between the natural background exposure and the exposure due to the use of the plant protection product (EFSA Supporting publication 2017:EN-1165).

For the renewal of eugenol, a waiver is requested for long-term reproductive toxicity data to birds as further vertebrate testing is not justified. Additional weight of evidence to support this waiver is presented below. Eugenol is a naturally occurring terpene oil found in a wide variety of plant species from 0.02 to 180000 mg/kg, for blueberry and clove respectively (Table B.9.1.1.3-1).

**Table B.9.1.1.3-1. Concentration of eugenol in vegetables, herbs and spices<sup>1</sup>**

Raw agricultural commodity	Plant part	Concentration of eugenol (ppm)
Clove	Flower	180 000
Pimento pepper	Fruit	36 000
Betel pepper	Leaf	17 850
Sweet basil	Leaf	8 575
Pimento (allspice)	Leaf	8 348
Carrot	Seed	7 000
Holy Basil	Leaf	4 200 – 4 970
Cinnamon	Bark	3 520
Bayleaf	Leaf	1 335
Sweet marjoram	Plant	1 152
Rockrose	Leaf	1 050
Hyssop	Leaf	443
Lesser galangal	Rhizome	400
Common violet	Flower	357
Mace	Seed	320
Oregano	Shoot	55-125
Spanish thyme	Leaf	0-21
<i>Micromeria congesta</i> – mint family	Leaf	5-15
Lavender	Plant	9
Hyacinth	Flower	4.6
French rose	Flower	4
Smooth licorice	Root	1
Blueberry	Fruit	0.02

Tan et al., (2012<sup>2</sup>) also conducted a literature review on occurrence of eugenol and its metabolite methyl-eugenol (ME) in plants. The metabolite can be found over 450 species of plants in essential oils from leaves, roots, stems, flowers, or whole plant extracts. The large number of families involved (Asteraceae, Apiaceae, Lamiaceae, Lauraceae, Aristolochiaceae, Rutaceae, Myrtaceae, Poaceae, Cupressaceae, Euphorbiaceae and Zingiberaceae) indicates that biosynthesis of ME evolved independently in many of the Plantae orders and families. The ME content varies greatly within and between species as well as within and between the plant families (i.e. 68 species possess ME content between 20 and 90% in essential oils). Eugenol and methyl-eugenol can be found in 13 species of grass family of Poaceae or Gramineae (for example, in *Lolium perenne*, methyl-eugenol yielded 16.6% of essential oils and eugenol 24.1%). These plant species involve ME in their chemical defence against pathogens and/or insect herbivores.

In accordance with Regulation (EU) No 283/2013, a test for the effects on reproduction in birds is currently requested if adult birds or nest sites are likely to be exposed during the breeding season. Following field application of the representative formulated product, Mevalone, initial environmental exposure of eugenol will decline rapidly in relation to the applied dose due to volatilisation and degradation. It is observed that the DT<sub>50</sub> in soil for eugenol is less than one day and the DT<sub>50</sub> in air obtained from the Atkinson model is 1.975 hours (Please, see Vol. 3 CA B.8).

Consequently, the duration of exposure under typical conditions will be very limited, particularly in relation to background levels of eugenol in the environment. This is also confirmed by the results of the residue trials conducted with Mevalone on grapevines and apples (please see Vol. 3 CA B.7.3 for further details). A total of 11 trials in grapes were conducted in Northern EU countries (Austria, Germany and Northern France) and in Southern EU countries (Spain, Portugal and Italy) in 2006 and 2020. All 2020 trials were conducted according to the critical GAP for the renewal and are therefore relevant to support the use of Mevalone in the EU. In the 2020 season trials in grapes, residues of eugenol were not detected in the untreated control samples and not detected or detected up to 0.02 mg/kg in the treated samples. All residues of eugenol in grapes had declined to not detectable by 1 day after the last application. Furthermore, a total of 6 trials in apples were conducted in Northern EU countries (Austria, Germany and Northern France) and in Southern EU countries (Spain, Southern France and Italy) in 2020 with an LOQ of 0.01 mg/kg. All trials were conducted according to the critical GAP

<sup>1</sup> Dr Duke's phytochemical and ethnobotanical databases, <http://www.ars-grin.gov/duke/highchem.html>

<sup>2</sup> Methyl eugenol: Its occurrence, distribution, and role in nature, especially in relation to insect behavior and pollination.

for the renewal and are therefore relevant to support the use of Mevalone in the EU. No residues of eugenol were detected at or above the LOQ of 0.01 mg/kg in any of the treated samples even on the day of the last application of Mevalone according to the critical GAP.

This indicates that even the acute exposure will be significantly less than that estimated by the shortcut value (SV). There is thus a clear pattern of exposure with very low acute levels and very short duration so that the long-term residue burden resulting from dietary exposure after application of Mevalone will be very limited.

Following treatment, elevated residues of eugenol in bird diet would be present for an extremely short period of time (a matter of hours) over the reproductive period of birds. It is considered that, given the potential for natural exposure to background levels in the diet, especially for herbivores, the exposure following a spray treatment would be negligible and does not merit the sacrifice of vertebrate animals. In contrast, the standard avian reproduction study (OECD test 206) involves at least 20 weeks of continued dietary exposure to the product. The exposure conditions of this test would bear no relation whatsoever to the potential exposure to eugenol following application of Mevalone to grapevine and pome fruit. There is thus a clear pattern of exposure with very short duration so that the long-term residue burden resulting from dietary exposure after application of Mevalone will be very limited with peaks of exposure that can be addressed with the acute toxicity data.

Eugenol is of low acute toxicity to birds (please see Vol. 3 CP B.9.1.1, Table 9.1.1-1). Two studies with the product Mevalone were conducted to address the acute and short-term toxicity. In the avian acute toxicity study with Mevalone and bobwhite quail (Vol. 3 CP Mevalone B.9.1.1) the acute oral LD<sub>50</sub> value was >10000 mg product/kg bw (corresponding to >320 mg eugenol/kg bw based on the nominal eugenol content of 3.2% w/w). In an 8-day dietary toxicity study (please see eugenol DAR, Volume 3, Annex B.9, 2011, B.9.1.2) with Mevalone there were no deaths or reductions in feed consumption or body weight at the maximum dose tested. The dietary LD<sub>50</sub> value was equivalent to 5866 mg product/kg bw/day (corresponding to > 187.7 mg eugenol/kg bw based on the nominal eugenol content of 3.2% w/w). It is also important to note no deaths or reductions in feed consumption or body weight at the maximum dose tested will occurred in short-term study with Mevalone on birds. As it can be expected nominal concentrations in diet were not maintained, likely due to high volatility of eugenol. However, according with the EFSA Conclusion of the active substance Eugenol (EFSA Journal, 2012 ; 10(11) :2914) this study « was considered no valid within the peer review of thymol and therefore, for consistency was not used for the short-term risk assessment for eugenol ». It should be noted that this study type is no longer required for the risk assessment.

Given the characteristics of the active substance, it is highly unlikely that a short-lived substance would result in any effects on reproduction. In the interests of minimising vertebrate testing, it is not justified to conduct a new reproductive avian toxicity study for an active substance that is ubiquitous in the environment and degrades rapidly following application as a plant protection product, and of known low acute oral avian toxicity. As stated in Section 4.3 of the EFSA Guidance Document on Risk Assessment for Birds and Mammals (EFSA Journal 2009; 7 (12): 1438), the lowest of the acute LD<sub>50</sub>/10 and the lowest NOAEL from avian reproduction studies should be used for the long-term screening assessment. Therefore, in light of all above information, the Applicant considers that the long-term risk assessment provided using the surrogate endpoint LD<sub>50</sub>/10 for a natural occurrence substance, with low persistence in the environment and low toxicity to birds is conservative enough and no chronic test on birds would therefore be necessary.

#### **RMS comments:**

No long-term toxicity data is available for the active substance eugenol or formulation Mevalone. Furthermore, the short-term study was considered as no valid according with the EFSA Conclusion of Eugenol. Therefore, a data gap for « further information to address the short-term and long-term risk to birds to insectivorous birds » was identified during the first EU review of eugenol (EFSA Journal 2012; 10(11):2914).

Since short-term study is no longer required for the risk assessment according to the current EFSA Guidance Document on Risk Assessment for Birds and Mammals (EFSA Journal 2009; 7(12): 1438), no further data are necessary to address short-term risk to birds.

The applicant has requested a waiver for long-term reproductive toxicity data to birds based on a weight of evidence (see above).

The applicant has also presented data of residues of eugenol in a range of plants other than grape or pome (0.02 – 180000 µg/kg, in plant tissue, see Table B.7.1.1.3-1). The data was sourced from “Dr Duke’s phytochemical and ethnobotanical databases” (<https://phytochem.nal.usda.gov/phytochem/search>). The database is an *ad-*

*hoc* inventory of agriculturally and medicinally relevant plant species and information regarding their phytochemical constituents. The data presented indicates that exposure can be expected when feeding on a range of plants.

However, there are significant uncertainties regarding the reliability of these data: The data do not provided a systematic assessment of the incidence of eugenol in plants. In particular, it does not identify how frequently the substances do not occur in plants relevant for the proposed use scenarios, which would result in no background exposure. The data are focused on agricultural and medicinal plants and read across to other relevant plant species has not been supported (for example grasses, acting as a food source in crop adjacent areas). Similarly the reported residues are stated for a range of plant tissues, residues are not necessarily evenly distributed within a plant so the quoted values may not reflect a diet unless composed of the specific plant tissues sampled in the underlying studies.

The range of estimated residues indicates that the dietary exposure, assuming 100% consumption of the food items, would be between 0.02 – 180000 mg/kg for eugenol. In most cases this would result in background exposure greater the predicted exposure following the proposed use.

This data were provided by the applicant during the confirmatory data requirement to address the long-term risk to insectivorous birds (EFSA Supporting publication 2017:EN-1165). The RMS (UK) concluded that :

*“The data from “Dr Duke’s phytochemical and ethnobotanical databases” indicates that eugenol and methyl eugenol are present in a range of plants. And based on the reported residues under some circumstances background exposure could exceed the predicted exposure based on the proposed use. However, the deficiencies identified above mean that this information is not sufficient to establish a background exposure for birds. The confirmatory data requirements have therefore not been met, and it cannot be concluded that background exposure will exceed the exposure based on the proposed use”.*

Furthermore, 11 residue trials are available for grapevines and 6 for apples. Five of the residue trials on grapevines were conducted in 2006 (Bailey, 2007 and 2008). These studies have been evaluated by the Residues section (Vol. 3 CA B.7.3.1). The study by Bailey (2008) included one residue trial conducted in Spain and it was not cover by intended GAP since only three applications were made. Furthermore, the study has been considered not to be acceptable by Residues Section. However, in the study by Bailey (2007) four residue trials were performed (2 in Spain and 2 in Italy) and they were conducted according with the proposed GAP. The study was accepted by Residues Section, but only 2 residue trials (one in Spain and one in Italy) were considered, due to the proximity of the trials, and the other two residue trials must be considered as replicates.

Two studies with 6 residues trials each on grapes (Chadwick, 2021a) and pomes (Chadwick, 2021b) on SEU and CEU countries were performed. The studies cover the GAP and were accepted by RMS. Residues of eugenol were not detected in at or above the LOQ of 0.01 mg/kg in any of the treated samples.

However, the residue trials data are relevant specifically to consideration of frugivorous birds. The conclusions for herbivorous birds and insectivorous birds are unaffected as no long-term endpoint are available. It should be noted that the data gap identified in Eugenol EFSA Conclusion (EFSA Journal 2012) was : «further consider the short-term and long-term risk to insectivorous birds. Further information on the background levels of eugenol in the environment might be useful for this purpose.». Information on natural background levels of eugenol was provided as confirmatory data (Eugenol Addendum – Confirmatory Data, August 2016). The RMS (UK) concluded that «... *the confirmatory data requirement are not met, as it cannot be established that background exposure is greater or similar to predicted exposures...*». EFSA agreed with RMS conclusion.

The applicant states that due to the high volatilisation ( $V_p = 2.7$  Pa at 20° C) and the rapid degradation ( $DT_{50}$  soil 1 < day) initial environmental exposure of eugenol will decline rapidly. However, these data are not sufficient to demonstrate a negligible exposure. Furthermore, the confirmatory data information on natural background levels of eugenol was not considered sufficient by EFSA to enable a comparison between the natural background exposure and the exposure due to the use of the plant protection product (EFSA Supporting publication 2017:EN-1165). Therefore, under RMS opinion a negligible exposure has not been demonstrated.

Moreover, the applicant proposed the use of  $LD_{50}/10$  as surrogate of the long-term endpoint for risk assessment since no avian reproduction study is available and a low acute toxicity was observed ( $LD_{50} > 320$  mg eugenol/kg bw). Birds and Mammals EFSA Guidance (2009) considers the use of the lowest acute  $LD_{50}/10$  and the NOAEL from avian reproduction studies for long-term risk assessment. However, no long-term endpoint on birds for eugenol is available for comparison. Therefore, the avian reproductive risk could not be considered addressed.

Under RMS opinion the weight of evidence submitted by the applicant is not sufficient to address the avian long term risk. Consequently, **further information should be submitted.**

The Co-RMS agrees with the RMS that the weight of evidence presented by the applicant is not sufficient to support a waiver for long-term reproductive toxicity data for birds due to the below uncertainties :

- The information of the Database presented is not sufficient to support the background exposure of eugenol.
- The residue studies do not cover all generic focal species identified at Tier 1.
- According to SANCO/11470/2012 on botanical active substances, the risk to non target organisms can be considered acceptable when estimated exposures are lower or similar to the natural exposure situations. Since negligible exposure of eugenol from the intended uses is not demonstrated, no unacceptable long-term reproductive risk to birds is supported.
- The chemical characteristics (i.w. high volatility) of eugenol may support a waiver for the avian reproductive test (OECD 206) since according to the test guidelines it cannot be used for highly volatile substances.
- On the other hand, possible effects on reproduction cannot be entirely excluded.
- According to the EFSA B & M Guidance 2009; 7(12):1438, the surrogate endpoint of LD50/10 should be compared with the NOAEL derived from the long-term reproductive study and the lowest endpoint should be used in the reproductive risk assessment for birds. Since no NOAEL is available, no safe conclusion on the reproductive risk to birds from the exposure to eugenol can be drawn.

Finally, the Co-RMS concluded that the OECD 206 test for investigating the long-term reproductive risk to birds may not be relevant for the compound eugenol. The RMS agrees that to perform an avian reproduction test could not be sufficiently justified. However, the weight of evidence submitted by the applicant is not sufficient to waive long term toxicity data. Therefore, further justification should be submitted.

RMS would appreciate the **opinion of other MMSS** regarding the acceptability of the weight of evidence proposed by the applicant to address the avian long-term risk.

### **B.9.1.2. Effects on terrestrial vertebrates other than birds**

#### ***B.9.1.2.1. Acute oral toxicity to mammals***

One acute oral toxicity study was previously evaluated as part of the EU review for the EU inclusion of eugenol (DAR, Volume 3, Annex B.9, 2011, B.9.3.1). No further studies are considered necessary for ecotoxicology. The full summary is provided in the mammalian toxicology section (Vol. 3 CA B.9.6.2.1.2). The acute oral **LD<sub>50</sub>** value for rat of **1930 mg eugenol/kg bw** is considered valid for use in the ecotoxicological risk assessment.

#### ***B.9.1.2.2. Long-term and reproduction toxicity to mammals***

One developmental toxicity study in the rat study was previously evaluated as part of the EU review for the EU inclusion of eugenol (DAR, Volume 3, Annex B.6, 2011, B.6.6.2). However, during the current renewal process, the study has not been accepted to set a NOAEL for developmental toxicity since was performed with clove oil instead of the active substance and the eugenol content in clove oil was not established (Vol. 3 CA B.6.6.2.1).

A developmental toxicity study in rats with eugenol is available (Wood and McKenzie 2004 ; Vol. 3 CA B.6.6.2.5) This study was not included in in the DRAR 2011 ; it was taken into account in the peer review of eugenol (EFSA Journal 2012 ; 10,11 : 2914). The study is considered acceptable, a developmental NOAEL value for rat of 250 mg eugenol/kg bw/day based on decreased mean foetal body weight and in the number of ossified metatarsals was derived from this study. No multi-generation studies with the active substance eugenol are available.

For the renewal of eugenol, a waiver is requested for long-term reproductive toxicity data to mammals as further vertebrate testing is not justified. Additional weight of evidence to support this waiver is presented below.

Eugenol is naturally occurring terpene oil found in a wide variety of plant species from 0.02 to 180000 mg/kg, for blueberry and clove respectively (please, see Table B.9.1.1.3-1, above).

In accordance with Regulation (EU) No 283/2013, a test for the effects on reproduction in mammals is currently requested if adult mammals are likely to be exposed during the breeding season. Following field application of the representative formulated product, Mevalone, initial environmental exposure of eugenol will decline rapidly in relation to the applied dose due to volatilisation and degradation. It is observed that the DT<sub>50</sub> in soil for eugenol is less than one day and the DT<sub>50</sub> in air obtained from the Atkinson model is 1.975 hours (Please, see Vol. 3 CA B.8).

Consequently, the duration of exposure under typical conditions will be very limited, particularly in relation to background levels of eugenol in the environment. This is also confirmed by the results of the residue trials conducted with Mevalone on grapevines and apples (please see Vol. 3 CA B.7.3 for further details). A total of 11 trials in grapes were conducted in Northern EU countries (Austria, Germany and Northern France) and in Southern EU countries (Spain, Portugal and Italy) in 2006 and 2020. All 2020 trials were conducted according to the critical GAP for the renewal and are therefore relevant to support the use of Mevalone in the EU. In the 2020 season trials in grapes, residues of eugenol were not detected in the untreated control samples and not detected or detected up to 0.02 mg/kg in the treated samples. All residues of eugenol in grapes had declined to not detectable by 1 day after the last application. Furthermore, a total of 6 trials in apples were conducted in Northern EU countries (Austria, Germany and Northern France) and in Southern EU countries (Spain, Southern France and Italy) in 2020 with an LOQ of 0.01 mg/kg. All trials were conducted according to the critical GAP for the renewal and are therefore relevant to support the use of Mevalone in the EU. No residues of eugenol were detected at or above the LOQ of 0.01 mg/kg in any of the treated samples even on the day of the last application of Mevalone according to the critical GAP.

This indicates that even the acute exposure will be significantly less than that estimated by the shortcut value (SV). There is thus a clear pattern of exposure with very low acute levels and very short duration so that the long-term residue burden resulting from dietary exposure after application of Mevalone will be very limited.

Following treatment, elevated residues of eugenol substances in mammal's diet would be present for an extremely short period of time (a matter of hours) over the reproductive period of mammals. It is considered that, given the potential for natural exposure to background levels in the diet, especially for herbivores, the exposure following a spray treatment would be negligible and does not merit the sacrifice of vertebrate animals. In contrast, the standard mammalian two-generation reproduction study (OECD test 416) involves at least 12 weeks of continued dietary exposure to the product. The exposure conditions of this test would bear no relation whatsoever to the potential exposure to eugenol following application to grapevine and pome fruit.

The active substance eugenol is of low acute toxicity to mammals (please see Vol. 3 CP B.9.1.2, Table 9.1.2-1)). One acute oral toxicity study was previously evaluated as part of the EU review for the EU inclusion of eugenol (DAR, Volume 3, Annex B.9, 2011, B.9.3.1). The acute **oral LD<sub>50</sub>** was **1930 mg eugenol/kg b.w.** (please see also Vol. 3 CA B.6.2.1).

The **NOAEL** value for rat of **250 mg eugenol/kg bw/day** is considered valid for use in the ecotoxicological risk assessment (please see Vol. 3 CA B.6.2.5). No further studies are considered necessary for ecotoxicology.

### **RMS comments:**

No multi-generation studies with the active substance eugenol or formulation Mevalone are available. However, in accordance with Regulation (EU) No 283/2013, a test for the effects on reproduction in mammals is currently requested if adult mammals are likely to be exposed during the breeding season.

The applicant has requested a waiver for long-term reproductive toxicity data to mammals based on a weight of evidence (see above).

The applicant has also presented data of residues of eugenol in a range of plants other than grape or pome (0.02 – 180000 µg/kg, in plant tissue, see Table B.7.1.1.3-1). The data was sourced from “Dr Duke’s phytochemical and ethnobotanical databases” (<https://phytochem.nal.usda.gov/phytochem/search>). The database is an *ad-hoc* inventory of agriculturally and medicinally relevant plant species and information regarding their phytochemical constituents. The data presented indicates that exposure can be expected when feeding on a range of plants.

However, there are significant uncertainties regarding the reliability of these data: The data do not provided a systematic assessment of the incidence of eugenol in plants. In particular, it does not identify how frequently the substances do not occur in plants relevant for the proposed use scenarios, which would result in no background exposure. The data are focused on agricultural and medicinal plants and read across to other relevant plant species has not been supported (for example grasses, acting as a food source in crop adjacent areas). Similarly the reported residues are stated for a range of plant tissues, residues are not necessarily evenly distributed within a plant so the quoted values may not reflect a diet unless composed of the specific plant tissues sampled in the underlying studies.

The range of estimated residues indicates that the dietary exposure, assuming 100% consumption of the food items, would be between 0.02 – 180000 mg/kg for eugenol. In most cases this would result in background exposure greater the predicted exposure following the proposed use.

This data were provided by the applicant during the confirmatory data requirement to address the long-term risk to insectivorous birds (EFSA Supporting publication 2017:EN-1165). The RMS (UK) concluded that :

*“The data from “Dr Duke’s phytochemical and ethnobotanical databases” indicates that eugenol and methyl eugenol are present in a range of plants. And based on the reported residues under some circumstances background exposure could exceed the predicted exposure based on the proposed use. However, the deficiencies identified above mean that this information is not sufficient to establish a background exposure for birds. The confirmatory data requirements have therefore not been met, and it cannot be concluded that background exposure will exceed the exposure based on the proposed use”.*

Furthermore, 11 residue trials are available for grapevines and 6 for apples. Five of the residue trials on grapevines were conducted in 2006 (Bailey, 2007 and 2008). These studies have been evaluated by the Residues section (Vol. 3 CA B.7.3.1). The study by Bailey (2008) included one residue trial conducted in Spain and it was not covered by intended GAP since only three applications were made. Furthermore, the study has been considered not to be acceptable by Residues Section. However, in the study by Bailey (2007) four residue trials were performed (2 in Spain and 2 in Italy) and they were conducted according with the proposed GAP. The study was accepted by Residues Section, but only 2 residue trials (one in Spain and one in Italy) were considered, due to the proximity of the trials, and the other two residue trials must be considered as replicates.

Two studies with 6 residues trials each on grapes (Chadwick, 2021a) and pomes (Chadwick, 2021b) on SEU and CEU countries were performed. The studies cover the GAP and were accepted by RMS. Residues of eugenol were not detected in at or above the LOQ of 0.01 mg/kg in any of the treated samples.

However, the residue trials data are relevant specifically to consideration of frugivorous mammals. The dietary exposure for other feeding guilds (insectivorous, granivorous, omnivorous...) are not covered by residue data. Furthermore, a purely frugivorous generic focal species is not considered at first tier for mammals in vineyard according to EFSA (2009). Also none of the first tier generic focal species relevant to vineyard with mixed diets include a percentage of the diet identified as ‘fruit’.

The applicant states that due to the high volatilisation ( $V_p = 2.7$  Pa at 20° C) and the rapid degradation ( $DT_{50}$  soil 1 < day) initial environmental exposure of eugenol will decline rapidly. However, these data are not sufficient to demonstrate a negligible exposure. Furthermore, the confirmatory data information on natural background levels of eugenol to address the short-term and long-term risk to birds to insectivorous birds was not considered sufficient by EFSA to enable a comparison between the natural background exposure and the exposure due to the use of the plant protection product (EFSA Supporting publication 2017:EN-1165). Therefore, under RMS opinion a negligible exposure has not been demonstrated.

The Co RMS agrees that the weight of evidence presented by the applicant to request for a waiver for long-term reproductive studies has some uncertainties.

Additionally, a two-generation reproduction study according to OECD test 416 are not available. However, developmental toxicity studies with the active substance eugenol according to OECD guideline 414 on rats and rabbits are available (Vol. 3 CA B.6.6.2). A developmental NOAEL of 250 mg eugenol/kg bw/day was set from this studies. Furthermore, according to EFSA conclusion based on the available data, no multi-generational studies were considered necessary (EFSA Journal 2012;10(11):2914, and “Peer review report on eugenol, October 2012”).

RMS agrees that considering the low acute toxicity of eugenol ( $LD_{50} = 1930$  mg eugenol/kg b.w.), the NOAEL value for rat of 250 mg eugenol/kg bw/day could be considered valid for use in the ecotoxicological risk assessment. Furthermore, the CoRMS agrees that since a valid endpoint from a developmental toxicity study on mammals, namely NOAEL 250 mg eugenol/kg bw is used in the reproductive risk assessment, no further studies are necessary for assessing the long-term reproductive risk to mammals.

### **B.9.1.3. Active substance bioconcentration in prey of birds and mammals**

The log  $P_{ow}$  values for eugenol are 2.47, 2.49 and 2.44 at pH values 4,7 and 9, respectively. These values are less than 3 and therefore do not trigger a need to assess the risk from bioaccumulation and secondary poisoning from consumption of contaminated fish or earthworms. Assessment of the risk of active substance bioconcentration in prey of birds and mammals is therefore not required, as previously agreed during the EU review for the EU inclusion of eugenol (EFSA Journal 2012;10(11):2914).

### **B.9.1.4. Other data on effects on terrestrial vertebrate wildlife (birds, mammals, reptiles and amphibians)**

It is noted that a literature paper was identified during the literature search, which assessed the use of eugenol (when mixed into grain pellets) as an attractant to trap pest voles (Appendix I, CA 9.6.3.4/01). Results of this

paper are summarised in the Appendix I for completeness, but this study is considered of limited relevance and reliability to the risk assessment of wild mammals following the plant protection spray application of eugenol. The risk assessments for birds and mammals performed according to current EU guidance are presented in Vol. 3 CP B.9. At the time of this submission there is no agreed guidance on the requirement for further data or risk assessments on other terrestrial vertebrate wildlife (including reptiles and amphibians). However, as stated in the guidance document for aquatic organisms (EFSA Journal 2013;11(7):3290 Section 7.2.4.) the rainbow trout *Oncorhynchus mykiss* is a good surrogate test species for predicting the acute toxicity of plant protection products for larval stages of amphibian species living in the aquatic compartment of the environment. Using the rainbow trout *Oncorhynchus mykiss* as a surrogate test species for predicting the toxicity of eugenol for larval stages of amphibian species a low toxicity can be concluded (B.9.2.1). It is also noted that two literature papers were identified during the literature search, which are related to the use of eugenol as a short-term anaesthetic during the handling and/or transport of *Xenopus* frogs by humans (Appendix I, CA 9.6.3.4/34 and CA 9.6.3.4/35). However, results of these anaesthesia studies are of limited relevance and reliability for the risk assessment due to the very short-term exposure (and lack of analytical verification to confirm exposure), followed by a recovery period in untreated water. The critical endpoints from the standard fish acute toxicity studies (B.9.2.1) are considered more relevant and reliable for predicting the low toxicity of eugenol to amphibians. No further relevant data on adverse effects to eugenol to other terrestrial wildlife were found during the open literature search and no further data are considered necessary.

#### **B.9.1.5. Potential for endocrine disruption**

The available data for eugenol do not show any adverse effects that are considered to be EAS-mediated. However, there are no available guideline studies with eugenol on birds to investigate the potential of endocrine disruption of eugenol. The available toxicology (mammalian) and ecotoxicology data for eugenol showed that the potential for endocrine disruption have not been sufficiently investigated, therefore, further data need to be generated before a conclusion on whether or not the ED criteria are met (Vol. 1, 2.10).

### **B.9.2. EFFECT ON AQUATIC ORGANISMS**

#### **B.9.2.1. Acute toxicity to fish**

Two acute fish toxicity studies with eugenol were previously evaluated as part of the EU review for the EU inclusion of eugenol (DAR, Volume 3, Annex B.9, 2011, B.9.2.1.1). Full summaries of these studies are provided below (Studies B.9.2.1/01 and B.9.2.1/02).

It is also noted that a large number of literature papers were identified during the literature search (see B.9.11.1), which assessed the very short-term anaesthesia effects of eugenol on various fish species and their subsequent recovery. Results of these papers are summarised in the Appendix I (B.10) for completeness, but as discussed further in the data point B.11.1 these are not considered reliable for use in the risk assessment, principally as analytical verification was not reported to confirm the actual exposure during the studies. The critical endpoints summarised below from the standard OECD 203 studies are considered more relevant and reliable for the acute fish risk assessment, and these OECD studies themselves will cover any possible short-term behavioural (e.g. anaesthesia) effects on fish. No further studies are considered necessary.

**Study B.9.2.1/01**

<b>Data point:</b>	CA 8.2.1/01
<b>Report author</b>	[REDACTED]
<b>Report year</b>	2008a
<b>Report title</b>	Acute toxicity of eugenol to rainbow trout ( <i>Oncorhynchus mykiss</i> ) in a 96-hour semi-static test
<b>Report No</b>	37984230
<b>Document No</b>	-
<b>Guidelines followed in study</b>	OECD Guideline 203 (1992) EPA Guideline 712-C-96-118: OPPTS 850.1075 (1996)
<b>Deviations from current test guideline</b>	None
<b>Previous evaluation</b>	Yes, evaluated and accepted in the DAR (Eugenol, Volume 3, Annex B.9, 2011, B.9.2.1.1) EU-agreed critical endpoint for EU inclusion of eugenol (EFSA Journal 2012;10(11):2914)
<b>GLP/Officially recognised testing facilities</b>	Yes, conducted under GLP/Officially recognised testing facilities
<b>Acceptability/Reliability:</b>	Yes

**Executive summary**

The 96-hour acute toxicity of eugenol to *Oncorhynchus mykiss* was studied under semi-static conditions in accordance with OECD 203. Juveniles of rainbow trout were exposed to eugenol at nominal concentrations of 1, 1.8, 3.2, 5.6 and 10 mg eugenol/L for 96 hours. Mortality and sub-lethal effects were observed daily and at test termination. Analytical verification of test concentrations confirmed measured concentrations were maintained between 85 and 102% of nominals throughout the exposure period and biological endpoints were based on nominal concentrations.

The 96-hour LC<sub>50</sub> value for rainbow trout (*Oncorhynchus mykiss*) was estimated to be >10 mg eugenol/L, the highest concentration tested based on nominal concentrations. The corresponding NOEC value based on sublethal symptoms was 1.8 mg eugenol/L, based on nominal concentrations. The sublethal effects included darker coloration at 3.2 mg eugenol/L and tumbling, exophthalmos, strong ventilation and lateral position at 5.6 and 10 mg eugenol/L.

This study is considered as acceptable and valid. The study satisfies the guideline requirements in place at the time the study was conducted (OECD test guideline 203, 1992), and also meets the validity criteria in the more recent OECD test guideline 203 update of 2019.

**Materials and methods***Test material*

Name: eugenol  
 Formulation type: -  
 Source and lot/batch no.: 95217  
 Active substance content: 98.8% w/w  
 Expiry date of lot/batch: March 2008  
 Storage conditions: In original container in the refrigerator (<5°C), under nitrogen, in the dark.

*Test organism*

Species: Rainbow trout (*Oncorhynchus mykiss*)  
 Age at study initiation: Juveniles  
 Weight/length at study initiation: 0.85 ± 0.16 g/4.57 ± 0.24 cm (mean ± SD)  
 Source: The test fish were obtained from [REDACTED], Germany.  
 Feeding during test: No

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Acclimation: For at least 12 days before the start of the test. The fish were fed with a commercial fish diet food until one day before the start of the test. During the last 7 days prior to test start no fish died in the test fish batch and all fish were healthy.

#### *Test conditions*

Hardness: 250 mg/L as calcium carbonate  
Test temperature: 14 - 15 °C  
pH: 7.7 - 7.9  
Dissolved oxygen: ≥95% of air saturation  
Photoperiod: 16 hours light: 8 hours dark  
Light intensity: 529 - 970 lux

Groups of seven rainbow trout (*Oncorhynchus mykiss*) were exposed to eugenol at nominal concentrations of 1, 1.8, 3.2, 5.6 and 10 mg eugenol/L for 96 hours in a semi-static system (22 L glass aquaria) under 16 hours light and 8 hours dark per day. There was also a control of test medium only, i.e. reconstituted water and a solvent control containing 100 mg dimethylformamide [DMF]/L. The test media were renewed after 48 hours. The temperature, was measured daily in all test units and the dissolved oxygen was measured in each vessel every 24 hours. Test concentrations were analysed at the start and the end of each renewal period.

Mortality and other observations were made after 1, 24, 48, 72 and 96 hours. Statistical analysis was performed if possible by appropriate statistical test, e.g. Probit analysis to determine LC<sub>50</sub> values. The values were calculated directly from the raw data.

## **Results**

#### *Analytical results*

The GS-MS analytical method for the determination of eugenol in test medium was validated with regards to specificity, linearity, accuracy and precision. Whilst the report does not refer to the current guideline, overall the validation results are in accordance with guideline SANCO/3029/99 rev. 4, 11/07/2000 and are considered fit for purpose. Specificity was demonstrated by the absence of a peak at the characteristic retention time for eugenol in the control sample. The analytical calibration was shown to be linear ( $r = 0.9989$ ) over the range of 0.3 to 3 mg reference item/L and 1 to 7 mg reference item/L (low range and high range respectively). Accuracy was confirmed with recovery determined by fortification of eugenol at 1, 3 and 10 mg test reference/L; all recoveries were within the range of 85-102% and mean recoveries were within 92-103 % (i.e. within the guideline range of 70-110%). Precision was confirmed with four determinations made at each fortification level (3 and 10 mg reference item/L); the relative standard deviation was between 3.9-5.9% (i.e. within the guideline limit of ≤20%). The limit of quantification (LOQ) was 1.482 mg eugenol/L (i.e. below the biological LC<sub>50</sub>/NOEC value). The limit of detection (LOD) was 0.075 mg/L in test medium.

A summary of the measured concentrations of eugenol in the test media is presented in the tables below.

**Table 9.2.1/01-01: Nominal and measured concentrations of eugenol in each replicate**

Nominal conc. (mg product/L)	Replicate	Measured conc. (mg eugenol/L)			Percentage of nominal		
		0 h	48 h	Mean*	0 h	48 h	Mean
Water control	1	4.672 <sup>a</sup>	<LOQ	n.a.	n.a.	n.a.	n.a.
	2	<LOQ	<LOQ		n.a.	n.a.	
Solvent control	1	<LOQ	n.a.	n.a.	n.a.	n.a.	n.a.
	2	n.a.	n.a.		n.a.	n.a.	
1.0	1	<LOQ	<LOQ	n.a.	n.a.	n.a.	n.a.
	2	<LOQ	<LOQ		n.a.	n.a.	
	3	<LOQ	<LOQ		n.a.	n.a.	
	4	n.a.	<LOQ		n.a.	n.a.	
1.8	1	<i>0.624</i>	<i>1.565</i>	1.726	35	88	85
	2	<i>1.603</i>	<i>1.691</i>		90	95	
	3	<i>1.388</i>	<i>1.388</i>		78	78	
	4	<i>0.476</i>	<i>1.471</i>		27	83	
3.2	1	3.205	2.833	2.535	101	90	102
	2	3.566	3.093		112	98	
	3	<i>0.773</i>	<i>3.511</i>		24	111	
	4	<i>0.170</i>	<i>3.125</i>		5	99	
5.6	1	5.981	4.933	4.944	107	89	97
	2	6.195	5.184		112	94	
	3	<i>1.765</i>	<i>5.438</i>		32	98	
	4	<i>4.518</i>	<i>5.537</i>		82	100	
10	1	10.355	8.687	7.728	105	88	95
	2	10.752	8.774		109	89	
	3	<i>4.363</i>	<i>8.664</i>		44	88	
	4	<i>1.272</i>	<i>8.959</i>		13	91	

<sup>a</sup> The high values is not result of a contamination of the sample, as could be concluded from the respective aged sample (collected after 48 hours).

LOQ = 3 mg product/L, corresponding to 1.482 mg eugenol/L after dilution 1:2

LOD = 0.075 mg eugenol/L

\* Arithmetic mean of the 8 measurements for each treatment group (duplicate samples at 0 and 48 hours)

n.a.: not applicable

Measures in *italics* was consider to be outliers (*Italic values were considered to be result of short-time inhomogeneity, as could be concluded from the respective aged samples (collected after 48hours, respectively)*)

Conc. concentration

**Table 9.2.1/01-02: Summary of nominal and mean measured concentrations of eugenol for 48 hours**

Nominal conc. (mg product/L)	Water control	Solvent control	Measured concentration (mg eugenol/L)				
			1.0	1.8	3.2	5.6	10
Range (min: max)	n.a.	n.a.	<LOQ	1.388-1.691	2.833-3.566	4.518-6.195	8.664-10.752
Median	n.a.	n.a.	n.a.	1.603	3.149	5.582	9.564
Mean*	n.a.	n.a.	n.a.	1.518	3.222	5.398	9.365
% of nominal (ref. to mean)	n.a.	n.a.	n.a.	85	102	97	95

\* Arithmetic mean of the 8 measurements for each treatment group (duplicate samples at 0 and 48 hours)

n.a.: not applicable

Conc. concentration

The results of the freshly prepared samples of Day 2 were not considered for the calculations as these test media were considered to have been temporarily not homogenous (sampling was performed directly after preparation of the test media). At the lowest concentration tested (nominally 1.0 mg eugenol/L), the measured concentrations were below the LOQ value of 1.482 mg eugenol/L. Excluding these results, analytical verification of test concentrations confirmed that measured concentrations were between 85 and 102% of nominals in fresh and aged media throughout the test. Biological endpoints are reported based on nominal concentrations of eugenol (not accounting for product purity).

*Biological results*

A summary of the effects of eugenol on mortality of *Oncorhynchus mykiss* after 96 hours exposure is presented in the table below.

**Table 9.2.1/01-03: Effect of eugenol on mortality of rainbow trout for 96 hours**

Nominal conc. (mg eugenol/L)	No. organisms at test start	Cumulative mortality (no. dead)				Cumulative mortality (%)			
		24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
Water control	7	0	0	0	0	0	0	0	0
Solvent control	7	0	0	0	1	0	0	0	14
1.0	7	0	0	0	0	0	0	0	0
1.8	7	0	0	0	0	0	0	0	0
3.2	7	0	0	0	0	0	0	0	0
5.6	7	0	0	0	0	0	0	0	0
10	7	0	1	3	3	0	14	43	43

Conc. concentration

In the control and up to the test nominal concentration of 5.6 mg product/L all fish survived until the end of the test. One fish died in the solvent control. The mortality in the solvent control was not significantly different compared to the control and is considered not to be caused by the solvent. In the test concentration of nominal 10.0 mg eugenol/L three fish died within the 96 hour exposure.

Sublethal symptoms were observed starting at the 3.2 mg eugenol/L nominal concentration, where specimens showed some darker colouration. Additional symptoms like tumbling, exophthalmos, strong ventilation or lateral position were observed at the test concentrations of 5.6 and 10 mg product/L. As a result the following endpoints were obtained, the 96-hour LC<sub>50</sub> (mortality) > 10 mg product/L and the 96-hour NOEC (sublethal effects) = 1.8 mg product/L (confidence intervals were not reported due to mathematical reasons).

*Validity*

All validity criteria were met in accordance with OECD test guideline 203 (1992 and current 2019):

- In the control, the mortality did not exceed one fish at the end of the test. (Actual values: no fish died during the test in the control, one fish died in the solvent control).
- The dissolved oxygen concentration in the test media was above 60% of air saturation value during the test (actual values: ≥95% of air saturation).
- Analytical measurement of test concentrations was included.

**Assessment and conclusion**

The 96-hour acute toxicity of eugenol to *Oncorhynchus mykiss* was studied under semi-static conditions in accordance with OECD 203. The 96-hour LC<sub>50</sub> value was estimated to be >10 mg eugenol/L based on nominal concentrations. The 96-hour NOEC value based on sublethal adverse effects was determined to be 1.8 mg eugenol/L based on nominal concentrations. The sublethal effects included tumbling, exophthalmos, strong ventilation or lateral position. This study is considered as acceptable and satisfies the guideline requirements for an acute toxicity study with fish.

**Assessment and conclusion by applicant:**

The study is acceptable.

*Oncorhynchus mykiss* 96-hour LC<sub>50</sub> (mortality) >10 mg eugenol/L based on nominal concentrations.

**Assessment and conclusion by RMS:**

This study was already evaluated and accepted during Annex I inclusion of Eugenol and it was included in the Eugenol Monograph (Volume 3, Annex B.9 Ecotoxicology, May 2011).

The validity criteria according to OECD guideline 203 (Fish, Acute Toxicity Test ; 1992, 2019) were met.

RMS agrees with the previous evaluation.

**Agreed endpoints :**

**96h-LC<sub>50</sub> > 10 mg eugenol/L (nom.) (based on mortality)**

**96h- NOEC = 1.8 mg eugenol/L (nom.) (based on sublethal effects)**

**Study B.9.2.1/02**

<b>Data point:</b>	CA 8.2.1/02
<b>Report author</b>	██████████
<b>Report year</b>	2008b
<b>Report title</b>	Acute toxicity of eugenol to Zebra fish ( <i>Danio rerio</i> ) in a 96-hour semi-static test
<b>Report No</b>	37983230
<b>Document No</b>	-
<b>Guidelines followed in study</b>	OECD Guideline 203 (1992) EPA Guideline 712-C-96-118: OPPTS 850.1075 (1996)
<b>Deviations from current test guideline</b>	None
<b>Previous evaluation</b>	Yes, evaluated and accepted in the DAR (Eugenol, Volume 3, Annex B.9, 2011, B.9.2.1.1)
<b>GLP/Officially recognised testing facilities</b>	Yes, conducted under GLP/Officially recognised testing facilities
<b>Acceptability/Reliability:</b>	Yes

**Executive summary**

The 96-hour acute toxicity of eugenol to *Danio rerio* was studied under semi-static conditions in accordance with OECD 203. Juveniles of zebra fish were exposed to eugenol at nominal concentrations of 1.8, 3.2, 5.6, 10.0 and 18.0 mg eugenol/L for 96 hours. Mortality and sublethal effects were observed daily and at test termination. Analytical verification of test concentrations confirmed measured concentrations were maintained within 89 and 112% of nominals throughout the exposure period and biological endpoints were based on nominal concentrations.

The 96-hour LC<sub>50</sub> value for *Danio rerio* (zebra fish) was calculated to be 11.9 mg eugenol/L based on nominal concentrations. The corresponding NOEC (mortality) value was 3.2 mg/L, based on nominal concentrations. Sublethal effects were observed at concentrations of 10.0 mg eugenol/L and higher and included tumbling, convulsions, strong ventilation, change of colour, apathy and reduced swimming behaviour.

This study is considered as acceptable and valid. The study satisfies the guideline requirements in place at the time the study was conducted (OECD test guideline 203, 1992), and also meets the validity criteria in the more recent OECD test guideline 203 update of 2019.

**Materials and methods***Test material*

Name: eugenol  
 Formulation type: -  
 Source and lot/batch no.: 95217

Active substance content: 98.8% w/w

Expiry date of lot/batch: March 2008

Storage conditions: In original container in the refrigerator (<5°C), under nitrogen, in the dark.

#### Test organism

Species: Zebra fish (*Danio rerio*)

Age at study initiation: Juveniles

Weight/length

at study initiation:  $0.15 \pm 0.03$  g /  $2.47 \pm 0.18$  cm (mean  $\pm$  SD)

Source: The test fish were obtained from profi aquarium [REDACTED], Germany.

Feeding during test: No

Acclimation: For at least 12 days before the start of the test. The fish were fed with a commercial fish diet food until one day before the start of the test. During the last 7 days prior to test start no fish died in the test fish batch and all fish were healthy.

#### Test conditions

Hardness: 250 mg/L as calcium carbonate

Test temperature: 22-23°C

pH: 7.8-7.9

Dissolved oxygen: at least 60% of the air saturation (actual values at least 94%)

Photoperiod: 16h light: 8 h dark

Light intensity: 330-520 lux

Groups of seven zebra fish (*Danio rerio*) were exposed to eugenol at nominal concentrations of 1, 1.8, 3.2, 5.6, 10.0 and 18.0 mg product/L for 96 hours in a semi-static system (22 L glass aquaria). There was also a control of test medium only, i.e. reconstituted water and a solvent control containing 100 mg dimethylformamide [DMF]/L. The test media were renewed after 48 hours. The temperature was measured daily in all test units, and the dissolved oxygen was measured in each vessel every 24 hours.

Test concentrations were analysed at the start and the end of each renewal period. Mortality and other observations were made after 1, 24, 48, 72 and 96 hours. Statistical analysis was performed using ToxRat Professional v2.09. EC<sub>50</sub> values and 95% confidence limits were calculated, where possible, by Probit analysis. The NOEC and LOEC values were determined directly from the raw data.

## Results

#### Analytical results

The GS-MS analytical method for the determination of eugenol in test medium was validated with regards to specificity, linearity, accuracy and precision. Whilst the report does not refer to the current guideline, overall the validation results are in accordance with guideline SANCO/3029/99 rev. 4, 11/07/2000 and are considered fit for purpose. Specificity was demonstrated by the absence of a peak at the characteristic retention time for eugenol in the control sample. The analytical calibration was shown to be linear ( $r = 0.9976$ ) over the range of 0.5 to 10 mg reference item/L. Accuracy was confirmed with recovery determined by fortification of eugenol at 1, 3 and 20 mg reference item/L; all recoveries were within the range of 78-166 % and mean recoveries were within 92-105 % (i.e. within the guideline range of 70-110%). Precision was confirmed with six determinations made at each fortification level (1, 3 and 20 mg reference item/L); the relative standard deviation was between 7-20 % (i.e. within the guideline limit of  $\leq 20\%$ ). The limit of quantification (LOQ) was 1.482 mg eugenol/L (i.e. below the biological LC<sub>50</sub>/NOEC value). The limit of detection (LOD) was 0.02 mg eugenol/L in test medium.

A summary of the measured concentrations of eugenol in the test media is presented in the tables below.

**Table 9.2.1/02-01: Nominal and measured concentrations of eugenol in each replicate**

Nominal conc. (mg product/L)	Replicate	Measured conc. (mg eugenol./L)			Percentage of nominal		
		0 h	48 h	Mean*	0 h	48 h	Mean
Water control	1	<LOQ	<LOQ	n.a.	n.a.	n.a.	n.a.
	2	<LOQ	<LOQ		n.a.	n.a.	
Solvent control	1	<LOD	<LOD	n.a.	n.a.	n.a.	n.a.
	2	<LOD	<LOD		n.a.	n.a.	
	2	<LOQ	<LOQ		n.a.	n.a.	
	3	<LOQ	<LOQ		n.a.	n.a.	
1.8	4	n.a.	<LOQ	n.a.	n.a.	n.a.	n.a.
	1	<LOQ	<LOQ		n.a.	n.a.	
	2	<LOQ	<LOQ		n.a.	n.a.	
	3	<LOQ	<LOQ		n.a.	n.a.	
3.2	4	<LOQ	<LOQ	3.341	n.a.	n.a.	106
	1	5.790	2.707		183	86	
	2	12.181	3.130		385	99	
	3	3.636	3.319		115	105	
5.6	4	4.017	3.236	4.944	127	102	89
	1	6.204	6.259		112	113	
	2	5.750	6.592		104	119	
	3	7.571	6.751		137	122	
10.0	4	6.434	6.141	11.06	116	111	112
	1	8.341	10.627		84	108	
	2	19.891	11.369		201	115	
	3	11.774	11.011		119	111	
18.0	4	12.572	8.959	16.479	127	119	93
	1	13.809	19.513		78	110	
	2	13.281	19.312		75	109	
		<b>0h</b>	<b>24h</b>		<b>0h</b>	<b>24h</b>	

LOQ = 3 mg product/L, corresponding to 1.482 mg eugenol/L after dilution 1:2

LOD = 0.02 mg eugenol./L

\* Arithmetic mean of the 8 measurements for each treatment group (duplicate samples at 0 and 48 hours).

Measures in *italics* were considered to be outliers

n.a.: not applicable

Conc. Concentration

**Table 9.2.1/02-02: Summary of nominal and mean measured concentrations of eugenol for 48 hours**

Nominal conc. (mg product/L)	Water control	Solvent control	Measured concentration (mg Eugenol/L)				
			1.8	3.2	5.6	10.0	18.0
Range (min: max)	n.a.	n..a	<LOQ	0.17-3.566	1.765-5.981	1.272-10.752	13.281-19.531
Median	n.a.	n..a	n.a.	2.918	5.582	9.654	16.56
Mean*	n.a.	n..a	n.a.	3.341	4.944	7.728	16.479
% of nominal (ref. to mean)	n.a.	n..a	n.a.	106	89	112	93

\* arithmetic mean of the 4-8 measurements for each treatment group (2-4 samples at 0 and 48 hours)

<sup>a</sup> Based on product purity of 98.8% w/w

n.a.: not applicable

Conc. concentration

The measured concentrations of the samples of nominal concentration of 1.8 mg/L were below the NOEC and below the limit of Quantification. Some analytical results of freshly prepared test media (day 0) deviated considerably from the nominal values. However, the analysis of the aged test media indicated correct dosing. The aberrant initial values are likely to have been the result of a temporary inhomogeneity of the test media, and they were not considered for the calculation of the mean exposure (outliers).

At the start of the test and after renewal of the test media 109 % of the nominal test concentrations were found (average for nominal concentrations of 3.2, 5.6, 10 and 18 mg eugenol/L). In the aged test media 109 % of the nominal values was found (average for nominal concentrations of 3.2, 5.6, 10 and 18 mg eugenol/L). Thus, during the test period the fish were exposed to an overall mean measured concentration of 109 % of nominal (average for nominal concentrations of 3.2, 5.6, 10 and 18 mg eugenol/L).

The active ingredient was found in concentrations close to the nominal concentrations at the start and the end of the test. Therefore, all reported results are expressed in terms of the nominal concentrations of the product.

### Biological results

A summary of the effects of eugenol on mortality of *Danio rerio* after 96 hours exposure is presented in the table below.

**Table 9.2.1/02-03: Effect of eugenol on mortality of Zebra fish for 96 hours**

Nominal conc. (mg eugenol/L)	No. organisms at test start	Cumulative mortality (no. dead)				Cumulative mortality (%)			
		24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
Water control	7	0	0	0	0	0	0	0	0
Solvent control	7	0	0	0	0	0	0	0	0
1.8	7	0	0	0	0	0	0	0	0
3.2	7	0	0	0	0	0	0	0	0
5.6	7	0	0	0	1	0	0	0	1
10.0	7	0	0	0	0	0	0	0	0
18.0	7	7	7	7	7	100	100	100	100

Conc. concentration

In the control and up to the test concentration of 3.2 mg eugenol/L all fish survived until the end of the test. At 5.6 mg eugenol/L one fish died after 96h. However at 10 mg product/L no mortality occurred, but sublethal symptoms like thumbling, reduce swimming behaviour were observed at 10 mg product/L and at higher concentration. At 18 mg product/L all fish were dead after 24 hours. As a result the following endpoints were obtained, the 96-h LC<sub>50</sub> (mortality) = 11.9 mg eugenol/L and the NOEC (96-hour) = 3.2 mg eugenol/L (confidence intervals were not reported due to mathematical reasons).

### Validity

All validity criteria were met.

- In the control, the mortality should not exceed one fish at the end of the test. (Actual values: no fish died).
- The dissolved oxygen concentration in the test media was above 60% of air saturation value during the test (actual values: at least 94%).
- Analytical measurement of test concentrations was included.

### Assessment and conclusion

The 96-hour acute toxicity of eugenol to *Danio rerio* was studied under semi-static conditions in accordance with OECD 203. The 96-hour LC<sub>50</sub> value was calculated to be 11.9 mg eugenol/L based on nominal concentrations. The 96-hour NOEC value based on sublethal adverse effects was determined to be 3.2 mg product/L based on nominal concentrations. The sublethal effects included were tumbling, reduce swimming behaviour and apathy. This study is considered as acceptable and satisfies the guideline requirements for an acute toxicity study with fish.

#### **Assessment and conclusion by applicant:**

The study is acceptable.

*Danio rerio* 96-hour LC<sub>50</sub> (mortality) = 11.9 mg eugenol/L based on nominal concentrations.

**Assessment and conclusion by RMS:**

This study was already evaluated and accepted during Annex I inclusion of Eugenol and it was included in the Eugenol Monograph (Volume 3, Annex B.9 Ecotoxicology, May 2011).

The validity criteria according to OECD guideline 203 (Fish, Acute Toxicity Test ; 1992, 2019) were met.

RMS agrees with the previous evaluation.

**Agreed endpoints :**

**96h-LC<sub>50</sub> = 11.9 mg eugenol/L (nom.) (based on mortality)**

**96h- NOEC = 3.2 mg eugenol/L (nom.) (based on sublethal effects)**

**B.9.2.2. Long-term and chronic toxicity to fish**

It is noted that a data gap for further information to address the chronic risk to aquatic organisms was identified during the first EU review of eugenol (EFSA Journal 2012; 10(11):2914). Further information on natural background levels of eugenol was provided as confirmatory data, but this was not considered sufficient by EFSA to enable a comparison between the natural background exposure and the exposure due to the use of the plant protection product (EFSA Supporting publication 2017:EN-1165).

For the renewal of eugenol, a waiver is requested for long-term toxicity data to fish as further vertebrate testing is not justified. Additional weight of evidence to support this waiver is presented below.

Significant long-term exposure of eugenol in surface waters is not expected due to its rapid volatilisation properties and ready biodegradation. Following field application of the representative formulated product, Mevalone, initial environmental exposure of eugenol will decline rapidly in relation to the applied dose. According to the acute toxicity endpoints obtained from EFSA Journal 2012; 10(11):2914, it is observed that *Daphnia magna* is more sensitive (up to 10 times more acutely toxic) than fish. Therefore, no new long-term toxicity studies have been carried out for fish to waive further vertebrate testing during active substance renewal. The new long-term study with *Daphnia magna* (Study B.9.2.5.1/01) is expected to be sufficient to address the long-term toxicity for aquatic organism.

No chronic test on fish is considered necessary as the chronic risk assessment for aquatic organisms can be derived from toxicity data on aquatic invertebrates. As already explained above, *Daphnia magna* was the most sensitive species in the acute tests.

It is also important to note that the chronic endpoint for *Daphnia magna* is likely to be overestimated, since this test was performed under semi-static conditions with three renewals of test medium per week. Please note that the number of intended applications is 4 treatments with an interval of 7 days, a much lower number than the renewals conducted in the chronic study.

The use of clove oil (main component: eugenol) as antifungal agent against *Saprolegnia* sp. during the incubation of rainbow trout eggs was investigated by Hoskonen, P., Heikkinen, J., Eskelinen, P., Pirhonen, J. (2015). For further details about this literature study, please refer Appendix I CA 9.6.3.4/29. Main conclusions of the study are shown below:

The treatments (exposure to clove oil at nominally 500 or 1000 mg/L for 15 minutes once or three times a week) did not influence the timing of the eyed stage or hatching. There were no significant differences in the survival to hatch or in the amount of visibly abnormal fry. The length of this study was 30 days.

Based on the above results, it is unlikely eugenol cause adverse effects on early life stages of fish. A priori the most sensitive stage of test organism.

According to the Regulation 283/2013 the long-term and chronic toxicity study on fish should be provided since eugenol was considered hydrolytically stable at pH 4 and 7. However, it is important to note the current legislation do not consider other relevant routes of degradation/dissipation to trigger the chronic data requirement to aquatic organisms. Please note the main route of dissipation of eugenol is via volatilization (DT<sub>50</sub> < 1 day).

Considering all the arguments provided above and in the interests of minimizing vertebrates testing, the Applicant thinks a FELS study would not be scientifically justified.

**RMS Comments:**

RMS agrees that the acute toxicity endpoint for *D. magna* is one order of magnitude below than the acute toxicity endpoint for fish (1.11 mg a.s./L and 10 mg a.s./L, respectively). Therefore, aquatic invertebrates are more sensitive than fish for acute risk (Table 9.3-1, Vol. 3 CP).

However, according to the Regulation 283/2013 the long-term and chronic toxicity study on fish is a data requirement. The study should be provided unless it is proved that the substance is stable in water, that is to say there is less than 90% loss of the original substance over 24 hours via hydrolysis. Since, the eugenol was considered hydrolytically stable at pH 4 and 7 (Vol. 3 CA Study B.8.2.1.1/01 ; Kelly 2021) the data gap for further information to address the chronic risk to aquatic organisms has not been addressed. Thus, a new study should be submitted.

Thus, under RMS opinion, an uncertainty has been arisen regarding aquatic invertebrates will remain the most sensitive species for chronic risk.

Moreover, under RMS opinion the chronic endpoint for *Daphnia* is not overestimated, since the study has been conducted according to OECD TG 211 (*Daphnia magna* reproduction test). In this test the endpoint should be set with a stable test substance concentration (i.e. in the range 80 - 120% of nominal or falling below 80% of the measured initial concentration). Furthermore, the guideline states that the frequency of medium renewal will depend on the stability of the test substance, but should be at least three times per week over the maximum renewal period (i.e. 3 days). Therefore, the endpoints is calculated according to the current guideline and it is not overestimated. Additionally, in PECsw/sed modelling the intended pattern of application is taken into account.

Furthermore, The study by Hoskonen *et al.* (2015) has been considered as not reliable by the applicant (Vol. 3 CA 9.6.3.4/29). The main reasons to support the unreliability of this study are that the analytical verification of test concentrations was not reported, and actual exposure is difficult to determine as only short 15-minute exposure periods once or twice a week in a study of approximately 30 days duration. Furthermore, all treatment groups also included exposure to a fungus, which grows on and inhibits fish eggs.

Considering the suggestion of the Co-RMS, an ELS test (OECD TG 210) could be performed to study the chronic risk to fish and EAS-adversity of the active substance eugenol.

Therefore, a **data gap** has been identified to **submit a chronic toxicity study on fish**.

***B.9.2.2.1. Fish early life stage toxicity test***

No further data are considered necessary. Please see point B.9.2.2 above.

***B.9.2.2.2. Fish full-life cycle test***

No further data are considered necessary. Please see point B.9.2.2 above.

***B.9.2.2.3. Bioconcentration in fish***

The log  $P_{ow}$  values for eugenol are 2.47, 2.49 and 2.44 at pH values 4, 7 and 9, respectively. These values are less than 3 and therefore do not trigger further consideration of bioconcentration in fish. Studies of the effects of active substance bioconcentration in fish are therefore not required, as previously agreed during the EU review for the EU inclusion of eugenol (EFSA Journal 2012;10(11):2914).

**B.9.2.3. Potential for endocrine disruption**

The available data for eugenol do not show any adverse effects that are considered to be EAS-mediated. However, there are no available guideline studies with eugenol on fish to investigate the potential of endocrine disruption of eugenol. The available toxicology (mammalian) and ecotoxicology data for eugenol showed that the potential for endocrine disruption has not been sufficiently investigated, therefore, further data need to be generated before a conclusion on whether or not the ED criteria are met (Vol. 1, 2.10).

### B.9.2.4. Acute toxicity to aquatic invertebrates

#### B.9.2.4.1. Acute toxicity to *Daphnia magna*

One acute *Daphnia* toxicity study with eugenol was previously evaluated as part of the EU review for the EU inclusion of eugenol DAR (Eugenol, Volume 3, Annex B.9, 2011, B.9.2.1.2). A full summary is provided below (Study B.9.2.4.1/01).

##### Study B.9.2.4.1/01

<b>Data point:</b>	CA 8.2.4.1/01
<b>Report author</b>	Pavić, B., Wydra, V.
<b>Report year</b>	2008
<b>Report title</b>	Acute toxicity of eugenol to <i>Daphnia magna</i> in a static 48-hour immobilization test.
<b>Report No</b>	37982220
<b>Document No</b>	-
<b>Guidelines followed in study</b>	OECD Guideline 202 (2004); Commission Directive 92/69/EEC, Annex Part C, C.2 (1992); EPA Guideline 712-C-96-114: OPPTS 850.1010 (1996)
<b>Deviations from current test guideline</b>	None
<b>Previous evaluation</b>	Yes, evaluated and accepted in the DAR (Eugenol, Volume 3, Annex B.9, 2011, B.9.2.1.2) EU-agreed critical endpoint for EU inclusion of eugenol (EFSA Journal 2012;10(11):2914)
<b>GLP/Officially recognised testing facilities</b>	Yes, conducted under GLP/Officially recognised testing facilities
<b>Acceptability/Reliability:</b>	Yes

#### Executive summary

The 48-hour acute toxicity of eugenol to *Daphnia magna* was studied under static conditions in accordance with OECD Guideline 202 (2004). *Daphnids* were exposed to nominal concentrations of 0.31, 0.63, 1.25, 2.5 and 5 mg eugenol/L and an untreated control and solvent control for 48 hours. Immobilization was observed after 24 and 48 hours of exposure. Analytical verification of test concentrations confirmed measured concentrations were maintained within 99-109 % of nominals throughout the exposure period and biological endpoints were based on nominal concentrations.

The 48-hour EC<sub>50</sub> value for *Daphnia magna* based on immobilisation was calculated to be 1.11 mg eugenol/L (nominal). The 48-hour NOEC value based on immobilisation was determined to be 0.63 mg eugenol/L based on nominal concentrations.

This study is considered acceptable and satisfies the guideline requirements for an acute toxicity study with freshwater invertebrates.

#### Materials and methods

##### *Test material*

Name: Eugenol  
 Formulation type: not applicable  
 Source and lot/batch no.: 95217  
 Active substance content: 98.8% w/w  
 Expiry date of lot/batch: March 2008  
 Storage conditions: In original container in the refrigerator (< 5 °C), under nitrogen, in the dark

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*Test organism*

Species:	<i>Daphnia magna</i> (water flea).
Strain/clone:	Clone 5
Age at study initiation:	< 24 hours old (6.5 – 20.75 hours old)
Source:	Originally sourced from ECT Oekotoxikologie GmbH, Germany. The <i>daphnids</i> were bred in the laboratories of IBACON under similar temperature and light conditions as used in the test. The test organisms were not first brood progeny. Once a week the <i>daphnids</i> in the stock culture were fed with Tetra Min-extract and at least all other working days with green algae ( <i>Desmodesmus subspicatus</i> ).
Feeding during test:	No
Acclimation:	6.5 hours under test conditions

*Test conditions*

Hardness:	250 mg/L as calcium carbonate
Test temperature:	19-20 °C. (Measures were obtained at the start of the study (0 hours) and at the end (48 hours))
pH:	6.8 to 7.8; and thus the pH-value did not vary by more than 1.5 units
Dissolved oxygen:	8.5 – 8.8 mg/L
Conductivity:	< 5 µS/cm
Photoperiod:	16 h light: 8 h dark
Light intensity:	220-300 lux

Test vessels comprised 100 mL glass beakers, each filled with approximately 80 mL of culture medium and covered with a lid to reduce the loss of water due to evaporation and to avoid the entry of dust into the culture medium. A stock solution was prepared by dissolving the product in dimethylformamid (DMF) p.A. (50.0 mg/L). The stock solutions were diluted in a series of sequential dilutions with the solvent to add the same volumes of solvent (0.1 mL/L) to each test solution. The final nominal concentrations of eugenol in the test media were: 0.31, 0.63, 1.25, 2.5 and 5 mg eugenol/L.

Five individual daphnids were added to each test vessel, with four replicate vessels per treatment group. The test solutions were prepared just before introduction of *Daphnia*. Since the product is stable in control medium, a static test was performed. Duplicate analytical samples were taken from all product concentrations at test start (0 hours) and after 48 hours. The contents of the test beakers of each treatment were poured together and afterwards duplicate samples from the pooled test media of all test concentrations and solvent control at both sampling times (0 and 48 hours) were analysed. From the control samples only one of the duplicate samples was analysed at both sampling times.

The immobility of daphnids was determined by visual assessments after 24 and 48 hours. Those animals not able to swim within 15 seconds after gentle agitation of the test beaker were considered to be immobile (even if they could still move their antennae). Statistical analysis was performed using ToxRat Professional v2.09. EC<sub>50</sub> values and 95% confidence limits were calculated, where possible, by Probit analysis. The NOEC and LOEC values were determined directly from the raw data.

## Results

*Analytical results*

The GC-MS analytical method for the determination of eugenol in test medium was validated with regards to specificity, linearity, accuracy and precision. Whilst the report does not refer to the current guideline, overall the validation results presented are in accordance with guideline SANCO/3029/99 rev. 4, 11/07/2000 and are considered fit for purpose. Specificity was demonstrated by the absence of a peak at the characteristic retention time for eugenol in the control sample. The analytical calibration was shown to be linear ( $r = 0.9999$ ) over the range of 0.1-3 mg reference item/L. Accuracy was confirmed with recovery determined by fortification of eugenol at 0.3, 1.0 and 5.0 mg/L; all recoveries at 1.0 and 5.0 mg/L were within the range of 75-117% and mean recoveries were within 91-109% (details for 0.3 mg/L are excluded as this is below the LOQ). Precision was confirmed with four determinations made at each fortification level; the relative standard deviation at 1.0 and

5.0 mg/L was between 7-12% (i.e. within the guideline limit of  $\leq 20\%$ ). The limit of quantification (LOQ) was 1 mg/L, corresponding to 0.494 mg eugenol/L after dilution 1:2 (i.e. below the biological  $EC_{50}/NOEC$  value). The limit of detection (LOD) was 0.007 mg/L in the test medium.

The measured concentrations of eugenol during the 48-hour exposure period of the *Daphnia magna* toxicity study are summarised in the table below.

**Table 9.2.4.1/01-01: Nominal and measured concentrations of eugenol in each replicate**

Nominal conc. (mg product/L)	Duplicate samples	Measured conc. (mg eugenol/L)			Percentage of nominal		
		0 h	48 h	Mean*	0 h	48 h	Mean*
Water control	1	<LOQ	<LOQ	n.a.	n.a.	n.a.	n.a.
Solvent control	1	<LOQ	<LOQ	<LOQ	n.a.	n.a.	n.a.
	2	<LOQ	<LOQ		n.a.	n.a.	
0.31	1	<LOQ	<LOQ	<LOQ	n.a.	n.a.	n.a.
	2	<LOQ	<LOQ		n.a.	n.a.	
0.63	1	<LOQ	<LOQ	<LOQ	n.a.	n.a.	n.a.
	2	<LOQ	<LOQ		n.a.	n.a.	
1.25	1	1.478	1.532	1.344	120	101	109
	2	1.533	1.214		124	101	
2.5	1	2.343	2.415	2.498	95	101	101
	2	2.503	2.731		101	101	
5.0	1	4.772	5.054	4.882	97	99	99
	2	4.610	5.141		93	99	

LOQ = 1 mg product/L, corresponding to 0.494 mg eugenol/L after dilution 1:2

LOD = 0.007 mg eugenol/L

\* arithmetic mean of the 4 measurements for each treatment group (duplicate samples at 0 and 48 hours)

n.a.: not applicable

Conc. concentration

**Table 9.2.4.1/01-02: Summary of nominal and mean measured concentrations of eugenol for 48 hours**

Nominal conc. (mg product/L)	Measured concentration (mg eugenol/L)						
	Water control	Solvent control	0.31	0.63	1.25	2.5	5.0
Range (min: max)	0	<LOQ	<LOQ	<LOQ	1.153-1.532	2.343-2.503	4.610-5.141
Median	n.a.	<LOQ	<LOQ	<LOQ	1.346	2.459	4.888
Mean*	n.a.	n.a.	n.a.	n.a.	1.344	2.498	4.882
% of nominal (ref. to mean)	0	n.a.	n.a.	n.a.	109	101	99

\* arithmetic mean of the 4 measurements for each treatment group (duplicate samples at 0 and 48 hours)

n.a.: not applicable

Conc. concentration

Analytical verification of test concentrations confirmed that measured concentrations of all fresh and aged samples were between 93 – 124 % of nominals. Biological endpoints are reported below based on nominal concentrations.

#### Biological results

A summary of the test effects of eugenol on immobilisation of *Daphnia magna* after 48 hours exposure is presented in the tables below.

**Table 9.2.4.1/01-03: Summary of effects of eugenol on immobilisation of *Daphnia magna* over 48 hours**

Nominal conc. (mg eugenol/L)	No. organisms at test start	Cumulative (no. dead) immobilised		Cumulative (%) immobilised	
		24 h	48 h	24 h	48 h
Water control	20	0	0	0	0
Solvent control	20	0	0	0	0
0.31	20	0	0	0	0
0.63	20	0	0	0	0
1.25	20	0	14	0	70
2.5	20	5	20	25	100
5.0	20	15	20	75	100

Conc. concentration

In the control and up to eugenol concentrations of nominally 0.63 mg eugenol/L, no significant immobility of the test animals or other signs of intoxication were observed during the test period of 48 hours. At the test concentration of 1.25 mg eugenol/L 14 (70%) *Daphnia* were immobile. At the two highest concentrations of 2.5 and 5.0 mg eugenol/L 20 (100%) *Daphnia* were immobile after 48 hours test duration. As a result the following endpoints were obtained, the 48-hour EC<sub>50</sub> (immobilisation) = 1.11 mg eugenol/L (nominal) and the 48-hour NOEC (immobilisation) = 0.63 mg eugenol/L (nominal) (confidence intervals were not reported due to mathematical reasons).

### Validity

All validity criteria were met in accordance with OECD test guideline 202 (2004):

- Immobilisation in the control group was  $\leq 10\%$  (actual value: 0%).
- The dissolved oxygen concentration at the end of the test was  $\geq 3$  mg/L in all test vessels (actual values:  $\geq 8.5$  mg/L).
- Analytical measurement of test concentrations was included.

### Assessment and conclusion

The 48-hour acute toxicity of eugenol to *Daphnia magna* was studied under static conditions in accordance with OECD 202. The 48-hour  $EC_{50}$  value was calculated to be 1.11 mg eugenol/L based on nominal concentrations. This study is considered as acceptable and satisfies the guideline requirements for an acute toxicity study with freshwater invertebrates.

#### **Assessment and conclusion by applicant:**

The study is acceptable.

*Daphnia magna* 48-hour  $EC_{50}$  (immobilisation) = 1.11 mg eugenol/L based on nominal concentrations.

#### **Assessment and conclusion by RMS:**

This study was already evaluated and accepted during Annex I inclusion of Eugenol and it was included in the Eugenol Monograph (Volume 3, Annex B.9 Ecotoxicology, May 2011).

The validity criteria according to OECD guideline 202 (*Daphnia* sp., Acute Immobilisation Test, 2004) were met.

RMS agrees with the previous evaluation.

#### **Agreed endpoints**

**48h- $EC_{50}$  = 1.11 mg eugenol/L<sub>(nom)</sub>**

**48h – NOEC = 0.63 mg eugenol/L<sub>(nom)</sub>**

#### ***B.9.2.4.2. Acute toxicity to an additional aquatic invertebrate species***

Since the active substance does not have an insecticidal mode of action, no further data are required.

It is also noted that several literature papers were identified during the literature search (see B.9.11.1), which assessed the very short-term anaesthesia effects of eugenol on various aquatic shrimp species and their subsequent recovery. Results of these papers are summarised in Appendix I (B.10) for completeness, but as discussed further in B.11.1 these are not considered reliable for use in the risk assessment, principally as analytical verification was not reported to confirm the actual exposure during the studies. The critical endpoint summarised above in B.9.2.4.1 from the standard OECD 202 study is considered more relevant and reliable for the acute risk assessment of aquatic invertebrates, and this OECD study covers any possible short-term behavioural (e.g. anaesthesia) effects on aquatic invertebrates. No further studies are considered necessary.

#### **B.9.2.5. Long-term and chronic toxicity to aquatic invertebrates**

##### ***B.9.2.5.1. Reproductive and development toxicity to *Daphnia magna****

A new chronic toxicity study with *Daphnia magna* has been provided to support the renewal of eugenol; a full summary is provided below.

#### Study B.9.2.5.1/01

<b>Data point:</b>	CA 8.2.5.1/01
<b>Report author</b>	Egeler, P.
<b>Report year</b>	2021
<b>Report title</b>	Eugenol: A Study on the Chronic Toxicity to <i>Daphnia magna</i>
<b>Report No</b>	20GC3DB
<b>Document No</b>	-
<b>Guidelines followed in study</b>	OECD Test Guideline 211 (2012)
<b>Deviations from current test guideline</b>	None
<b>Previous evaluation</b>	No
<b>GLP/Officially recognised testing facilities</b>	Yes, conducted under GLP/Officially recognised testing facilities
<b>Acceptability/Reliability:</b>	Yes

#### Executive summary

The chronic toxicity of eugenol to *Daphnia magna* was studied under static-renewal conditions in accordance with OECD Test Guideline 211 (2012). Daphnids were exposed to nominal concentrations of 7.81, 15.6, 31.3, 62.5 and 125 µg eugenol/L (corresponding to mean measured concentrations of 4.50, 10.8, 21.0, 47.1 and 95.9 µg eugenol/L) and an untreated control (Elendt medium M4) for 21 days.

Adult daphnids were observed daily for immobility, presence of eggs and mortality. They were transferred to fresh media 3 times per week. Any juveniles produced were counted and removed daily.

Analytical verification of the test item concentrations indicated they were unstable in the test solutions, decreasing to <80% of the nominal concentrations. Therefore, biological endpoints are based on mean measured concentrations.

No concentration-response relationship was observed for reproduction, therefore, EC<sub>x</sub> values could not be calculated. The 21-day NOEC value for *Daphnia magna*, based on the total number of living offspring per surviving *Daphnia magna* parental daphnids, was determined to be 95.9 µg eugenol/L and the corresponding 21-day LOEC value was estimated to be >95.9 µg eugenol/L (mean measured).

This study is considered acceptable and satisfies the requirements for a chronic toxicity study with freshwater invertebrates (OECD test guideline 211, 2012).

#### Materials and methods

##### *Test material*

Name:	Eugenol
Chemical Name:	2-Methoxy-4-(2-propenyl)phenol
Formulation type:	not applicable
Source and lot/batch no.:	40002011619
Active substance content:	99.74%
Appearance:	Pale yellow liquid
Expiry date of lot/batch:	31 July 2021
Storage conditions:	In original container at ambient temperature, in the dark

##### *Test organism*

Species:	<i>Daphnia magna</i> (water flea).
Strain/clone:	M10
Age at study initiation:	<24 hours old

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Source:	Originally supplied by KU Leuven, Belgium, cultured at ECT Oekotoxikologie GmbH since December 22, 2011
Feeding during test:	Three times per week with fresh algae suspension
Acclimation:	Not applicable

#### *Test conditions*

Test medium:	Elendt medium M4
Hardness:	250 - 268 mg/L as CaCO <sub>3</sub>
Test temperature:	19.7 – 21.6 °C* (manual measurement)
pH:	7.7 – 8.9*
Dissolved oxygen:	9.2 – 11.2 mg/L*
Photoperiod:	16 hours of light / 8 hours of dark
Light intensity:	16.45 – 17.36 μE m <sup>-2</sup> s <sup>-1</sup>

\*Measured (x 2) on days 0, 3, 10, 12, 17 and 19 of the test

Ten replicate vessels, each a glass beaker containing a single daphnid in 50-60 mL medium, were allocated to each test concentration and control. Test solutions were renewed three times per week (semi-static test system). The daphnids were fed three times per week after transfer to fresh test solutions. Daily observations were made of the parental daphnids in all test vessels; immobile parental daphnids were removed upon recording. From day 8, onwards, the live offspring (F1 generation) was counted daily and removed from the vessels. Deviations in behaviour compared to the control animals, presence of aborted eggs or dead offspring were recorded.

Samples of test media were taken at the start and end of media renewal cycles, during the 21-day exposure period, and analysed for eugenol by GC-MS. Water quality parameters (dissolved oxygen concentration, pH, water hardness and temperature (manual measurement)) were determined once per test week in fresh and aged solutions of the control and the highest test item concentration.

The Cochran-Armitage test procedure was applied with immobility at 21 days to detect an increasing trend in responses (Alpha: 0.050; one-sided greater). Determination of EC<sub>x</sub> values for parental immobility by Probit analysis was not possible due to the lacking concentration-response relationship. Parental immobility was additionally corrected for control immobility using Abbott's formula. Determination of EC<sub>x</sub> values for reproduction, using non-linear regression analysis, was not possible due to the poor concentration-response relationship. Prior to threshold concentration testing, a qualitative trend analysis by contrasts was applied to check for monotonicity of the concentration-response relationship. Dunnett's multiple t-test procedure (p≤0.05) was used to determine the threshold concentrations for reproduction. Fisher's Exact Binomial Test with Bonferroni correction was used to determine the threshold concentration for parental immobility. The statistical software package ToxRat Professional 3.3.0 (ToxRat Solutions GmbH, Naheweg 15, D-52477 Alsdorf) was used for these calculations using the nominal concentrations.

## **Results**

### *Analytical results*

The GC-MS analytical method for the determination of eugenol in test medium was validated with regards to specificity, linearity, accuracy and precision in accordance with guideline SANCO/3029/99 rev. 4, 11/07/2000. Specificity was demonstrated by the absence of a peak at the characteristic retention time for eugenol in the control sample. The analytical calibration was shown to be linear (r = 0.9951) over the range of 1 - 200 μg eugenol/L. Accuracy was confirmed with recovery determined by fortification of eugenol at 3.5 and 150 μg eugenol/L; all recoveries were within the range of 81-110% and mean recoveries were within 82-107% (i.e. within the guideline range of 70-110%). Precision was confirmed with five determinations made at each fortification level; the relative standard deviation was between 0.9-4.9% (i.e. within the guideline limit of ≤20%). The limit of quantification (LOQ) was 3.50 μg eugenol/L (i.e. below the biological NOEC value). The limit of detection (LOD) was 1.05 μg eugenol/L in test medium. All samples were analysed within 24 h after extraction, therefore the stability of eugenol in the final extracts was not assessed.

The measured concentrations of eugenol during the 21-day exposure period of the *Daphnia magna* toxicity study are summarised in the tables below.

Table 9.2.5.1/01-1: Nominal and measured concentrations of eugenol in each replicate during the 21-day study

Nominal concentration (µg eugenol/L)	Measured concentration (µg eugenol/L)							Percentage of nominal (%)						
	Day						Mean <sup>a</sup>	Day						Mean <sup>a</sup>
	0F	3A	10F	12A	17F	19A		0F	3A	10F	12A	17F	19A	
Control	<LOD	-	-	-	-	<LOQ (1.36)*	-	-	-	-	-	-	-	-
7.81	6.82	3.83	7.58	<LOQ (1.93)	8.01	<LOQ (1.47)	4.50	87.3	49.0	97.1	n.a	103.0	n.a	57.6
15.6	14.0	10.5	16.5	4.41	15.2	6.66	10.8	89.7	67.3	106	28.3	97.4	n.a	69.2
31.3	27.3	19.5	28.6	9.78	31.6	13.4	21.0	87.2	62.3	91.4	31.2	101	42.7	67.1
62.5	63.2	40.7	64.0	29.1	65.7	27.5	47.1	101	65.1	102	46.6	105	42.8	75.4
125	127	81.2	130	65.1	135	53.9	95.9	102	65.0	104	52.1	108	44.0	76.7

<sup>a</sup> time weighted mean

n.a. = not applicable

\*both qualifier mass fragments yielded a residue &lt; LOD

F: fresh media; A: aged media

The measured concentrations of 7.81 µg eugenol/L (nominal) were below LOQ but above LOD (measured values shown in brackets in table above) on days 12 and 19. The measured values were used for calculation of the time-weighted mean (TWM) as shown in the following table.

**Table 8.2.5.1/01-2: Summary of nominal and time-weighted mean measured concentrations of eugenol during the 21-day study**

Nominal concentration (µg eugenol/L)	Measured concentration (µg eugenol/L)					
	Control	7.81	15.6	31.3	62.5	125
Range (min - max)	-	4.41 – 16.5	9.78 – 31.6	13.4–28.6	27.5–65.7	53.9 –135
Mean*	-	4.50	10.8	21.0	47.1	95.9
% of nominal	-	57.6	69.2	67.1	75.4	76.7

\* time weighted mean

Since the analytical verification of the test item concentrations confirmed that measured concentrations were unstable and below 80% of nominal concentrations, the biological endpoints are therefore based on mean measured (time-weighted) concentrations.

#### *Biological results*

Summaries of the effects of eugenol on parental daphnid survival/immobility and the fecundity of the introduced and surviving parent daphnids are presented in the tables below.

**Table 9.2.5.1/01-3: Total mobility/immobility of parental daphnids at the end of the 21-day study**

Nominal concentration (µg eugenol/L)	Mean measured concentration (µg eugenol/L)	Number of daphnids			% Immobility
		Introduced	Mobile	Immobile	
Control	Control	10	9	1	10.0
7.81	4.50	9 <sup>1</sup>	9	0	0.0
15.6	10.8	10	7	3	30.0
31.3	21.0	9 <sup>1</sup>	8	1	11.1
62.5	47.1	10	9	1	10.0
125	95.9	10	9	1	10.0

<sup>1</sup> dead parental daphnid after unintended handling error: excluded from all further analysis

One parental daphnid at nominal test concentrations of 7.81 µg eugenol/L and 31.3 µg eugenol/L died following documented unintended handling errors, before first production of offspring. These replicates were excluded from all further analysis. For any other immobile parental daphnids, a concentration-response relationship could not be confirmed.

A few sublethal effects were observed in the living parental daphnids at all concentration levels, but no concentration-response relationship based on sublethal effects in living parental daphnids was determined.

Table 9.2.5.1/01-4: Summary of effects of eugenol on the total number of living offspring per surviving *Daphnia magna* parent after 21-days' exposure

Nominal concentration (µg eugenol/L)	Mean measured concentration (µg eugenol/L)	Replicate	Cumulative number of live juveniles per surviving parent at 21 days		
			Per test vessel	Mean	% reduction relative to control*
Control	-	1	132	129.4	-
		2	152		
		3	157		
		4	102		
		5	179		
		6	108		
		7	102		
		8	125		
		9	-		
		10	108		
7.81	4.50	1	74	128.1	1.0
		2	108		
		3	114		
		4	+		
		5	150		
		6	157		
		7	111		
		8	152		
		9	163		
		10	124		
15.6	10.8	1	-	133.7	-3.3
		2	-		
		3	92		
		4	123		
		5	177		
		6	146		
		7	61		
		8	185		
		9	152		
		10	-		
31.3	21.0	1	173	156.1	-20.6
		2	-		
		3	+		
		4	97		
		5	195		
		6	145		
		7	159		
		8	191		
		9	124		
		10	165		
62.5	47.1	1	131	138.2	-6.8
		2	-		
		3	102		
		4	166		
		5	135		
		6	131		
		7	163		
		8	125		
		9	131		
		10	160		

Nominal concentration (µg eugenol/L)	Mean measured concentration (µg eugenol/L)	Replicate	Cumulative number of live juveniles per surviving parent at 21 days		
			Per test vessel	Mean	% reduction relative to control*
125	95.9	1	117	135.4	-4.6
		2	126		
		3	127		
		4	107		
		5	-		
		6	161		
		7	163		
		8	93		
		9	149		
		10	176		

+ = documented handling accident: excluded from all evaluation

- = inadvertent mortality (unknown cause): offspring excluded from statistical analysis

\* % offspring reduction compared to control (negative values = higher number than control)

The total number of living offspring was evaluated per surviving parent daphnid and per introduced parent daphnid, which did not die accidentally or inadvertently during the test. No concentration-response relationship was observed for reproduction.

Since no concentration-response relationship was observed for reproduction, EC<sub>x</sub> values could not be calculated, but are estimated to be greater than the highest concentration tested (i.e. >95.9 µg eugenol/L (mean measured)). The 21-day NOEC value for *Daphnia magna*, based on the total number of living offspring per surviving *Daphnia magna* parental daphnids, was determined to be 95.9 µg eugenol/L and the corresponding LOEC value was estimated to be >95.9 µg eugenol/L (mean measured).

#### Validity

All validity criteria were met in accordance with OECD test guideline 211 (2012):

- The mortality of the parent animals (female *Daphnia*) in the controls does not exceed 20% at the end of the test; (actual value: 10%)
- The mean number of living offspring produced per surviving parent animal in the controls at the end of the test is >60 (actual value: 134.5%)
- Analytical measurement of test concentrations was included.

#### Assessment and conclusion

The 21-day chronic toxicity of eugenol to *Daphnia magna* was studied under static-renewal conditions in accordance with OECD test guideline 211 (2012). Since no concentration-response relationship was observed for reproduction, EC<sub>x</sub> values could not be calculated.

The 21-day NOEC value for *Daphnia magna*, based on the total number of living offspring per surviving *Daphnia magna* parental daphnids, was determined to be 95.9 µg eugenol/L and the corresponding 21-day LOEC value was estimated to be >95.9 µg eugenol/L (mean measured).

**Assessment and conclusion by applicant:**

The study is acceptable.

*Daphnia magna* 21-day NOEC (reproduction) = 95.9 µg eugenol/L (mean measured), based on the total number of living offspring per surviving *Daphnia magna* parental daphnids

Since no concentration-response relationship was observed for reproduction, EC<sub>x</sub> values could not be calculated, but are estimated to be greater than the highest concentration tested (i.e. >95.9 µg eugenol/L (mean measured)).

**Assessment and conclusion by RMS:**

This study was submitted for the current renewal proposal of Eugenol.

The validity criteria according to OECD guideline 211 (*Daphnia magna* Reproduction Test, 2012) were met.

The endpoints evaluated were mortality (F<sub>0</sub>) and fecundity (total number of living offspring (F<sub>1</sub>) per parental animal (F<sub>0</sub>)). No statistically significant differences were observed in mortality (immobility) or reproduction: Furthermore, no dose-response relationship could be established for the measured endpoints, thus no reliable EC<sub>x</sub> values could be calculated.

Therefore, the NOEC of this study was the highest tested concentration, and the EC<sub>50</sub> was considered to be higher than the highest tested concentration.

Since test item concentration were not maintained ± 20%, the endpoints were referred to time-weighted means of the measured concentrations.

The study is considered acceptable

**Agreed endpoints**

**21d-EC<sub>50</sub> > 95.9 µg eugenol/L<sub>(mm)</sub>**

**21d – NOEC = 95.9 µg eugenol/L<sub>(mm)</sub>**

***B.9.2.5.2. Reproductive and developmental toxicity to an additional aquatic invertebrate species***

Since the active substance does not have an insecticidal mode of action, no further data are required.

***B.9.2.5.3. Development and emergence in *Chironomus riparius****

Since the active substance does not have an insecticidal mode of action, no further data are required.

***B.9.2.5.4. Sediment dwelling organisms***

Accumulation of eugenol in aquatic sediment is not expected due to its rapid volatilisation properties and ready biodegradation. No further toxicity data on sediment dwelling organisms are required, as previously agreed during the EU inclusion of eugenol (EFSA Journal 2012; 10(11):2914).

**B.9.2.6. Effects on algal growth*****B.9.2.6.1. Effects on growth of green algae***

One green algal study with eugenol was previously evaluated as part of the EU review for the EU inclusion of eugenol (DAR, Volume 3, Annex B.9, 2011, B.9.2.1.3). This study is considered appropriate for the current assessment to support renewal of eugenol; full summary is provided below.

#### Study B.9.2.6.1/01

<b>Data point:</b>	CA 8.2.6.1/01
<b>Report author</b>	Meister Werner, A., Wydra, V.
<b>Report year</b>	2008
<b>Report title</b>	Toxicity of eugenol to <i>Pseudokirchneriella subcapitata</i> in an algal growth inhibition test.
<b>Report No</b>	37981210
<b>Document No</b>	-
<b>Guidelines followed in study</b>	OECD Guideline for Testing of Chemicals, Section 2, No. 201: "Freshwater Alga and Cyanobacteria, Growth Inhibition Test", (2006) EPA Guideline 712-C-96-164: OPPTS 850.5400, "Algal Toxicity, Tiers I and II", (1996)
<b>Deviations from current test guideline</b>	No
<b>Previous evaluation</b>	Yes, evaluated and accepted in the DAR (Eugenol, Volume 3, Annex B.9, 2011, B.9.2.1.3) EU-agreed critical endpoint for EU inclusion of eugenol (EFSA Journal 2012;10(11):2914)
<b>GLP/Officially recognised testing facilities</b>	Yes, conducted under GLP/Officially recognised testing facilities
<b>Acceptability/Reliability:</b>	Yes

#### Executive summary

The purpose of this test was to determine the inhibitory effect of the product eugenol on the growth of the freshwater green algal species *Pseudokirchneriella subcapitata*. The 96-hour toxicity of eugenol to *Pseudokirchneriella subcapitata* was studied in an algal growth inhibition test in accordance with OECD 201. Test species were exposed to control, solvent control, and test chemical at nominal concentrations of 4, 8, 16, 32 and 64 mg eugenol/L for 96 hours. Analytical verification of test concentrations confirmed measured concentrations were maintained within 95-106 % of nominals at the start of the exposure period. After 96 hours of exposure the mean measured product concentrations were 55% (22-89%) of the nominal values (average for the test concentrations of 16, 32 and 64 mg/L; at lower test concentrations the measured values were below the LOQ at the end of exposure). The product was not stable over the test period of 96 hours under the test conditions. The average exposure was calculated to be 76% of nominal (geometric mean of measured values at start and end); therefore the endpoints based on mean measured concentrations are given in the report.

The 72-hour  $E_rC_{50}$  value for *Pseudokirchneriella subcapitata* was calculated to be 15.4 mg eugenol/L based on mean measured concentrations. The 72-hour  $E_bC_{50}$  (biomass) was calculated to be 10.0 mg eugenol/L (mean measured). The 72-hour  $E_yC_{50}$  (yield) was calculated to be 10.8 mg eugenol/L (mean measured). The 72-hour  $EC_{10}$  values were calculated to be 11.1, 4.9 and 6.1 mg eugenol/L for the growth rate, biomass and yield, respectively (values based on mean measured concentrations). The 72-hour NOEC value based on growth rate was determined to be 12.2 mg eugenol/L based on mean measured concentrations. This study is considered as acceptable and satisfies the guideline requirements for an inhibition study with algae species (OECD test guideline 201 (2006)).

#### Materials and methods

##### Test material

Name: Eugenol  
 Formulation type: Not applicable  
 Source and lot/batch no.: 95217  
 Active substance content: 98.8 % w/w  
 Expiry date of lot/batch: March 2008 (sponsor information)  
 Storage conditions: In original container in the refrigerator (< 5°C), under nitrogen. in the dark.

*Test organism*

Species:	Alga ( <i>Pseudokirchneriella subcapitata</i> )
Strain/clone:	Strain No. 61, 81 SAG
Source:	Supplied by the “Sammlung von Algenkulturen, Pflanzenphysiologisches Institut der Universität Göttingen”, 37073 Göttingen, Germany. The algae were cultivated in the laboratories of Ibacon under standardised conditions according to the test guidelines.
Feeding during test:	Not applicable
Acclimation:	Algal cells were taken from an exponentially growing pre-culture, which was set up 3 days prior to the test start under the same conditions as in the test.

*Test conditions*

Hardness:	24 mg/L as calcium carbonate
Test temperature:	21-24 °C
pH:	7.9 – 8.1 at test start; 7.5 – 7.6 at test end
Photoperiod:	Continuous illumination
Light intensity:	4580 – 5260 lux; 4946 lux (mean)

The toxicity of eugenol to *Pseudokirchneriella subcapitata* was tested in an algal growth inhibition test. A stock solution of eugenol was prepared by dissolving it in the solvent dimethylformamide (DMF); the stock solution was then serially diluted with DMF to obtain a set of solvent solutions. The final test concentrations were prepared by adding these solvent solutions to the culture media.

There were five test concentrations: 4, 8, 16, 32 and 64 mg eugenol/L, a control containing culture medium only, and a solvent control containing 100 mg DMF/L.

Exponentially growing cultures of *P. subcapitata* were inoculated at 5000 cells/mL, and cultured for 96 hours. Measured concentrations of eugenol were determined at 0 and 96 hours. Algal cell numbers were determined spectrophotometrically after approximately 24, 48, 72 and 96 hours. Cell densities were determined by means of a regression between absorption and cell densities, which had been calculated previously using a light microscope on a dilution series of control samples. A sample was taken from the test concentration of 32 mg/L and examined microscopically.

Based on the calculated cell densities, the corresponding EC<sub>50</sub> values and where possible their 95%-confidence limits were calculated by Probit analysis.

For the determination of the LOEC and NOEC, the calculated growth rates, areas under the growth curve and yields at each test concentration were tested for significant differences compared to the control values with Bonferroni-t Test (growth rate), Williams test (yield) and Bonferroni t-Test (area under the growth curve) respectively. The software used to perform the statistical analysis was ToxRat Professional, Version 2.09, ToxRat® Solutions GmbH.

## Results

*Analytical results*

The GC-MS analytical method for the determination of eugenol in test medium was validated with regards to specificity, linearity, accuracy and precision. Whilst the report does not refer to the current guideline, overall the validation results are in accordance with guideline SANCO/3029/99 rev. 4, 11/07/2000 and are considered fit for purpose. Specificity was demonstrated by the absence of a peak at the characteristic retention time for eugenol in the control sample. The analytical calibration was shown to be linear ( $r = 0.9982$ ) over the range of 3-20 mg product/L and 5-30 mg product/L (low range and high range respectively). Accuracy was confirmed with recovery determined by fortification of eugenol at 4, 8 and 60 mg reference item/L; all recoveries were within the range of 80-117% and mean recoveries were within 83-114% (i.e. very slightly above the guideline range of 70-110%, but considered fit for purpose). Precision was confirmed with two determinations made at each fortification level; the relative standard deviation was between 2 - 4.3% (i.e. within the guideline limit of  $\leq 20\%$ ). The limit of quantification (LOQ) was 4 mg product/L after dilution 1:2 (i.e. below the biological ErC<sub>50</sub>/NOEC value). The limit of detection (LOD) was 0.075 mg/L in test medium.

**Table 9.2.6.1/01-01: Nominal and measured concentrations of eugenol in each replicate**

Nominal conc. (mg product/L)	Duplicate samples	Measured conc. (mg eugenol/L)			Percentage of nominal		
		0 h	96h	Mean*	0 h	96 h	Mean*
Water control	1	<LOD	<LOD	n.a.	n.a	n.a.	n.a
	2						
Solvent control	1	<LOQ	<LOQ	n.a.	n.a.	n.a.	n.a.
	2						
4	1	4.550	<LOD	4.635 <sup>a</sup>	114	n.a.	116
	2	4.720	<LOD		118	n.a.	
8	1	8.184	<LOD	8.505 <sup>a</sup>	102	n.a.	106
	2	8.862	<LOD		111	n.a.	
16	1	15.220	3.562	12.268 <sup>a</sup>	95	22	77
	2	18.021	<LOD		113	n.a.	
32	1	32.715	15.768	25.282	102	49	79
	2	34.209	18.435		107	58	
64	1	60.700	57.019	58.625	95	89	92
	2	60.539	56.240		95	88	

<sup>a</sup> the aged test media with concentrations <LOD were not considered for the calculations.

LOQ = 4 mg product/L, corresponding to 2 mg eugenol/L after dilution 1:2

LOD = 0.075 mg eugenol./L

\* arithmetic mean of the 2-4 measurements for each treatment group (duplicate samples at 0 and 96 hours)

n.a.: not applicable

Conc. concentration

**Table 9.2.6/01-02: Summary of nominal and mean measured concentrations of eugenol for 96 hours**

Nominal concentration (mg eugenol/L)	Measured concentration (mg eugenol/L)						
	Water control	Solvent control	4	8	16	32	64
Range (min: max)	n.a.	n.a.	<LOD – 4.720	<LOD – 8.862	<LOD – 18.021	<LOD – 34.209	<LOD – 60.539
Median	n.a.	n.a.	4.635	8.505	15.22	25.575	58.779
Mean*	n.a.	n.a.	4.635 <sup>a</sup>	8.505 <sup>a</sup>	12.268 <sup>a</sup>	25.282	58.625
% of nominal (ref. to mean)	n.a.	n.a.	116	106	77	79	92

<sup>a</sup> the aged test media with concentrations <LOD were not considered for the calculations.

\* Arithmetic mean of the 2-4 measurements for each treatment group (duplicate samples at 0 and 96 hours)

n.a.: not applicable

At test start (0 hours) all measured concentrations were within 95 – 118% of nominals (mean value of all freshly prepared media = 105% of nominal concentrations). After 96 hours, the measured concentrations were below the limit of detection at the two lowest nominal test concentrations of 4 and 8 mg eugenol/L. At the three highest nominal test concentrations of 16, 32 and 64 mg eugenol/L (which cover the range of the EC<sub>50</sub> values), measured concentrations in the 96-hour aged media ranged between 22 and 89% of nominals) (mean value of the aged media = 55% of nominal concentrations). The geometric mean of all test media as a % of nominals across the 96-hour exposure period was calculated from the mean value of the freshly prepared media (105% of nominal) and the mean value of the aged media (55% of nominal), to give an overall geometric mean of 76% of nominal concentrations. Biological results are presented based on the geometric mean measured test concentrations.

#### Biological results

A summary of the effects of eugenol on biomass, growth rate and yield of *Pseudokirchneriella subcapitata* after 96 hours exposure are presented in the tables below.

**Table 9.2.6/01-03: Algal cell densities during the test period 96 hours.**

Nominal conc. (mg eugenol/L)	Mean measured conc. (mg eugenol/L)	Rep	Cell density (10000 cells/mL)					Mean biomass <sup>a</sup>	Mean biomass <sup>b</sup>
			0 h	24 h	48 h	72 h	96 h		
Control	Control	1	0.5	3.951	14.580	77.131	266.417	233.847	83.433
		2	0.5	3.951	15.194	93.893	250.473		
		3	0.5	4.973	9.879	69.976	209.191		
		4	0.5	4.155	12.332	96.550	257.426		
		5	0.5	3.747	14.785	89.804	216.131		
		6	0.5	4.564	14.989	73.247	203.458		
Solvent control	Solvent control	1	0.5	3.338	11.541	72.225	263.964	226.011	67.217
		2	0.5	3.747	10.697	57.303	225.534		
		3	0.5	2.316	6.813	49.535	210.612		
		4	0.5	2.929	14.172	86.125	251.495		
		5	0.5	2.316	14.580	72.020	201.005		
		6	0.5	2.316	10.083	66.092	203.458		
4	4.635	1	0.5	3.747	13.558	6.2413	203.658	267.711	97.527
		2	0.5	0.680	10.288	79.992	273.162		
		3	0.5	3.747	9.675	7.9175	271.936		
8	8.505	1	0.5	1.702	10.697	62.413	240.456	229.214	73.860
		2	0.5	0.476	10.288	79.992	215.927		
		3	0.5	0.680	9.675	79.175	231.258		
16	12.268	1	0.5	0.751	5.177	37.066	180.768	183.153	34.204
		2	0.5	0.680	4.973	34.817	182.608		
		3	0.5	-0.546	4.973	30.729	186.083		
32	25.282	1	0.5	1.702	2.929	0.272	40.950	30.456	0.340
		2	0.5	2.520	3.542	1.702	15.602		
		3	0.5	0.680	-0.342	-0.955	34.817		
64	58.625	1	0.5	1.702	3.133	0.067	12.536	9.266	-0.069
		2	0.5	-0.137	1.294	-0.546	10.697		
		3	0.5	1.498	2.111	0.272	4.564		

a mean values after 96 hours

b mean values after 72 hours

at the start of 5000 cells/mL

**Table 9.2.6/01-04: Effect of eugenol on biomass (area under the growth curve) for *Pseudokirchneriella subcapitata***

Nominal conc. (mg product/L)	Mean measured conc. (mg eugenol/L)	Area under the growth curves A [ $\times 10^4$ ]				% of inhibition of $\mu$			
		0 – 24 hours	0 – 48 hours	0 - 72 hours	0 - 96 hours	0 – 24 hours	0 – 48 hours	0 - 72 hours	0 - 96 hours
Control	Control	1.862	10.287	58.317	216.457	0.0	0.0	0.0	0.0
Solvent control	Solvent control	1.163	7.732	46.495	192.609	37.5	24.8	20.3	11.0
4	4.635	1.112	10.798	67.907	250.049	40.3	-5.0	-16.4	-15.5
8	8.505	0.226	5.313	46.853	197.889	87.8	48.4	19.7	8.6
16	12.268	-0.137	1.997	21.120	129.298	107.3	80.6	63.8	40.3
32	25.282	0.567	1.963	2.870	17.928	69.5	80.9	95.1	91.7
64	58.625	0.283	1.407	2.053	6.242	84.8	86.3	96.5	97.1

Conc.: concentration; Rep.: replicate

<sup>a</sup> Treatment mean area under the growth curve (biomass) to 96 hours

<sup>b</sup> % inhibition relative to the control. Negative values indicate an increase relative to the control.

\* Statistically significant difference compared to the control (Williams' test,  $p < 0.05$ ).

**Table 9.2.6/01-05: Effect of eugenol on the growth rate of *Pseudokirchneriella subcapitata* during the test period**

Nominal conc. (mg product/L)	Mean measured conc. (mg eugenol/L)	Growth rates $\mu$ [1/day]				% of inhibition of $\mu$			
		0 – 24 hours	0 – 48 hours	0 - 72 hours	0 - 96 hours	0 – 24 hours	0 – 48 hours	0 - 72 hours	0 - 96 hours
Control	Control	2.129	1.647	1.703	1.536	0.0	0.0	0.0	0.0
Solvent control	Solvent control	1.713	1.544	1.629	1.527	19.5	6.2	4.4	0.6
4	4.635	1.445	1.769	1.758	1.571	32.1	-7.4	-3.2	-2.3
8	8.505	0.495	1.508	1.663	1.532	76.8	8.4	2.4	0.2
16	12.268	-5.575	1.155	1.407	1.476	361.9	29.9	17.4	3.9
32	25.282	1.050	-0.799	-0.878	1.007	50.7	148.5	151.6	34.4
64	58.625	-2.065	0.704	-1.237	0.708	197.0	57.2	172.6	53.9

Negative% inhibition indicates an increase in growth rate relative to that of the control.

**Table 9.2.6/01-06: Effect of eugenol on the yield of *Pseudokirchneriella subcapitata* during the test period**

Nominal conc. (mg eugenol/L)	Mean measured conc. (mg eugenol/L)	Yield y [mg/L]				% of inhibition of y			
		0 – 24 hours	0 – 48 hours	0 - 72 hours	0 - 96 hours	0 – 24 hours	0 – 48 hours	0 - 72 hours	0 - 96 hours
Control	Control	3.72	13.13	82.93	233.35	0.0	0.0	0.0	0.0
Solvent control	Solvent control	2.327	10.810	66.717	225.511	37.5	17.6	19.6	3.4
4	4.635	2.224	17.147	97.072	267.211	40.3	-30.6	-17.0	-14.5
8	8.505	0.453	9.720	73.360	228.714	87.8	26.0	11.5	2.0
16	12.268	-0.273	4.541	33.704	182.653	107.3	65.4	59.4	21.7
32	25.282	1.134	1.657	0.158	29.956	69.5	87.4	99.8	97.2
64	58.625	0.567	1.679	-0.387	8.799	84.4	87.2	100.5	96.2

Negative % inhibition indicates increase in growth rate relative to that of the control.

The control and the solvent control did not show any significant difference in cell densities over the 96-hour test period (Student t-test).

The 72-hour  $E_xC_{50/10}$  values for *Pseudokirchneriella subcapitata* were calculated to be (mean measured based on 76% of nominal values):

The 72-hour  $E_rC_{50}$  (growth rate) was calculated to be 15.4 mg eugenol/L (mean measured).

The 72-hour  $E_bC_{50}$  (biomass) was calculated to be 10.0 mg eugenol/L (mean measured).

The 72-hour  $E_yC_{50}$  (yield) was calculated to be 10.8 mg eugenol/L (mean measured).

The 72-hour  $E_rC_{10}$  (growth rate) was calculated to be 11.1 mg eugenol/L (mean measured).

The 72-hour  $E_bC_{10}$  (biomass) = 4.9 mg eugenol/L (mean measured).

The 72-hour  $E_yC_{10}$  (yield) = 6.1 mg eugenol/L (mean measured).

The 72-hour NOEC values for *Pseudokirchneriella subcapitata* were determined to be:

The 72-hour  $NOE_rC$  was determined to be 12.2 mg product/L for growth rate (mean measured).

The 72-hour  $NOE_bC$  was determined to be 3.0 mg eugenol/L for biomass (mean measured).

The 72-hour  $NOE_yC$  was determined to be 6.1 mg eugenol/L for yield (mean measured).

The 72-hour LOEC values for *Pseudokirchneriella subcapitata* were determined to be:

The 72-hour  $LOE_rC$  was determined to be 24.3 mg eugenol/L for growth rate (mean measured).

The 72-hour  $LOE_bC$  was determined to be 6.1 mg eugenol/L for biomass (mean measured).

The 72-hour  $LOE_yC$  was determined to be 12.2 mg eugenol/L for yield (mean measured)

For completeness, the 96-hour  $E_xC_{50/10}$  values for *Pseudokirchneriella subcapitata* were calculated to be:

The 96-hour  $E_rC_{50}$  (growth rate) was calculated to be 41.6 mg eugenol/L (mean measured).

The 96-hour  $E_bC_{50}$  (biomass) was calculated to be 13.4 mg eugenol/L (mean measured).

The 96-hour  $E_yC_{50}$  (yield) was calculated to be 16.1 mg eugenol/L (mean measured).

The 96-hour  $E_rC_{10}$  (growth rate) = 12.6 mg eugenol/L (mean measured).

The 96-hour  $E_bC_{10}$  (biomass) = 7.22 mg eugenol/L (mean measured).

The 96-hour  $E_yC_{10}$  (yield) = 10.1 mg eugenol/L (mean measured).

The 96-hour NOEC values for *Pseudokirchneriella subcapitata* were determined to be:

The 96-hour  $NOE_rC$  was determined to be 12.2 mg product/L for growth rate (mean measured).

The 96-hour  $NOE_bC$  was determined to be 6.1 mg eugenol/L for biomass (mean measured).

The 96-hour  $NOE_yC$  was determined to be 6.1 mg eugenol/L for yield (mean measured).

The 96-hour LOEC values for *Pseudokirchneriella subcapitata* were determined to be:

The 96-hour  $LOE_rC$  was determined to be 24.3 mg eugenol/L for growth rate (mean measured).

The 96-hour  $LOE_bC$  was determined to be 12.2 mg eugenol/L for biomass (mean measured).

The 96-hour  $LOE_yC$  was determined to be 12.2 mg eugenol/L for yield (mean measured)

#### Validity

All validity criteria were met in accordance with OECD test guideline 201 (2006):

- The biomass in the control cultures increased exponentially by a factor of at least 16 within 72 hours (actual value: increased by a factor of 167)
- The mean coefficient of variation for section-by-section specific growth rates in the control cultures did not exceed 35% (actual value: 34.1%).
- The coefficient of variation of average specific growth rates during the whole test period in control replicates did not exceed 7% (actual value: 2.7% (0-72h)).
- Analytical measurement of test concentrations was included.

#### Assessment and conclusion

In a 96-hour toxicity study, cultures of *Pseudokirchneriella subcapitata* were exposed to eugenol at nominal concentrations of 0, 4, 8, 16, 32 and 64 mg eugenol/L under static-dose response test conditions in accordance with the OECD 201 (2006). Analytical verification of test concentrations confirmed that measured concentrations of all fresh samples were between 95 and 118% of nominal and the mean value of the aged media (55% of nominal), to give an overall geometric mean of 76% of nominal concentrations. Biological results are presented based on the geometric mean measured test concentrations.

The 72-hour  $E_rC_{50}$  (growth rate) for *Pseudokirchneriella subcapitata* was calculated to be 15.4 mg eugenol/L (mean measured), 72-hour  $E_bC_{50}$  (biomass) was calculated to be 10.0 mg eugenol/L (mean measured) and the 72-hour  $E_yC_{50}$  (yield) was calculated to be 10.8 mg eugenol/L (mean measured), and this study is considered acceptable and valid.

#### **Assessment and conclusion by applicant:**

The study is acceptable.

##### *Pseudokirchneriella subcapitata*

72-hour  $E_rC_{50}$  (growth rate) = 15.4 mg eugenol/L (mean measured).

72-hour  $E_bC_{50}$  (biomass) = 10.0 mg eugenol/L (mean measured).

72-hour  $E_yC_{50}$  (yield) = 14.2 mg eugenol/L (mean measured).

72-h  $EC_{10}$  (growth rate) = 11.1 mg eugenol/L (mean measured).

72-h  $EC_{10}$  (biomass) = 4.9 mg eugenol/L (mean measured).

72-h  $EC_{10}$  (yield) = 6.1 mg eugenol/L (mean measured).

72-hour  $E_xC_{20}$  values were not calculated in the original report but further calculation is not considered necessary as these endpoints will not be used in the risk assessment.

**Assessment and conclusion by RMS:**

This study was already evaluated and accepted during Annex I inclusion of Eugenol and it was included in the Eugenol Monograph (Volume 3, Annex B.9 Ecotoxicology, May 2011).

The validity criteria according to OECD guideline 201 Freshwater Alga and Cyanobacteria, Growth Inhibition Test ; 2006, 2011) were met.

RMS agrees with the previous evaluation.

**Agreed endpoints :**

**72-h E<sub>r</sub>C<sub>50</sub> = 15.4 mg eugenol/L (mm.)**

**72-h E<sub>b</sub>C<sub>50</sub> = 10.0 mg eugenol/L (mm.)**

**72-h E<sub>y</sub>C<sub>50</sub> =14.2 mg eugenol/L (mm.)**

***B.9.2.6.2. Effects on growth of an additional algal species***

Since the active substance does not have an herbicidal mode of action, no further data are required.

**B.9.2.7. Effects on aquatic macrophytes**

Eugenol does not exhibit herbicidal activity or a plant growth regulator mode of action. Therefore, data on the effects on aquatic macrophytes are not required in accordance with Regulation (EU) No 283/2013.

**B.9.2.8. Further testing on aquatic organisms**

It is noted that two literature papers were identified during the literature search, which are related to the use of eugenol as a short-term anaesthetic during the handling and/or transport of *Xenopus* frogs by humans (Appendix I, CA 9.6.3.4/34 and CA 9.6.3.4/35). However, results of these anaesthesia studies are of limited relevance and reliability for the risk assessment due to the very short-term exposure (and lack of analytical verification to confirm exposure), followed by a recovery period in untreated water. The critical endpoints from the standard fish acute toxicity studies (B.9.2.1) are considered more relevant and reliable for predicting the low toxicity of eugenol to amphibians. No further relevant data on adverse effects to eugenol to other aquatic organisms were found during the open literature search and no further data are considered necessary. No further studies are considered necessary.

**B.9.3. EFFECTS ON ARTHROPODS****B.9.3.1. Effects on bees*****B.9.3.1.1. Acute toxicity to bees*****B.9.3.1.1.1. Acute oral toxicity**

The data on the representative formulation, Mevalone, are sufficient to address the active substance data requirement. An acute oral honey bee toxicity study with Mevalone was previously evaluated as part of the EU review for the EU inclusion of eugenol (DAR, Volume 3, Annex B.9, 2011, B.9.4.1). A full summary is provided in document Vol. 3 CP B.9.5.1.1/01.

**B.9.3.1.1.2. Acute contact toxicity**

The data on the representative formulation, Mevalone, are sufficient to address the active substance data requirement. An acute contact honey bee toxicity study with Mevalone was previously evaluated as part of the EU review for the EU inclusion of eugenol (DAR, Volume 3, Annex B.9, 2011, B.9.4.1). A full summary is provided in document Vol. 3 CP B.9.5.1.1/01.

#### ***B.9.3.1.2. Chronic toxicity to bees***

New data on the representative formulation, Mevalone are sufficient to address the active substance data requirement. A new chronic oral honey bee toxicity study with Mevalone has been provided to meet new data requirements under Regulation (EU) No 284/2013; a full summary is provided in Vol. 3 CP B.9.5.1.2/01.

#### ***B.9.3.1.3. Effects on honeybee development and other honeybee life stages***

New data on the representative formulation, Mevalone are sufficient to address the active substance data requirement. A new repeated exposure honey bee larval toxicity study with Mevalone has been provided to meet new data requirements under Regulation (EU) No 284/2013; a full summary is provided in Vol. 3 CP B.9.5.1.3/01.

#### ***B.9.3.1.4. Sub-lethal effects***

No further studies are considered necessary

### **B.9.3.2. Effects on non-target arthropods other than bees**

It is noted that one literature paper was identified during the literature search, assessing the effects of exposure to dry residues of clove oil (containing 87.4% eugenol and 11.5%  $\beta$ -caryophyllene) on a *Coleomegilla* (ladybird) species. Results of this paper are summarised in Appendix I CA 9.6.3.4/36, but these are considered of limited relevance and reliability for use in the risk assessment, principally as a non-standard study design was used and the test item was clove oil (albeit of high eugenol content (87.4% eugenol)). The critical endpoints summarised in Vol. 3 CP B.10.5.2.1 from the standard glass plate (IOBC) studies with *Typhlodromus* and *Aphidius* species, testing the representative product, Mevalone, are considered more relevant and reliable for the risk assessment. No further studies are considered necessary.

#### ***B.9.3.2.1. Effects on *Aphidius rhopalosiphi****

The data on the representative formulation, Mevalone are expected to be sufficient to address the active substance data requirement. An *Aphidius rhopalosiphi* glass plate laboratory study with Mevalone was previously evaluated as part of the EU review for the EU inclusion of eugenol (DAR, Volume 3, Annex B.9, 2011, B.9.5.1). A full summary is provided in Vol. 3 CP B.10.5.2.1/02.

#### ***B.9.3.2.2. Effects on *Typhlodromus pyri****

The data on the representative formulation, Mevalone are expected to be sufficient to address the active substance data requirement. A *Typhlodromus pyri* glass plate laboratory study with Mevalone was previously evaluated as part of the EU review for the EU inclusion of eugenol (DAR, Volume 3, Annex B.9, 2011, B.9.5.2). A full summary is provided in Vol. 3 CP B.10.5.2.1/01.

## **B.9.4. EFFECTS ON NON-TARGET SOIL MESO- AND MACROFAUNA**

### **B.9.4.1. Earthworm – sub-lethal effects**

The data on the representative formulation, Mevalone are expected to be sufficient to address the active substance data requirement. One acute earthworm toxicity study with Mevalone was previously evaluated as part of the EU review for the EU inclusion of eugenol (DAR, Volume 3, Annex B.9, 2011, B.9.6.1). The requirement for acute toxicity data for earthworms is now obsolete under Regulation (EU) No 283/2013. This study is therefore not directly relevant for the current assessment, but is provided in Vol. 3 CP B.9.7.1 as supporting information to support renewal of eugenol. In addition, a new chronic toxicity study with formulation Mevalone has been provided to meet new data requirements under Regulation (EU) No 284/2013; a full summary is provided in Vol 3 CP B.9.7.1.1/02.

### **B.9.4.2. Effects on non-target soil meso- and macrofauna (other than earthworms)**

#### ***B.9.4.2.1. Species level testing***

The data on the representative formulation, Mevalone, are expected to be sufficient to address the active substance data requirement. It is noted that *Hypoaspis acuelifer* and *Folsomia candida* studies are not formally required as there is no

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direct application to soil and a low risk is concluded at Tier 1 with *T. pyri* and *A. rhopalosiphi*, but a new *Folsomia candida* study is provided for completeness. A new chronic toxicity study in *Folsomia candida* with formulation Mevalone has therefore been provided, a full summary is provided in Vol. 3 CP B.9.7.2.1.

### **B.9.5. EFFECTS ON SOIL NITROGEN TRANSFORMATION**

The data on the representative formulation, Mevalone, are expected to be sufficient to address the active substance data requirement. One mineralisation study with Mevalone was previously evaluated as part of the EU review for the EU inclusion of eugenol (DAR, Volume 3, Annex B.9, 2011, B.9.8.1). A full summary is provided in Vol. 3 CP B.9.9.

### **B.9.6. EFFECTS ON TERRESTRIAL NON-TARGET HIGHER PLANTS**

#### **B.9.6.1. Summary of screening data**

The available screening data on the representative formulation, Mevalone, are expected to be sufficient to address the active substance data requirement. A summary is provided in Vol. 3 CP B.9.11.1.

In addition, it is noted that several literature papers were identified during the literature search, assessing the effects of eugenol on various plant terrestrial species. Results of these papers are summarised in Appendix I (CA 9.6.3.4), but these are considered of limited relevance and reliability for use in the risk assessment, principally as non-standard study designs were used. The available screening data summarised in Vol. 3 CP B.9.11.1., testing the representative product, Mevalone, are considered more relevant and reliable for the risk assessment of terrestrial non-target higher plants. No further studies are considered necessary.

#### **B.9.6.2. Testing on non-target plants**

Since the screening data confirmed that the representative formulation, Mevalone, does not have an herbicidal mode of action, no further data are required.

### **B.9.7. EFFECTS ON OTHER TERRESTRIAL ORGANISMS (FLORA AND FAUNA)**

No further studies are considered necessary.

During the EU review for the EU inclusion of eugenol, it was concluded that pending on the outcome in the fate and behaviour risk assessment, the ecotoxicological risk assessment should be reconsidered for methyleugenol' (EFSA Journal 2012;10(11):2914). As noted in Vol. 3 CA B.8, methyleugenol is not considered a relevant metabolite for the environmental risk assessment. Therefore, no further consideration of the ecotoxicity of methyleugenol is considered necessary. Nevertheless, for completeness a literature search has been conducted for methyleugenol. Four literature papers were identified which included some potentially relevant parameters of interest to the ecotoxicity of methyleugenol and a reliability assessment was therefore conducted for completeness. Following detailed assessment (Please, see Appendix I), overall it was concluded that these studies are of limited reliability for use in the ecotoxicological risk assessment of eugenol or methyleugenol and therefore have not been considered further.

### **B.9.8. EFFECTS ON BIOLOGICAL METHODS FOR SEWAGE TREATMENT**

#### **Study B.9.8/01**

<b>Data point:</b>	CA 8.8/01
<b>Report author</b>	Hammesfahr, U.
<b>Report year</b>	2020
<b>Report title</b>	Mevalone: Toxicity to Activated Sludge in a Respiration Inhibition Test
<b>Report No</b>	155781171
<b>Document No</b>	-
<b>Guidelines followed in study</b>	OECD Guideline 209 (2010) and ISO 8192 (2007)
<b>Deviations from current test guideline</b>	None
<b>Previous evaluation</b>	No, not previously submitted at EU level
<b>GLP/Officially recognised testing facilities</b>	Yes, conducted under GLP/Officially recognised testing facilities
<b>Acceptability/Reliability:</b>	Yes

### Executive summary

The influence of the product Mevalone on the activity of activated sludge was evaluated by measuring the respiration rate under defined conditions. The respiration rate (oxygen consumption) of an aerobic activated sludge fed with a standard amount of synthetic sewage feed was measured in the presence of various concentrations of the product after an incubation period of 3 hours.

The test was performed in accordance with OECD guideline 209 (2010). Five test concentrations were tested: 10, 32, 100, 320 and 1000 mg product/L. The product Mevalone showed significant effects on total and heterotrophic respiration at the highest tested concentration of 1000 mg product/L, corresponding to 31.5 mg eugenol/L, 69.3 mg geraniol/L and 62.3 mg thymol/L.

The critical (lowest) 3-hour NOEC value, based on nitrification respiration, was determined to be 32 mg product/L, corresponding to 1.0 mg eugenol/L, 2.2 mg geraniol/L, and 2.0 mg thymol/L. The critical (lowest) 3-hour EC<sub>50</sub> value, based on nitrification respiration, was calculated to be 204.9 mg product/L (CI: 115.5 – 361.7 mg product/L), corresponding to 6.5 mg eugenol/L, 14.2 mg geraniol/L and 12.8 mg thymol/L.

This study is considered as acceptable and satisfies the requirements established in OECD Guideline 209 (2010).

### Materials and methods

#### *Test material*

Name: Mevalone  
 Formulation type: CS  
 Source and lot/batch no.: 11001  
 Active substance content: 3.15%, 6.93 and 6.23% w/w (analysed) for eugenol, geraniol and thymol respectively  
 Expiry date of lot/batch: August 31, 2021  
 Storage conditions: At 20 ± 5 °C in the dark

#### *Toxic reference*

Name: 3,5-Dichlorophenol  
 Formulation type: Not relevant  
 Source and lot/batch no.: MKBZ0947V  
 Active substance content: 100%  
 Expiry date of lot/batch: June 28, 2022  
 Storage conditions: At 20 ± 5 °C in the dark

#### *Nitrification inhibitor*

Name: N-allylthiourea (ATU)  
 Formulation type: Not relevant  
 Source and lot/batch no.: S7709958

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Active substance content: 100%  
Expiry date of lot/batch: Not reported  
Storage conditions: At 20 ± 5 °C in the dark

#### *Test organism*

Species: Activated sludge, microorganisms  
Source: From a domestic waste water treatment plant (municipal sewage treatment plant Bensheim, Germany).

#### *Test conditions*

Test temperature: 20 ± 2°C during pre-incubation (3 hours) and evaluation period. Recorded continuously  
pH: At start 6.6 - 6.8 at the end 6.8 - 6.9  
Oxygen concentration: At start 4.4 – 5.0 at the end 6.0- 8.2 mg O<sub>2</sub>/L

Five nominal test concentrations were tested: 10, 32, 100, 320 and 1000 mg product/L, with five replicates for each test concentration. The reference item 3,5-Dichlorophenol was tested at the nominal test concentrations of 1, 4, and 16 mg/L (five replicates for each test concentration) under otherwise identical test conditions. Six controls (pure water, synthetic sewage feed and inoculum, but without addition of the product) were tested in parallel. A nitrification inhibitor N-allylthiourea (ATU) was tested in the same way with six separate controls and at the identical nominal concentrations of the product and also the reference item 3,5-dichlorophenol under otherwise identical test conditions.

For each replicate a test solution with a final volume of 500 mL was tested per treatment in a glass flask. 16 mL synthetic sewage feed and an adequate amount of the product or an adequate volume of the stock solution of the reference item were filled up with pure water to 250 mL before the start of the test. At the start of the test 250 mL activated sludge inoculum with a sludge concentration of 3.0 g/L suspended solids was added, first to two controls, then to the test solutions of the reference item in increasing concentrations, to a further two controls, then to the product in increasing concentrations and finally to an additional two controls. During the 3 hour aeration period the flasks were stirred on a magnetic stirrer to maintain sludge flocs in suspension. For the measurement of the respiration rate a well-mixed sample of each test medium was poured into a Karlsruher flask after exactly 3 hours incubation time. The oxygen concentration was then measured with an oxygen electrode and recorded for about ten minutes. During measurement, the samples were continuously stirred on a magnetic stirrer.

For the statistical determination of NOEC values, data were analysed using a Williams Multiple Sequential t-test ( $\alpha=0.05$ , one-sided smaller). Regression analysis was used to calculate EC<sub>x</sub> values. The software used to perform the statistical analysis was ToxRat Professional, Version 3.3.0, ToxRat Solutions GmbH.

## **Results**

### *Biological results*

The influence of Mevalone on the total respiration rate of activated sludge is presented below:

#### **Table 9.8/01-1. Influence of Mevalone and the Reference Item 3,5-Dichlorophenol on the Inhibition of the Total Respiration Rate of Aerobic Waste Water Microorganisms after 3 Hours of Exposure**

Treatment	Nominal Conc.	Respiration Rate [mean] <sup>a</sup>	Standard deviation <sup>a</sup>	Inhibition [mean] <sup>a</sup>	Significant effect compared to control <sup>b</sup>
	[mg/L]	[mg O <sub>2</sub> /g*h]		[%]	
Control	---	22.9	3.3		
3,5-DCP	1	13.6	3.1	40.7	*
	4	12.5	2.6	45.4	*
	16	4.9	0.4	78.7	*
Mevalone	10	26.8	3.0	-17.2	n.s.
	32	28.7	2.2	-25.6	n.s.
	100	26.2	1.9	-14.5	n.s.
	320	22.4	1.7	2.0	n.s.
	1000	6.6	0.7	71.0	*

Coefficient of Variation of control: 14.6%

a: mean value of 5 replicates, 6 replicates in case of control

b: significance according to Williams Multiple Sequential t-test, one-sided smaller,  $\alpha = 0.05$  (\* = significant; n. s.: not significant)

Inhibition [mean] % = 100-(Respiration Rate [mean] [mg O<sub>2</sub>/g\*h])\*100 / mean value respiration rate in controls)

In comparison to the inoculum controls total respiration was not inhibited up to and including a product concentration of 320 mg product/L. The highest product concentration of 1000 mg product/L strongly inhibited the total respiration. For total respiration, the 3-hour NOEC value was determined to be at a Mevalone concentration of 320 mg product/L, corresponding to 10.1 mg eugenol/L, 22.2 mg geraniol/L and 19.9 mg thymol/L.

The 3-hour EC<sub>10</sub> value for total respiration was established to be 282.6 mg product/L (CI: 193.4 – 413.1 mg product/L), corresponding to 8.9 mg eugenol/L, 19.6 mg geraniol/L and 17.6 mg thymol/L.

The 3-hour EC<sub>20</sub> value for total respiration was established to be 376.5 mg product/L (CI: 263.7 – 543.4 mg product/L), corresponding to 8.9 mg eugenol/L, 19.6 mg geraniol/L and 17.6 mg thymol/L.

The 3-hour EC<sub>50</sub> value for total respiration was established to be 651.4 mg product/L (CI: 422.0 – >1000 mg product/L), corresponding to 20.5 mg eugenol/L, 45.1 mg geraniol/L and 40.6 mg thymol/L.

The influence of Mevalone on the heterotrophic respiration rate of activated sludge is presented below:

**Table 9.8/01-2. Influence of Mevalone and the Reference Item 3,5-Dichlorophenol on the Inhibition of the Heterotrophic Respiration Rate of Aerobic Waste Water Microorganisms after 3 Hours of Exposure**

Treatment	Nominal Conc.	Respiration Rate [mean] <sup>a</sup>	Standard deviation <sup>a</sup>	Inhibition [mean] <sup>a</sup>	Significant effect compared to control <sup>b</sup>
	[mg/L]	[mg O <sub>2</sub> /g*h]		[%]	
Control	---	14.7	3.8	---	
3,5-DCP	1	12.0	2.2	18.2	n.s.
	4	16.3	1.2	-11.3	n.s.
	16	5.0	0.6	65.6	*
Mevalone	10	18.8	2.3	-28.2	n.s.
	32	19.8	1.9	-34.7	n.s.
	100	21.0	1.4	-43.0	n.s.
	320	18.3	1.6	-24.9	n.s.
	1000	9.4	2.2	36.2	*

Coefficient of Variation of control: 26.1%

a: mean value of 5 replicates, 6 replicates in case of control

b: significance according to Williams Multiple Sequential t-test, one sided smaller,  $\alpha = 0.05$  (test item) or Welsh t-test after Bonferroni Holm, one sided smaller,  $\alpha = 0.05$  (reference item), \* = significant; n. s.: not significant

Inhibition [mean] % = 100-(Respiration Rate [mean] [mg O<sub>2</sub>/g\*h])\*100 / mean value respiration rate in controls)

In comparison to the inoculum controls the heterotrophic respiration was not inhibited up to and including a product concentration of 320 mg product/L. The highest product concentration of 1000 mg product/L inhibited the total respiration.

For heterotrophic respiration, the 3-hour NOEC value was determined to be at a product concentration of 320 mg product/L, corresponding to 10.1 mg eugenol/L, 22.2 mg geraniol/L and 19.9 mg thymol/L.

The 3-hour EC<sub>10</sub> value based on heterotrophic respiration was determined to be 599.5 mg product/L (CI: 8.2 – >1000 mg product/L), corresponding to 18.9 mg eugenol/L, 41.5 mg geraniol/L and 37.3 mg thymol/L.

The 3-hour EC<sub>20</sub> value based on heterotrophic respiration was determined to be 717.0 mg product/L (CI: 9.8 – >1000 mg product/L), corresponding to 22.6 mg eugenol/L, 49.7 mg geraniol/L and 44.7 mg thymol/L.

The 3-hour EC<sub>50</sub> value based on heterotrophic respiration was determined to be 1010.0 mg product/L (CI: 3.0 – >1000 mg product/L), corresponding to 31.8 mg eugenol/L, 70.0 mg geraniol/L and 62.9 mg thymol/L.

The influence of Mevalone on the respiration rate based on nitrification of activated sludge is presented below:

**Table 9.8/01-3. Influence of Mevalone and the Reference Item 3,5-Dichlorophenol on the Inhibition of the Nitrification Respiration Rate of Aerobic Waste Water Microorganisms after 3 Hours of Exposure**

Treatment	Nominal Concentration	Respiration Rate [mean] <sup>a</sup>	Standard Deviation <sup>a</sup>	Inhibition [mean] <sup>a</sup>	Significant effect compared to control <sup>d</sup>
	[mg/L]	[mg O <sub>2</sub> /g*h]		[%]	
Control	---	8.2	3.3	---	---
3,5-DCP	1	1.6	3.1	81.0	---
	4	-3.8	2.6	146.8	---
	16	-0.2	0.4	102.0	---
Mevalone	10	8.0	3.0	2.3	n.s.
	32	8.9	2.2	-9.1	n.s.
	100	5.2	1.9	36.5	*
	320	4.1	1.7	50.2	*
	1000	-2.7	0.7	133.2	*
Coefficient of Variation of control: 40.8%					
<sup>a</sup> : mean value of 5 replicates, 6 replicates in case of control					
<sup>d</sup> significance according Williams Multiple t-test, one-sided smaller, $\alpha = 0.05$ (* = significant; n. s.: not significant)					
Inhibition [mean] % = 100-(Respiration Rate [mean] [mg O <sub>2</sub> /g*h]*100 / mean value respiration rate in controls)					

In comparison to the inoculum controls, the respiration rates of the activated sludge based on nitrification were inhibited starting from a product concentration of 100 mg product/L.

For nitrification respiration the 3-hour NOEC value was determined to be at a product concentration of 32 mg product/L, corresponding to 1.0 mg eugenol/L, 2.2 mg geraniol/L and 2.0 mg thymol/L.

The 3-hour EC<sub>10</sub> value based on nitrification respiration was determined to be 64.7 mg product/L (CI: 40.3 – 103.9 mg product/L), corresponding to 2.0 mg eugenol/L, 4.5 mg geraniol/L and 4.0 mg thymol/L.

The 3-hour EC<sub>20</sub> value based on nitrification respiration was determined to be 96.1 mg product/L (CI: 60.9 – 153.3 mg product/L), corresponding to 3.0 mg eugenol/L, 6.7 mg geraniol/L and 6.0 mg thymol/L.

The 3-hour EC<sub>50</sub> value based on nitrification respiration was determined to be 204.9 mg product/L (CI: 115.5 – 361.7 mg product/L), corresponding to 6.5 mg eugenol/L, 14.2 mg geraniol/L and 12.8 mg thymol/L.

#### Validity

All validity criteria were met in accordance with OECD guideline 209 (2010):

- The blank controls oxygen uptake was  $\geq 20$  mg oxygen per one gram of activated sludge (dry weight of suspended solids) in an hour (actual value 22.9 mg O<sub>2</sub>/g/h).
- The coefficient of variation of oxygen uptake rate in control replicates was  $\leq 30\%$  at the end of the definitive test (actual values 14.6% for the total respiration and 26.1% for the heterotrophic respiration).
- The reference item 3,5-dichlorophenol EC<sub>50</sub> values were within the range of 2 mg/L to 25 mg/L for total respiration (actual value 2.8 mg/L), 5 mg/L to 40 mg/L for heterotrophic respiration (actual value 15.0 mg/L) and 0.1 mg/L to 10 mg/L for nitrification respiration (actual value 0.8 mg/L).

#### Assessment and conclusion

In a 3-hour activated sludge respiration inhibition test, Mevalone showed significant effects on total and heterotrophic respiration at concentrations of 1000 mg product/L, corresponding to 31.5 mg eugenol/L, 69.3 mg geraniol/L and 62.3 mg thymol/L. For nitrification respiration, significant effects were determined starting from a test item concentration of 100 mg product/L, corresponding to 3.2 mg eugenol/L, 6.9 mg geraniol/L, and 6.2 mg thymol/L.

For total and heterotrophic respiration, the 3-hour NOEC value was determined to be at a test item concentration of 320 mg product/L, corresponding to 10.1 mg eugenol/L, 22.2 mg geraniol/L and 19.9 mg thymol/L. For nitrification respiration the 3-hour NOEC value was determined to be at a test item concentration of 32 mg product/L, corresponding to 1.0 mg eugenol/L, 2.2 mg geraniol/L and 2.0 mg thymol/L.

The critical (lowest) 3-hour NOEC value, based on nitrification respiration, was determined to be 32 mg product/L, corresponding to 1.0 mg eugenol/L, 2.2 mg geraniol/L, and 2.0 mg thymol/L. The critical (lowest) 3-hour EC<sub>50</sub> value, based on nitrification respiration, was calculated to be 204.9 mg product/L (CI: 115.5 – 361.7 mg product/L), corresponding to 6.5 mg eugenol/L, 14.2 mg geraniol/L and 12.8 mg thymol/L. This study is considered as acceptable and satisfies the requirements established in OECD Guideline 209 (2010).

This study is considered as acceptable and satisfies the requirements established in OECD Guideline 209 (2010).

#### **Assessment and conclusion by applicant:**

The study is acceptable.

The critical (lowest) 3-hour NOEC value, based on nitrification respiration, was determined to be 32 mg product/L, corresponding to 1.0 mg eugenol/L, 2.2 mg geraniol/L and 2.0 mg thymol/L.

The critical (lowest) 3-hour EC<sub>50</sub> value, based on nitrification respiration, was calculated to be 204.9 mg product/L (CI: 115.5 – 361.7 mg product/L), corresponding to 6.5 mg eugenol/L, 14.2 mg geraniol/L and 12.8 mg thymol/L.

#### **Assessment and conclusion by RMS:**

The test was performed according to OECD 209 (2010). The validity criteria are met:

- Control O<sub>2</sub> uptake was 22.9 mg O<sub>2</sub>/g/h (it should be at least 20 mg O<sub>2</sub>/g/h);
- The coefficient of variation in the controls was 14.6% for the total respiration and 26.1% for heterotrophic respiration (it should be less than 30%);
- The EC<sub>50</sub> for 3,5-DCP was:
  - o For the total respiration: 2.8 mg/L (the expected range is 2 – 25 mg/L).
  - o For heterotrophic respiration: 15.0 mg/L (the expected range is 5 - 40 mg/L).
  - o For the oxygen uptake due to nitrification: 0.8 mg/L (the expected range is 0.1 - 10 mg/L).

The study was conducted with the formulated product (Mevalone) instead of the active substance.

The study is considered acceptable.

#### **Agreed endpoints**

**Based on nitrification respiration:**

**3-h EC<sub>50</sub> = 204.9 (CI: 115.5-361.7) mg Mevolane/L; 6.5 mg eugenol/L**

**3-h EC<sub>10</sub> = 64.7 (CI: 40.3-103.9) mg Mevolane/L; 2.0 mg eugenol/L**

**3-h EC<sub>20</sub> = 96.1 (CI: 60.9-153.3) mg Mevolane/L; 3.0 mg eugenol/L**

**3-h NOEC = 32 mg Mevolane/L; 1.0 mg eugenol/L;**

#### **B.9.9. MONITORING DATA**

No further studies are considered necessary.

#### **B.9.10. BIOLOGICAL ACTIVITY OF METABOLITES POTENTIALLY OCCURRING IN GROUNDWATER**

No further studies are considered necessary since the residue definition does not include any relevant metabolite in groundwater.

#### **B.9.11. REFERENCES RELIED ON**

##### **B.9.11.1. Literature search**

### **B.9.11.1.1. Summary**

This data point reviewed the literature data relating to the active substance eugenol, as required by Article 8(5) of Regulation (EC) No 1107/2009 on the placing of plant protection products on the market.

It has been written according to:

- EFSA (2011). Guidance of EFSA, Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009, EFSA Journal 2011;9(2):2092.
- AGES (2013). External scientific report, Case studies for the application of the Guidance of EFSA on Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009, using substances for which dossiers are submitted under Regulation (EU) No 1141/2010, EFSA supporting publication 2013:EN-511.

In this literature search, the aim was to find scientific peer-reviewed open literature on eugenol dealing with toxicological and toxicokinetic studies, operator exposure, residues, fate and behaviour in the environment and ecotoxicological studies which were published between 1/1/2005 and 16/02/2021 for eugenol and between 1/1/1995 and 16/02/2021 for methyleugenol.

To achieve this goal, a wide range of reference collections was consulted utilising the online various databases.

The following initial search strategy was used, in line with Section 5.2.2 of EFSA Journal 2011;9(2):2092:

- The search terms were defined, initially using terms related to the active substance, its metabolites and plant protection products containing the active substance, including synonyms.
- Specific search terms were then used relating to the specific data requirements in question (i.e. for toxicology, human exposure, residues, environmental fate and behaviour and ecotoxicology) to refine the search.
- The search of publications was conducted, using the two-stage approach outlined above. A list of titles, authors and dates were extracted. Any duplicate summary records were removed, before proceeding.

The remaining summary records were then assessed for relevance in a two-step process, in line with Section 5.3 of EFSA Journal 2011;9(2):2092:

- Firstly, a rapid assessment for relevance was conducted based on the titles and abstracts, to exclude summary records which were obviously irrelevant to the specific data requirements in question.
- Secondly, a detailed assessment of full-text documents was conducted to identify potentially relevant, irrelevant or relevant and reliable records that were not excluded in the first step.

All literature papers that were considered relevant to the risk assessment following this process are summarised in the Appendix I, where an assessment of their reliability is also presented in line with Section 5.4 of EFSA Journal 2011;9(2):2092.

The results of this search were as follows:

Study selection process	Eugenol			
	Toxicology (including human exposure)	Residues	Environmental fate and behaviour	Ecotoxicology
Total number of documents retrieved (with duplicates) (global search results related to the active substance, its metabolites and plant protection products containing the active substance)	28 270			
Total number of documents retrieved (without	1191	487	733	1865

duplicates) after focused search strategy (combining terms for active substance and specific data requirements)				
Number of publications excluded after rapid assessment for relevance (non-relevant, excluded literature)	1166	473	730	1756
Number of publications for which detailed assessment of full-text required after rapid assessment for relevance	25	13	3	109
Number of publications excluded from further consideration after detailed assessment for relevance (non-relevant, excluded literature)	13	3	1	66
<b>Number of publications considered relevant following the two-step process (i.e. after detailed assessment of full-text)</b>	12	10	2	<b>43</b>

For ecotoxicology, as the reliability of relevant papers is limited for use in the ecotoxicological risk assessment of eugenol they have not been considered further in this document and brief summaries of these papers are provided below in the Appendix I for transparency.

Study selection process	Methyl-eugenol			
	Toxicology (including human exposure)	Residues	Environment al fate and behaviour	Ecotoxicology
Total number of documents retrieved (with duplicates) (global search results related to the active substance, its metabolites and plant protection products containing the active substance)	4 133			
Total number of documents retrieved (without duplicates) after focused search strategy (combining terms for active substance and specific data requirements)	191	102	147	329
Number of publications excluded after rapid assessment for relevance (non-relevant, excluded literature)	176	98	147	275
Number of publications for which detailed assessment of full-text required after rapid assessment for relevance	15	4*	0	54
Number of publications excluded from further consideration after detailed assessment for relevance (non-relevant, excluded literature)	1	1*	0	50
<b>Number of publications considered relevant following the two-step process (i.e. after detailed assessment of full-text)</b>	14	3*	0	<b>4</b>

\* The same papers were also identified in the eugenol literature search.

For ecotoxicology, as the reliability of relevant papers is limited for use in the ecotoxicological risk assessment of methyl-eugenol they have not been considered further in this document and brief summaries of these papers are provided below in the Appendix I for transparency.

Aim is to find scientific peer-reviewed open literature, as required by Article 8(5) of Regulation (EC) No 1107/2009 on the placing of plant protection products on the market, on Eugenol dealing with toxicological and toxicokinetic studies, operator exposure, residues, fate and behavior in the environment and ecotoxicological articles which are published within the last fifteen years from a wide range of reference collections consulted through online wide and various databases.

Details on these various reference collections are provided hereafter (B.11.1.2) fulfilling requirements from EFSA and AGES on the bibliographic database (in terms of quality and quantity). The search strategy leading to the inclusion or exclusion of publications is described below (B.9.11.1.3 and B.9.11.1.4).

#### ***B.9.11.1.2. Search strategy***

The following search strategy was used, in line with Section 5.2.2 of EFSA Journal 2011;9(2):2092:

The following initial search strategy was used, in line with Section 5.2.2 of EFSA Journal 2011;9(2):2092:

- The search terms were defined using terms related to the active substance, its metabolites and plant protection products containing the active substance, including synonyms.
- Specific search terms relating to the data requirements in question (i.e. for toxicology, human exposure, residues, environmental fate and behaviour and ecotoxicology) were then used to refine the search.
- These search terms are listed in Section B.9.11.1.2.2 below.

The literature search was conducted on 2<sup>nd</sup> July 2020 and 16 February 2021. The search identified scientific peer-reviewed open literature published in the last 15 years (from 1/1/2005 to 16/02/2021) for eugenol and in the last 25 years (from 1/1/1995 to 16/02/2021) for methyleugenol.

##### **B.9.11.1.2.1. Bibliographic database used in the literature review**

In line with Section 5.2.1 of EFSA Journal 2011;9(2):2092, a number of different sources were used in the literature search with the aim to locate all relevant scientific peer-reviewed open literature. The reference collections searched were AGRICOLA, AGRIS (FAO), PASCAL, PubMed (MEDLINE) inc. TOXNET, Science Direct, Springer, and Wiley. All sources and the rationale for their inclusion in the search are presented in the table below.

**Table 9.11.1.2.1-1: Bibliographic databases used in the literature search**

Database	Description/Rationale for Inclusion
1. AGRICOLA	Coverage of the database includes agricultural economics and rural sociology, agricultural production, animal sciences, chemistry, entomology, food and human nutrition, forestry, natural resources, pesticides, plant science, soils and fertilizers, and water resources. More than 6.7 million records (09/2019). 1970-present.
2. AGRIS (FAO)	AGRIS is one of the most comprehensive search engines in food and agricultural scientific literature providing free access to millions of bibliographic records in 90 different languages. Up to hundreds of organisations worldwide contribute to knowledge and data to the AGRIS platform, resulting in a multilingual bibliographic collection of food and agricultural scientific research with special attention to scientific information produced in the global south. Therefore, AGRIS is used by whoever is inclined to find literature on any of FAO's areas of interest.
3. PASCAL	The site is an archive of the PASCAL and FRANCIS bibliographic databases in exact, human and social sciences, produced by the Inist-CNRS from 1972 to 2015. It provides access to more than 20 millions references (articles, patents, maps, conferences, books, reports and thesis) in exact sciences and technology, in biological and medical sciences, in art, archaeology, economics, ethnology, geography, history of science and technology, literature, linguistics, administrative and legal sciences, education and religion sciences.
4. PubMed MEDLINE (including TOXNET)	MEDLINE contains information on every area of medicine. More than 30 million records (08/2019). 1946-present.
5. Science Direct	ScienceDirect provides access to more than 16 million articles, 2,500 journals, 370 full open access journals, 39,000 books and 330,000 topic pages to help researchers discover more insights, achieve more breakthroughs and move their research forward.
6. Springer	Springer is a leading global scientific, technical and medical portfolio, providing researchers in academia, scientific institutions and corporate R&D departments with quality content through innovative information, products and services. It handles more than 2,900 journals and 300,000 book.
7. Wiley	Wiley is one of the largest and most authoritative collections of online journals, books, and research resources, covering life, health, social, and physical sciences.

**B.9.11.1.2.2. Input parameters (search terms) for the literature search**

Any documents relating to the essential oils, their synonyms and other common names were collected. The reference collections were queried by name e.g. "eugenol" in addition to common variants such as eugenic acid, and "clove oil". Where the number of records for, say, eugenol alone exceeded individual reference collection download limits additional specific topics of interest, ecotoxicity, fate, residues, etc., were included. The tables below present the general search terms used:

**Table 9.11.1.2.2-1: List search terms used to identify all literature related to the active substance (eugenol), its metabolites and plant protection products containing the active substance**

<b>Common name</b>	Eugenol Eugenol acid Eugenol methyl ether Allylguaiacol Eugenol acid Caryophyllic acid p-Allylguaiacol p-Eugenol 1,3,4-Eugenol allylveratrol Caryophyllic acid Clove oil Nutmeg oil Cinnamon oil Basil oil Bay leaf Phenol, 2-methoxy-4-(2-propenyl)-
<b>Chemical name (IUPAC and CA)</b>	2-methoxy-4-prop-2-enylphenol 2-methoxy-4-(2-propen-1-yl)phenol 2-Methoxy-4-(2-propenyl)phenol 2-Methoxy-4-allylphenol 4-Allyl-2-methoxyphenol 4-Allylguaiacol 1-Hydroxy-2-methoxy-4-allylbenzene 1-Hydroxy-2-methoxy-4-prop-2-enylbenzene 1-Hydroxy-2-methoxy-4-propenylbenzene 2-Methoxy-1-hydroxy-4-allylbenzene 2-Methoxy-4-(2-propen-1-yl)phenol 2-Methoxy-4-allylphenol 2-methoxy-4-(2-propenyl)phenol 2-Methoxy-4-prop-2-enylphenol 2-Metoksy-4-allilofenol 4-Allyl-1-hydroxy-2-methoxybenzene 4-Allyl-2-methoxyphenol 4-Allylcatechol 2-methyl ether 4-Allylcatechol-2-methyl ether 4-Allylguaiacol 4-Hydroxy-3-methoxy-1-allylbenzene 4-Hydroxy-3-methoxyallylbenzene 5-Allylguaiacol 1-Allyl-3-methoxy-4-hydroxybenzene
<b>Metabolite(s)</b>	-
<b>Product name</b>	Mevalone, Andromeda, 3Logy, ES-00108, Trigemol, Cagenoletta, Eugeti
<b>CAS No.</b>	97-53-0
<b>EC No.</b>	202-589-1

**Table 9.11.1.2.2-2: List search terms used to identify all literature related to the compound methyleugenol**

<b>Common name</b>	Methyl Eugenol Methyl eugenol Methyleugenol methyl-eugenol ME Benzene, 1,2-dimethoxy-4-(2-propenyl)- O-Methyl eugenol O-Methyleugenol
<b>Chemical name (IUPAC and CA)</b>	1,2-dimethoxy-4-prop-2-enylbenzene 1,2-Dimethoxy-4-(2-propen-1-yl)benzene 1,2-Dimethoxy-4-(2-propenyl)benzene 1,2-Dimethoxy-4-allylbenzene 1,3,4-Eugenol methyl ether 1-(3,4-Dimethoxyphenyl)-2-propene 1-Allyl-3,4-dimethoxybenzene 3,4-Dimethoxyallylbenzene 4-Allyl-1,2-dimethoxybenzene 4-Allylveratrole Benzene, 1,2-dimethoxy-4-(2-propenyl)- Benzene, 4-allyl-1,2-dimethoxy- 4-Allyl-1,2-dimethoxybenzene
<b>Metabolite(s)</b>	-
<b>Product name</b>	Mevalone
<b>CAS No.</b>	93-15-2
<b>EC No.</b>	202-223-0

Often an event or outcome is not explicitly described by the subject at the title or abstract level and it would be difficult to adequately describe the individual ecotox effects one can envisage using key words and/or subject headings in a complex search query. This granular information was captured during the text processing phase using customised gazetteer lists such as the extracts given in Table 9.11.1.2.2-3 below for aquatic invertebrates and birds. These case insensitive lists contain both generic terms such as “mollusc(s)” and both common and scientific names at the species level, e.g., pond snail (*Lymnaea*). In total the gazetteer list of terms describing aquatic invertebrates contained 47 entities and the birds gazetteer contained 63 entities (All subject gazetteer list files used in this project have been provided to Staphyt as a separate zip file KCA 9.2.4/01). On another hand, the subject lists were crossed with Effects lists for each section.

The tables below present examples of the subject and effect section specific search terms used:

**Table 9.11.1.2.2-3: List of subject search terms used specific to a section (example)**

<b>Aquatic Invertebrate list (selected entries)</b>	<b>Birds list (selected entries)</b>
chironomid	Anas
chironomids	avian
Chironomus	bird
Crassostrea	birds
crustacea	blackcap
crustacean	blackcaps
crustaceans	bluetit
Daphnia	bluetits
daphnid	bobwhite
...	...

**Table 9.11.1.2.2-4: List of effect search terms used specific to a section (example)**

<b>Effect (ecotox)</b>
absorption
acute
adrenal

adsorption
adverse
allergen
allergenic
allergens
allergic
...

### ***B.9.11.1.3. Search results***

The total numbers of records (before removing duplicates) for the search are summarised in the table below.

The peer-reviewed literature search strategy highlighted 28 270 documents of potential interest for eugenol and 4 133 documents of potential interest for methyleugenol to this review before the removal of duplicates occurring as a result of searching the individual reference collections separately.

**Table 9.11.1.3-1: Numbers of records (before removing duplicates) for the search (based on search terms according to Table 9.11.1.2.2-1)**

<b>Databases (through online STN)</b>	<b>Number of hits Eugenol</b>
1. AGRICOLA	97
2. AGRIS (FAO)	423
3. PASCAL	508
4. PubMed MEDLINE (including TOXNET)	4 509
5. Science Direct	10 512
6. Springer	7 801
7. Wiley	4 420
Total number of documents retrieved (with duplicates) (global search results related to the active substance, its metabolites and plant protection products containing the active substance)	<b>28 270</b>

**Table 9.11.1.3-2: Numbers of records (before removing duplicates) for the search (based on search terms according to Table 9.11.1.2.2-2)**

Databases (through online STN)	Number of hits Methyleugenol
1. AGRICOLA	18
2. AGRIS (FAO)	128
3. PASCAL	-
4. PubMed MEDLINE (including TOXNET)	500
5. Science Direct	1 599
6. Springer	900
7. Wiley	988
Total number of documents retrieved (with duplicates) (global search results related to the active substance, its metabolites and plant protection products containing the active substance)	<b>4 133</b>

To identify which of these records mentioned the aspects of interest to this particular project a text mining application was built and applied to each document in turn and only those which specifically mentioned, for example fate and behavior or the specified ecotox terms together with an essential oil or its common variants, were identified as a positive result.

The text mining application comprises a number of different steps each performing a different function in the application and in general terms the approach taken was to Tokenise (identify individual words and features) and Sentence split the documents; use the Gazetteer lists to identify any important key words and phrases such as sediment dwelling organisms; identify the Title and Abstract part of the document; look within the Title and Abstract for patterns matching the natural language expressions for, say, the ecotoxicity of thymol, an example being “Acaricidal activities of *Santolina africana* and *Hertia cheirifolia* essential oils against the two-spotted spider mite (*Tetranychus urticae*).”; and index the results.

After removing duplicates and text mining process, the total number of hits potentially relevant is presented below:

**Table 9.11.1.3-3: Number of documents identified as potentially relevant after text mining and removal of duplicates**

All databases	Number of hits* Eugenol	Number of hits* Methyleugenol
Mammalian Toxicity	1 191	191
Residues	487	102
Environmental fate and behaviour	733	147
Ecotox	<b>1 865</b>	<b>329</b>

\*Total number of documents retrieved after removal of duplicates/triplicates (using general plus section specific terms)

#### ***B.9.11.1.4. Study Selection***

The remaining summary records were assessed for relevance in a two-step process, in line with Section 5.3 of EFSA Journal 2011;9(2):2092:

- Firstly, a rapid assessment for relevance was conducted based on the titles and abstracts, to exclude summary records which were considered irrelevant and the justification was recorded.
- Secondly, a detailed assessment relevance and reliability of full-text documents was conducted for any remaining summary records that were not excluded in the first step.

All literature papers that were considered relevant to the risk assessment following this two-step process are summarised in the corresponding MCA/MCP sections of this supplementary dossier, where an assessment of their reliability is also presented in line with Section 5.4 of EFSA Journal 2011;9(2):2092.

**B.9.11.1.4.1. Rapid assessment**

The total number of publications excluded after rapid assessment for relevance and the number of publications for which a detailed assessment of the full-text was required are summarised in the table below.

**Table 9.11.1.4.1-1: Numbers of publications excluded/identified after rapid assessment for relevance for eugenol**

Study selection process	Eugenol			
	Toxicology (including human exposure)	Residues	Environmental fate and behaviour	Ecotoxicology
Number of publications excluded after rapid assessment for relevance (non-relevant, excluded literature)	1166	474	730	1756
Number of publications for which detailed assessment of full-text required after rapid assessment for relevance	25	13	3	109

**Table 9.11.1.4.1-2: Numbers of publications excluded/identified after rapid assessment for relevance for methyleugenol**

Study selection process	Methyleugenol			
	Toxicology (including human exposure)	Residues	Environmental fate and behaviour	Ecotoxicology
Number of publications excluded after rapid assessment for relevance (non-relevant, excluded literature)	176	98	147	275
Number of publications for which detailed assessment of full-text required after rapid assessment for relevance	15	4*	0	54

\* The same papers were also identified in the eugenol literature search.

**B.9.11.1.4.2. Detailed assessment**

The total number of publications excluded and the final number of relevant papers identified after detailed assessment of full-texts for relevance are summarised in the tables below.

**Table 9.11.1.4.2-1: Criteria for Relevance – Detailed Screening**

Data requirement	Justification for Search Strategy
<b>Ecotoxicological studies (OECD IIA 8.1 to 8.8 and IIIA 10.1 to 10.7)</b>	
Effects on birds (OECD IIA 8.1.1 and IIA 8.1-3 – 8.1.5; OECD IIIA 10.1.1)  Effects on aquatic organisms (OECD II 8.2 and IIIA 10.2)  Effects on other terrestrial vertebrates (OECD IIA 8.1.2 and IIA 8.1-3 – 8.1.5; IIIA 10.1.2, IIIA 10.1.3)  Effects on bees (OECD IIA 8.3.1 and OECD IIIA 10.3.1)  Effects on other arthropod species (OECD IIA 8.3.2 and OECD IIIA 10.3.2)  Effects on earthworms (OECD IIA 8.4.1 and OECD IIIA 10.4.1)	Relevance check based on information given in the title and the abstract:  1. Ecotoxicological studies conducted with the active substance, metabolites or product containing the active substance (defined test material) addressing any of the data requirements. 2. Field studies relevant for European conditions (climate, species, ...) 3. Papers which are dealing with the effects on mammals were not included in the relevance assessment because this was considered to be covered in the section toxicology (see 2.4.1 Toxicology). However, studies on other non-target vertebrate species (e.g. fish, birds, other mammals than relevant for toxicological testing) or studies which are not covered by the data requirements of the section toxicology, e.g. field studies, avoidance studies, were considered in the relevance and reliability check. 4. Literature reviews were excluded where clear from the title and abstract. 5. Literature coping with the general production/presence of essential oils in plants were excluded. 6. Literature coping with effects only at a molecular level (e.g. gene expression) were excluded, unless where potentially related to endocrine activity.
Effects on soil non-target macro-organisms (OECD IIA 8.4.2 and OECD IIIA 10.4.2)  Effects on soil non-target micro-organisms (OECD IIA 8.5 and OECD IIIA 10.5)  Effects on other non-target organisms (flora and fauna) believed to be at risk (OECD IIA 8.6 and OECD IIIA 10.7)  Effects on biological methods of sewage treatment (OECD IIA 8.8)	Relevance check based on full-text:  1. Literature reviews were excluded. 2. Literature coping with combined and/or mixture toxicity were excluded (no tests were conducted with the active substance and/or a solo formulation). 3. Papers were excluded if they only assessed effects of plant essential oils containing several constituents. If an active substance test item had a purity of <70% of the active substance of interest (i.e. <70% eugenol, geraniol or thymol), literature were excluded. 4. Literature coping with effects on target pests (i.e. efficacy data), including effects on fungi, insect pests (mites, mosquitoes, ticks) and nematodes were excluded

**Table 9.11.1.4.2-2: Numbers of publications excluded/identified after detailed assessment for relevance for eugenol**

Study selection process	Eugenol			
	Toxicology (including human exposure)	Residues	Environmental fate and behaviour	Ecotoxicology
Number of publications excluded from further consideration after detailed assessment for relevance (non-relevant, excluded literature)	13	3	1	66
<b>Number of publications considered relevant following the two-step process (i.e. after detailed assessment of full-text)</b>	<b>12</b>	<b>10</b>	<b>2</b>	<b>43</b>

**Table 9.11.1.4.2-3: Numbers of publications excluded/identified after detailed assessment for relevance for methyleugenol**

Study selection process	Methyleugenol			
	Toxicology (including human exposure)	Residues	Environmental fate and behaviour	Ecotoxicology
Number of publications excluded from further consideration after detailed assessment for relevance (non-relevant, excluded literature)	1	1*	NA**	50
<b>Number of publications considered relevant following the two-step process (i.e. after detailed assessment of full-text)</b>	<b>14</b>	<b>3*</b>	<b>0</b>	<b>4</b>

\* The same papers were also identified in the eugenol literature search.

\*\* Not applicable as none of the publications were considered as relevant following rapid assessment of the title or abstract.

All publications excluded following the detailed assessment are presented in the tables below, with a reason for not including in the dossier.

All relevant publications identified following the detailed assessment are presented in the tables below, in order of data requirement and author, respectively. These relevant papers and reliable are summarised in the corresponding MCA/MCP sections of this supplementary dossier, where an assessment of their reliability is also presented in line with Section 5.4 of EFSA Journal 2011;9(2):2092.

#### *Eugenol*

For ecotoxicology, as the reliability of relevant papers is limited for use in the ecotoxicological risk assessment of eugenol they have not been considered further in the Appendix I of this document and brief summaries of these papers are included.

#### *Methyleugenol*

For ecotoxicology, as the reliability of relevant papers is limited for use in the ecotoxicological risk assessment of eugenol and methyl-eugenol they have not been considered further and brief summaries of these papers are provided below in the Appendix I.

**Table 9.11.1.4.2-4: Relevant studies included in the supplementary dossier after detailed assessment of full-text documents for relevance in the Ecotoxicology Section for eugenol**

Data requirement (indicated by the corresponding CA and CP data point)	Author(s)	Year	Title	Source
CA 9.6.3.4/01 [8.1.2]	Annika Schläpfer <sup>a</sup> ; c; Gerhard Jakob <sup>b</sup> ; Sonoko Bellingrath-Kimura <sup>c</sup> ; Jens Jacob <sup>a</sup>	2018	Natural bait additives improve trapping success of common voles <i>Microtus arvalis</i> .	Applied Animal Behaviour Science Volume 208 November 2018 Pages 75-81
CA 9.6.3.4/02 [8.2.1]	Thanapat Pattanasiri; Wara Taparhudee; Panuwat Suppakul	2017	Acute toxicity and anaesthetic effect of clove oil and eugenol on Siamese fighting fish <i>Betta splendens</i>	Aquaculture International volume 25 pages 163 -“ 175 ( 2017 )
CA 9.6.3.4/03 [8.2.1]	Crislaine Palmeira Barbosa de Oliveira <sup>a</sup> ; Carlos Henrique da Paixão Lemos <sup>a</sup> ; Luiz Vitor Oliveira Vidal <sup>a</sup> ; Ricardo David Couto <sup>b</sup> ; Denise Soledade Peixoto Pereira <sup>c</sup> ; Carlos Eduardo Copatti <sup>a</sup>	2019	Anaesthesia with eugenol in hybrid Amazon catfish ( <i>Pseudoplatystoma reticulatum</i> – <i>Leirius marmoratus</i> ) handling: Biochemical and haematological responses.	Aquaculture Volume 501 25 February 2019 Pages 255-259
CA 9.6.3.4/04 [8.2.1]	Reza Tarkhani; Ahmad Imani; Hadi Jamali; Kouros Sarvi Moghanlou; Reza Tarkhani; Ahmad Imani; Hadi Jamali; Kouros Sarvi Moghanlou	2017	Anaesthetic efficacy of eugenol on Flowerhorn ( <i>Amphilophus labiatus</i> – <i>Amphilophus trimaculatus</i> )	Aquaculture Research Volume 48 Issue 6
CA 9.6.3.4/05 [8.2.1]	Seyyed Morteza Hoseini; Hamid Rajabiesterabadi; Reza Tarkhani; Seyyed Morteza Hoseini; Hamid Rajabiesterabadi; Reza Tarkhani	2015	Anaesthetic efficacy of eugenol on iridescent shark <i>Pangasius hypophthalmus</i> (Sauvage 1878) in different size classes	Aquaculture Research Volume 46 Issue 2
CA 9.6.3.4/06 [8.2.1]	Hamed Ghafari Farsani; Reza Tarkhani; Ahmad Imani; Hadi Jamali; Hamed Ghafari Farsani; Reza Tarkhani; Ahmad Imani; Hadi Jamali	2017	Anaesthetic efficacy of eugenol on various size classes of angelfish ( <i>Pterophyllum scalare</i> Schultze 1823)	Aquaculture Research Volume 48 Issue 10
CA 9.6.3.4/07 [8.2.1]	Park In-Seok ; Park Sung Jun ; Gil Hyun Woo ; Nam Yoon	2011	Anesthetic effects of clove oil and lidocaine-HCl on marine	Lab Anim (NY). 2011 Feb;40(2):45-51. doi: 10.1038/labani0211-45

Data requirement (indicated by the corresponding CA and CP data point)	Author(s)	Year	Title	Source
	Kwon ; Kim Dong Soo		medaka ( <i>Oryzias dancena</i> ).	
CA 9.6.3.4/08 [8.2.1]	In-Seok Park; Tae Ho Lee; Sang Gu Lim	2018	Anesthetic efficacy and physiological responses of clove oil on juvenile and adult red spotted grouper <i>Epinephelus akarra</i>	Fisheries and Aquatic Sciences volume 21 Article number: 25 ( 2018 )
CA 9.6.3.4/09 [8.2.1]	Bruce W. Menzel e; James A. Roth d; DuÅ;an PaliÅ; a d; Dawn M. Herolt c; Claire B. Andreasen b	2006	Anesthetic efficacy of tricaine methanesulfonate metomidate and eugenol: Effects on plasma cortisol concentration and neutrophil function in fathead minnows ( <i>Pimephales promelas Rafinesque 1820</i> ).	Aquaculture Volume 254 Issues 1-“4 28 April 2006 Pages 675-685
CA 9.6.3.4/10 [8.2.1]	Ozório R A ; Tsuzuki M Y ; Correia A M ; Pedrazzani A S ; Mendonça R C ; Massucatto A	2018	Basil tea tree and clove essential oils as analgesics and anaesthetics in <i>Amphiprion clarkii</i> (Bennett 1830).	Braz J Biol. 2018 Aug;78(3):436-442. doi: 10.1590/1519-6984.166695.
CA 9.6.3.4/11 [8.2.1]	Macova Stanislava ; Dolezelova Petra ; Pistekova Vladimira ; Svobodova Zdenka ; Bedanova Iveta ; Voslarova Eva	2008	Comparison of acute toxicity of 2-phenoxyethanol and clove oil to juvenile and embryonic stages of <i>Danio rerio</i> .	Neuro Endocrinol Lett. 2008 Oct;29(5):680-4
CA 9.6.3.4/12 [8.2.1]	Luiz VÃ;tor Oliveira Vidal Wilson Massamitu Furuya ThÃ;mis Sakaguti Graciano Christiano Rodrigues Chamber et al.	2013	ConcentraÃ;Ã;es de Eugenol para anestesia profunda e toxidade aguda em juvenis de piavuÃ;su ( <i>Leporinus macrocephalus</i> ) = Eugenol concentrations for deep anesthesia and acute toxicity in piavuÃ;su ( <i>Leporinus macrocephalus</i> ) juveniles	2013/AV/AV2013_0 rdf
CA 9.6.3.4/13 [8.2.1]	Ruipeng He a; Bo Lei a; Yuepeng Su b; Anli Wang c; Kuopeng Cui b; Xiaokun Shi b; Xiaoming Chen b	2020	Effectiveness of eugenol as an anesthetic for adult spotted sea bass ( <i>Lateolabrax maculatus</i> ).	Aquaculture Volume 523 30 June 2020 735180

Data requirement (indicated by the corresponding CA and CP data point)	Author(s)	Year	Title	Source
CA 9.6.3.4/14 [8.2.1]	Romaneli Rafael de Souza ; Boaratti André Zuffo ; Rodrigues Andressa Tellechea ; Queiroz Daniel Monge de Almeida ; Khan Kifayat Ullah ; Nascimento Thiago Matias Torres ; Fernandes João Batista Kochenborger ; Mansano Cleber Fernando Menegasso	2018	Efficacy of Benzocaine Eugenol and Menthol as Anesthetics for Freshwater Angelfish.	J Aquat Anim Health. 2018 Sep;30(3):210-216. doi: 10.1002/aah.10030.
CA 9.6.3.4/15 [8.2.1]	Kristan Jiri ; Stara Alzbeta ; Polgesek Miroslav ; Drasovean Alexandru ; Kolarova Jitka ; Priborsky Josef ; Blecha Miroslav ; Svacina Petr ; Policar Tomas ; Velisek Josef	2014	Efficacy of different anaesthetics for pikeperch ( <i>Sander lucioperca</i> L.) in relation to water temperature.	Neuro Endocrinol Lett. 2014;35 Suppl 2:81-5
CA 9.6.3.4/16 [8.2.1]	Uton Charoendat(Kasetsart University Bangkok (Thailand). Faculty of Fisheries. Department of Aquaculture) Nontawith Areechon(Kasetsart University Bangkok (Thailand). Faculty of Fisheries. Department of Aquaculture) Prapansak Srisapoome(Kasetsart University Bangkok (Thailand). Faculty of Fisheries. Department of Aquaculture) Doungdaw Chantasart(Mahidol University Bangkok (Thailand). Faculty of Pharmacy. Department of Pharmacy)	2009	Efficacy of synthetic eugenol as an anesthetic for tilapia ( <i>Oreochromis niloticus</i> Linn.) fry	2009/TH/TH2009_0 rdf

Data requirement (indicated by the corresponding CA and CP data point)	Author(s)	Year	Title	Source
CA 9.6.3.4/17 [8.2.1]	Ribeiro Paula A P ; Miranda-Filho Kleber C ; Melo Daniela C de ; Luz Ronald K	2015	Efficiency of eugenol as anesthetic for the early life stages of Nile tilapia ( <i>Oreochromis niloticus</i> ).	An Acad Bras Cienc. 2015 Mar;87(1):529-35. doi: 10.1590/0001-3765201520140024
CA 9.6.3.4/18 [8.2.1]	Rodrigo Roubach; Levy Carvalho Gomes; Flavio Augusto Leão Fonseca; Adalberto Luiz Val; Rodrigo Roubach; Levy Carvalho Gomes; Flavio Augusto Leão Fonseca; Adalberto Luiz Val	2005	Eugenol as an efficacious anaesthetic for tambaqui <i>Colossoma macropomum</i> (Cuvier)	Aquaculture Research Volume 36 Issue 11
CA 9.6.3.4/19 [8.2.1]	Stevens E Don ; Baldisserotto Bernardo ; Parodi Thaylise V	2018	Lack of postexposure analgesic efficacy of low concentrations of eugenol in zebrafish.	Vet Anaesth Analg. 2018 Jan;45(1):48-56. doi: 10.1016/j.vaa.2017.08.009.
CA 9.6.3.4/20 [8.2.1]	André Fernando Nascimento Gonçalves Elane Cristine Correia Santos João Batista Kochenborger Fernandes Leonardo Susumu Takahashi	2012	Mentol e eugenol como substitutos da benzocaína na indução anestésica de juvenis de pacu = Menthol and eugenol as benzocaine substitutes in anesthetic induction of pacu juveniles	[Acta Scientiarum : Animal Sciences]; 2012/DJ/DJ2012_0.rdf
CA 9.6.3.4/21 [8.2.1]	Perdikaris C.Technological Educational Inst. of Epirus Igoumenitsa (Greece). Dept. of Aquaculture and Fisheries Nathanailides C.Technological Educational Inst. of Epirus Igoumenitsa (Greece). Dept. of Aquaculture and Fisheries Gouva E.Technological Educational Inst. of Epirus Igoumenitsa (Greece). Dept. of Aquaculture and Fisheries Gabriel U.U.Rivers State Univ. of Science and Technology	2011	Size-relative effectiveness of clove oil as an anaesthetic for rainbow trout ( <i>Oncorhynchus mykiss</i> Walbaum 1792) and goldfish ( <i>Carassius auratus</i> Linnaeus 1758)	[Acta Veterinaria (Czech Republic)]; 2011/CZ/CZ2011_0.rdf

Data requirement (indicated by the corresponding CA and CP data point)	Author(s)	Year	Title	Source
	Port Harcourt (Nigeria). Dept. of Fisheries and Aquatic Environment et al.			
CA 9.6.3.4/22 [8.2.1]	C G Soto; J L Keene; D L G Noakes; R D Moccia; C G Soto; J L Keene; D L G Noakes; R D Moccia	1998	The efficacy of clove oil as an anaesthetic for rainbow trout <i>Oncorhynchus mykiss</i> (Walbaum)	Aquaculture Research Volume 29 Issue 2
CA 9.6.3.4/23 [8.2.1]	Wenhao Wang; Hongbiao Dong; Yongxu Sun; Ming Cao; Yafei Duan; Hua Li; Qingsong Liu; Qunhong Gu; Jiasong Zhang; Wenhao Wang; Hongbiao Dong; Yongxu Sun; Ming Cao; Yafei Duan; Hua Li; Qingsong Liu; Qunhong Gu; Jiasong Zhang	2019	The efficacy of eugenol and tricaine methanesulphonate as anaesthetics for juvenile Chinese sea bass ( <i>Lateolabrax maculatus</i> ) during simulated transport	Journal of Applied Ichthyology Volume 35 Issue 2
CA 9.6.3.4/24 [8.2.1]	Morteza Yousefi a; Seyyed Morteza Hoseini b; Yury Anatolyevich Vatnikov a; Alexandr Alexeevich Nikishov a; Evgeny Vladimirovich Kulikov a	2018	Thymol as a new anesthetic in common carp ( <i>Cyprinus carpio</i> ): Efficacy and physiological effects in comparison with eugenol	Aquaculture Volume 495 1 October 2018 Pages 376-383
CA 9.6.3.4/25 [8.2.1]	Xiaohuan Cao; Yajun Wang; Na Yu; Qijun Le; Jiabao Hu; Yang Yang; Siwen Kuang; Man Zhang; Yibo Sun; Weiwei Gu; Xiaojun Yan; Xiaohuan Cao; Yajun Wang; Na Yu; Qijun Le; Jiabao Hu; Yang Yang; Siwen Kuang; Man Zhang; Yibo Sun; Weiwei Gu; Xiaojun Yan	2019	Transcriptome analysis reveals the influence of anaesthetic stress on the immune system of crucian carp ( <i>Carassius auratus</i> ) under the process of treatment and low concentration transport by MS-• 222 and Eugenol	Aquaculture Research Volume 50 Issue 11
CA 9.6.3.4/26 [8.2.1]	Fatih A-Äretmen; Selami GÄ¶lbaÄyi; Burak E. Inanan; Volkan Kizak; Murathan	2014	Use of Clove Oil and Eugenol to Anesthetize Fingerling Shabut <i>Barbus grypus</i>	North American Journal of Aquaculture Volume 76 Issue 1

Data requirement (indicated by the corresponding CA and CP data point)	Author(s)	Year	Title	Source
	Kayim; Fatih A-Åretmen; Selami GÄ¶lbaÅyi; Burak E. Inanan; Volkan Kizak; Murathan Kayim			
CA 9.6.3.4/27 [8.2.1]	Costa D G C ; Oliveira N Y ; Sanches E G ; Azevedo V G ; Takatsuka V	2019	Use of eugenol for anesthesia of lesser guitarfish <i>Zapteryx brevirostris</i> (Rhinobatidae).	Braz J Biol. 2019 Jul-Sep;79(3):516-520. doi: 10.1590/1519-6984.186755.
CA 9.6.3.4/28 [8.2.1]	Diego Prestes Gomes; Brunele Weber Chaves; Alexssandro Geferson Becker; Bernardo Baldisserotto; Diego Prestes Gomes; Brunele Weber Chaves; Alexssandro Geferson Becker; Bernardo Baldisserotto	2011	Water parameters affect anaesthesia induced by eugenol in silver catfish <i>Rhamdia quelen</i>	Aquaculture Research Volume 42 Issue 6
CA 9.6.3.4/46 [CA 8.2.1]	Eman Zahran a; Engy Risha b; Awad Rizk c	2021	Comparison propofol and eugenol anesthetics efficacy and effects on general health in Nile Tilapia	Aquaculture Volume 534 15 March 2021 736251
CA 9.6.3.4/47 [CA 8.2.1]	Phillipe Thiago Leite Barbosa; Jayme Aparecido Povh; Laice Menes Laice; Gabrielly Cristina Teodoro; et al.	2020	Essential oil of <i>Ocimum basilicum</i> and Eugenol as Sedatives for Nile Tilapia	[Journal of Agricultural Studies]; 2020/US/US2020_6 rdf
CA 9.6.3.4/29 [8.2.2.1]	Petri Hoskonen; Jouni Heikkinen; PÄivi Eskelinen; Juhani Pirhonen; Petri Hoskonen; Jouni Heikkinen; PÄivi Eskelinen; Juhani Pirhonen	2015	Efficacy of clove oil and ethanol against <i>Saprolegnia</i> sp. and usability as antifungal agents during incubation of rainbow trout <i>Oncorhynchus mykiss</i> (Walbaum) eggs	Aquaculture Research Volume 46 Issue 3
CA 9.6.3.4/30 [8.2.4.2]	Falin Zhou; Wanli Yang; Zhigang Wu; Yin Le; Qibin Yang; Yebing Yu; Shigui Jiang; Song Jiang; Falin Zhou; Wanli Yang; Zhigang Wu; Yin Le; Qibin Yang; Yebing Yu; Shigui Jiang; Song Jiang	2020	Anaesthetic effect of eugenol at different concentrations and temperatures on black tiger shrimp ( <i>Penaeus monodon</i> )	Aquaculture Research Early View

Data requirement (indicated by the corresponding CA and CP data point)	Author(s)	Year	Title	Source
CA 9.6.3.4/31 [8.2.4.2]	Li Yingdong ; She Qiuxin ; Han Zhibin ; Sun Na ; Liu Xu ; Li Xiaodong	2018	Anaesthetic Effects of Eugenol on Grass Shrimp ( <i>Palaemonetes sinensis</i> ) of Different Sizes at Different Concentrations and Temperatures.	Sci Rep. 2018 Jul 20;8(1):11007. doi: 10.1038/s41598-018-28975-w
CA 9.6.3.4/32 [8.2.4.2]	Darbyshire Amanda K ; Oliver Kendra H ; Dupont William D ; Plummer W Dale ; Jones Carissa P ; Boyd Kelli L	2019	Anesthesia and Euthanasia of Brine Shrimp ( <i>Artemia franciscana</i> ).	J Am Assoc Lab Anim Sci. 2019 Jan 1;58(1):58-64. doi: 10.30802/AALAS-JAALAS-18-000040.
CA 9.6.3.4/33 [8.2.4.2]	Cansian R L ; Vanin A B ; Orlando T ; Piazza S P ; Puton B M S ; Cardoso R I ; Gonçalves I L ; Honaiser T C ; Paroul N ; Oliveira D	2017	Toxicity of clove essential oil and its ester eugenyl acetate against <i>Artemia salina</i> .	Braz J Biol. 2017 Mar;77(1):155-161. doi: 10.1590/1519-6984.12215.
CA 9.6.3.4/34 [8.2.8]	Beaudry Francis ; Vachon Pascal ; Guénette Sarah A ; Hélie Pierre	2007	Eugenol for anesthesia of African clawed frogs ( <i>Xenopus laevis</i> ).	Vet Anaesth Analg. 2007 May;34(3):164-70. doi: 10.1111/j.1467-2995.2006.00316.x
CA 9.6.3.4/35 [8.2.8]	Hélie P ; Goulet F ; Vachon P	2011	Evaluation of the toxicity of eugenol at anesthetic doses in African clawed frogs ( <i>Xenopus laevis</i> ).	Toxicol Pathol. 2011 Apr;39(3):471-7. doi: 10.1177/0192623311399785.
CA 9.6.3.4/36 [8.3.2]	Toledo Pedro F S ; Viteri Jumbo Luis O ; Rezende Sarah M ; Haddi Khalid ; Silva Bruno A ; Mello Tarcísio S ; Della Lucia Terezinha M C ; Aguiar Raimundo W S ; Smagghe Guy ; Oliveira Eugenio E	2020	Disentangling the ecotoxicological selectivity of clove essential oil against aphids and non-target ladybeetles.	Sci Total Environ. 2020 May 20;718:137328. doi: 10.1016/j.scitotenv.2020.137328.
CA 9.6.3.4/37 [8.6]	Xuan Tran Dang ; Toyama Tsuneaki ; Fukuta Masakazu ; Khanh Tran Dang ; Tawata Shinkichi	2009	Chemical interaction in the invasiveness of cogongrass ( <i>Imperata cylindrica</i> (L.) Beauv.).	J Agric Food Chem. 2009 Oct 28;57(20):9448-53. doi: 10.1021/jf902310j

Data requirement (indicated by the corresponding CA and CP data point)	Author(s)	Year	Title	Source
CA 9.6.3.4/38 [8.6]	Hossein Reza Darabi; Shabnam Mohandessi; Yadollah Balavar; Mojtaba Mirhosseini Moghaddam; Kioumars Aghapoor; Farshid Mohsenzadeh; Abbas Ali Nourinia	2011	Clove bud oil: an efficient economical and widely available oil for the inhibition of wheat seed germination	Environmental Chemistry Letters volume 9 pages 519 -“ 524 ( 2011 )
CA 9.6.3.4/39 [8.6]	Nitina Ahuja; Daizy R. Batish; Harminder Pal Singh; Ravinder K. Kohli	2015	Herbicidal activity of eugenol towards some grassy and broad-leaved weeds	Journal of Pest Science volume 88 pages 209 -“ 218 ( 2015 )
CA 9.6.3.4/40 [8.6]	Waliwitiya Ranil ; Isman Murray B ; Vernon Robert S ; Riseman Andrew	2005	Insecticidal activity of selected monoterpenoids and rosemary oil to <i>Agriotes obscurus</i> (Coleoptera: Elateridae).	J Econ Entomol. 2005 Oct;98(5):1560-5. doi: 10.1093/jee/98.5.1560
CA 9.6.3.4/41 [8.6]	Jana Kalinova; Jan Triska; Nadezda Vrchatova	2011	Occurrence of eugenol coniferyl alcohol and 3,4,5-trimethoxyphenol in common buckwheat ( <i>Fagopyrum esculentum</i> Moench) and their biological activity	Acta Physiologiae Plantarum volume 33 pages 1679 -“ 1685 ( 2011 )

**9.11.1.4.2-5: Relevant studies included in the supplementary dossier after detailed assessment of full-text documents for relevance: sorted by data requirement(s) for methyleugenol in the Ecotoxicology Section**

Data requirement (indicated by the corresponding CA and CP data point)	Author(s)	Year	Title	Source
CA 9.6.3.4/42 [CA 8.6]	Amri Ismail ; Mancini Emilia ; De Martino Laura ; Marandino Aurelio ; Lamia Hamrouni ; Mohsen Hanana ; Bassem Jamoussi ; Scognamiglio Mariarosa ; Reverchon Ernesto ; De Feo Vincenzo	2012	Chemical composition and biological activities of the essential oils from three <i>Melaleuca</i> species grown in Tunisia.	Int J Mol Sci. 2012 Dec 5;13(12):16580-91. doi: 10.3390/ijms131216580
CA 9.6.3.4/43 [CA 8.6]	Lal Mohan ; Gogoi Roktim ; Loying Rikraj ; Sarma Neelav ; Begum Twahira ; Pandey Sudin Kumar	2020	Comparative in-vitro biological activities of methyl eugenol rich <i>Cymbopogon khasianus</i> Hack. leaf essential oil with pure methyl eugenol compound.	Curr Pharm Biotechnol. 2020 Feb 16. doi: 10.2174/1389201021666200217113921
CA 9.6.3.4/44 [CA 8.2.1]	Cecilia Soares Vilhena a; Luís Adriano Santos do Nascimento a; Eloísa Helena de Aguiar Andrade b; Joyce Kelly do Rosário da Silva b; Moisés Hamoy c; Marcelo Ferreira Torres d; Luis André Luz Barbas d	2019	Essential oil of <i>Piper divaricatum</i> induces a general anaesthesia-like state and loss of skeletal muscle tonus in juvenile tambaqui <i>Colossoma macropomum</i>	Aquaculture Volume 510 15 August 2019 Pages 169-175
CA 9.6.3.4/45 [CA 8.3.2]	Keng-Hong Tan b; Kong-Luen Heong c; Zhong-Xian Lu a; Hong-Xing Xu a; Xu-Song Zheng a; Ya-Jun Yang a; Jun-Ce Tian a; Yan-Hui Lu a	2015	Methyl eugenol bioactivities as a new potential botanical insecticide against major insect pests and their natural enemies on rice ( <i>Oriza sativa</i> )	Crop Protection Volume 72 June 2015 Pages 144-149

***B.9.11.1.5. Conclusion***

In this literature search for the active substance eugenol and methyleugenol, 28 270 references (for eugenol) and 4 133 references (for methyleugenol) were identified and evaluated for their potential relevance for data requirements “toxicological and toxicokinetic studies, human exposure, residues, fate and behaviour in the environment and ecotoxicological studies”.

After rapid and detailed assessments for relevance, the following publications were considered to be relevant:

	<b>Number of publications considered to be relevant Eugenol</b>	<b>Number of publications considered to be relevant Methyleugenol</b>
Toxicology, including human exposure	12	14
Residues	10	3*
Environmental fate	2	0
Ecotoxicology	<b>43</b>	<b>4</b>

\* The 3 papers were also identified in the eugenol literature search

All relevant and reliable publications identified following the detailed assessment are summarised in the corresponding MCA/MCP sections of this supplementary dossier, where an assessment of their reliability is also presented in line with Section 5.4 of EFSA Journal 2011;9(2):2092.

***Eugenol***

For ecotoxicology, as the reliability of relevant papers is limited for use in the ecotoxicological risk assessment of eugenol they have not been considered further in this document and brief summaries of these papers are provided below in the Appendix I for transparency.

***Methyleugenol***

For ecotoxicology, as the reliability of relevant papers is limited for use in the ecotoxicological risk assessment of eugenol and methyl-eugenol they have not been considered further in this document and brief summaries of these papers are provided below in the Appendix I for transparency.

**Assessment and conclusion by RMS:**

The RMS has checked the review of literature data submitted by the applicant and considers the databases used for the search are acceptable, and the search strategy as well. Furthermore, the justifications given by the applicant for non-relevance seemed acceptable.

Therefore, RMS considered acceptable the review literature submitted by the applicant.

## B.9.11.2. Reference Submitted in the Renewal Dossier

Data Point	Author(s)	Year	Title Report No. Document No. Source (where different from company) GLP/ Officially recognised testing facilities <sup>2,3</sup> Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously used <sup>1</sup>  Y/N  If yes, for which data point?
Study B.9.2.1/01  CA 8.2.1/01	██████████ ██████████ ██████████	2008a	Acute toxicity of eugenol to rainbow trout ( <i>Oncorhynchus mykiss</i> ) in a 96-hour semi-static test Report No. 37984230 ██████████ ██████████, Germany GLP Unpublished	Y	N	-	Eden Research plc	Y In DAR (2011) IIA 8.2.1.1/01
Study B.9.2.1/02  CA 8.2.1/02	██████████ ██████████ ██████████	2008b	Acute toxicity of eugenol to zebra fish ( <i>Danio rerio</i> ) in a 96-hour semi-static test Report No. 37983230 ██████████ ██████████, Germany GLP Unpublished	Y	N	-	Eden Research plc	Y In DAR (2011) IIA 8.2.1.1/02
Study B.9.2.4.1/01  CA 8.2.4.1/01	Pavić, B., Wydra, V.	2008	Acute toxicity of eugenol to <i>Daphnia magna</i> in a static 48-hour immobilization test Report No. 37982220 IBACON GmbH, Germany GLP Unpublished	N	N	-	Eden Research plc	Y In DAR (2011) 8.3.1.1/01
Study B.9.2.5.1/01  CA 8.2.5.1/01	Egeler, P.	2021	Eugenol: A study on the chronic toxicity to <i>Daphnia magna</i> Report No. 20GC3DB ECT Oekotoxikologie GmbH, Germany GLP	N	Y	Study not previously submitted	Eden Research plc	N Submitted for the purpose of renewal

			Unpublished					
Study B.9.2.6.1/01  CA 8.2.6.1/01	Meister- Werner, A., Wydra, V.	2008	Toxicity of eugenol to <i>Pseudokirchneriella</i> <i>subcapitata</i> in an algal growth inhibition test Report No. 37981210 IBACON GmbH, Germany GLP Unpublished	N	N	-	Eden Research plc	Y In DAR (2011) IIA 8.4/01
Study 9.8.1/01  CA 8.8/01	Hammesfahr, U.	2020	Mevalone: Toxicity to activated sludge in a respiration inhibition test Report No. 155781171 IBACON GmbH, Germany GLP Unpublished	N	Y	Study not previously submitted	Eden Research plc	N Submitted for the purpose of renewal

<sup>1</sup> In order to facilitate the compilation of the final list of the tests and studies relied upon and the corresponding data protection, indicate whether the study was used in the previous DAR/RAR or, when the information is available, whether the study was already submitted in the framework of national authorisations.

<sup>2</sup> See Art.3 of Annex of Regulation No 283/2013 and 284/2013

<sup>3</sup> The RMS shall check that the GLP statement has been properly signed in the study report, that the study results are properly reported in accordance with GLP standards and following the relevant guidance by OECD on the review of the GLP status of non-clinical safety data (currently under development).

## APPENDIX I.

### STUDY SUMMARIES OF RELEVANT PUBLICATIONS ACCORDING FULL-TEXT

As some potentially relevant parameters were included in the following studies, a reliability assessment was also conducted. Following detailed assessment, overall it was concluded that these studies are of limited reliability for use in the ecotoxicological risk assessment of eugenol. Whilst some may be considered as supporting information, they are of limited added value to the risk assessment and therefore have not been considered further in the corresponding data point. Since these studies are considered as not reliable they have not been evaluated by the RMS, brief summaries of these papers with the applicant's assessment have been included below for transparency.

In particular, it is noted that the majority of papers are related to the use of eugenol as a short-term anaesthetic during the handling and/or transport of fish or other aquatic organisms by humans. As highlighted in the summaries below, these papers are not considered reliable for use in the risk assessment, principally as analytical verification was not reported to confirm the actual exposure during the studies (as required as standard in all aquatic ecotoxicology studies). Furthermore, results of these anaesthesia studies are of limited relevance for a quantitative risk assessment due to the very short-term exposure, followed by a recovery period in untreated water. The critical endpoints from the standard aquatic ecotoxicity studies summarised in the corresponding data point are considered more relevant and reliable for the aquatic risk assessment, and these OECD studies themselves will cover any possible short-term behavioural (e.g. anaesthesia) effects.

*Ecotoxicology: Eugenol (41 literature papers)*

<b>Data point:</b>	CA 9.6.3.4/01 [CA 8.1.2]
<b>Report author</b>	Schlötterburg, A.; Jakob, G.; Bellingrath-Kimura, S.; Jacob, J.
<b>Report year</b>	2018
<b>Report title</b>	Natural bait additives improve trapping success of common voles, <i>Microtus arvalis</i>
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 247 Applied Animal Behaviour science, Volume 208, Pages 75-81 (2018)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	Not reliable

#### Abstract (copied from original literature)

Common voles are serious pests in European agriculture, damaging cereals, rapeseed and other crops and causing substantial losses per outbreak. Not only might the usual approach of applying rodenticides for population management have disadvantages for non-target species, these rodenticides also cannot be used in organic farming. An alternative solution could be an approach related to the concept of ecologically-based rodent management combining environmentally sustainable methods based on knowledge about the target species. Such a method inhibits the dispersal of common voles from field margins to crop habitats via a trap-barrier system. However, little is known about attractants, which could increase the trapping success of common voles. We screened 22 natural substances in T-maze trials. The three most successful substances were further tested during enclosure trials under semi-natural conditions. Bisabolol, eugenol and maltol attracted six of eight voles during the T-maze trials and were mixed into grain pellet bait. Bait containing maltol caught significantly more common voles in enclosures than plain control bait or bait containing bisabolol or eugenol. Of all individuals, 21% chose exclusively baits with maltol. Generalized Linear Mixed Model (GLMM) indicated that every individual would be trapped once in 6 h with this bait type and GLMM predicted that 63% of females and 56%

of males would choose this bait over plain control bait. Maltol bait could help either by more efficiently trapping common voles in a trap-barrier system or improving bait acceptance when rodenticides are applied.

## Materials and methods

### Test material

Name: Eugenol  
 Formulation type: Not relevant  
 Source and lot/batch no.: Sigma-Aldrich GmbH

Active substance content: Not reported  
 Expiry date of lot/batch: Not reported  
 Storage conditions: Not reported

### Test organism

Species: common vole, *Microtus arvalis*  
 Strain/clone: Not reported  
 Age at study initiation: adults  
 Weight/length/height at study initiation: 15 – 39 g for screening test  
 Source: captured on agricultural fields near Münster (North Rhine-Westphalia, Germany) and Bernburg (Saxony-Anhalt, Germany)  
 Feeding during test: Yes, 15 g of wheat and 15 g of pellets were offered in two metal feeding racks (120×42×35 mm) for four 24 h periods  
 Acclimation: Voles were fed wood shavings, hay, water *ad libitum* and standard rodent chow (Altromin 1324, Altromin Spezialfutter GmbH & Co. KG, Lage, Germany), supplemented with slices of apple or carrot once a week. Voles were maintained on a 12:12 h light:dark cycle at 18–21°C for at least one week before trials.

### Test conditions

Test temperature: 18–21 °C  
 Photoperiod: 12:12 h light:dark  
 Light intensity: Not reported

#### Test system

Study type: T-maze trials  
 Duration of study: 20 min for screening tests, 12 hours for enclosure tests  
 Treatments: Not reported  
 Analytical determination of test concentrations: No  
 Negative control included: Yes, distilled water  
 Positive control included: Yes, standard bait (3 g of apple slices, two peanut curls and 5 g of oat flakes) and a commercial product for baiting voles  
 Parameters measured: Mortality/behaviour  
 Validity criteria: Not reported

The trial was conducted in three steps, step 1 initial screening, step 2 preliminary feeding trials and step 3 enclosure trials. The screening step was performed using T-maze trials in the laboratory facility of the Julius Kühn Institute in Münster, Germany, from May to July 2015. Each substance was tested with four different

female and male common voles ( $n = 8$  per substance). Then, feeding trials were conducted to ensure that grain pellets were as attractive as wheat because grain pellets seemed to be more appropriate for mixing with additives than wheat kernels. For the enclosure trials, eugenol was mixed into pellets. Enclosure trials were run under semi-natural conditions at the Julius Kühn Institute in September and October 2015. Five females and three males were released in each enclosure.

#### Statistics

The uptake of wheat kernels and vole in 6 h during the enclosure trials were integrated in linear mixed models (LMM), respectively generalized linear mixed models (GLMM) using the statistical program R (version 3.5.0) (RCoreTeam, 2018) and the R package “lme4” fitting models by maximum likelihood (Laplace approximation). Using LMMs, it was analyzed whether the consumed grain quantities were dependent on food type (wheat kernels or grain pellets), body weight, sex of the voles (fixed effects), trial day or experimental animal (random effects). In the GLMMs, to know if trapping success was depended on the bait type, a Poisson error distribution and log link were used.

#### Results

During the initial screening, eugenol lured six of eight voles (75%) within 20 min into the T-maze trap. In comparison, during the enclosure trials (under semi-natural conditions), eugenol was not found to attract voles.

#### Assessment and conclusion

##### Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Non-standard study design testing eugenol (purity not reported) mixed into grain pellets (of limited relevance to spray application). Volume of eugenol applied (0.3 mL) is reported, but a corresponding air concentration (considering study was testing effects of odour) was not reported.

#### Assessment and conclusion by applicant:

The study is considered not reliable.

*Microtus arvalis*: Eugenol (0.3 mL applied to cotton pads) attracted voles in the T-maze trials, but during the enclosure trials, eugenol was not found to attract voles.

<b>Data point:</b>	CA 9.6.3.4/02 [8.2.1]
<b>Report author</b>	Pattanasiri, T., Taparhudee, W., Suppaku, P.
<b>Report year</b>	2016
<b>Report title</b>	Acute toxicity and anaesthetic effect of clove oil and eugenol on Siamese fighting fish, <i>Betta splendens</i>
<b>Report No</b>	Internal reference: Study 20 Aquaculture international: Journal of the European Aquaculture Society Volume 25, Pages 163-175.
<b>Document No</b>	-
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

#### Abstract (copied from original literature)

With brilliant colouration and long, flowing fins, the Siamese fighting fish (*Betta splendens*) is one of the most popular species of freshwater aquarium fish. In the ornamental Siamese fighting fish business, stress is one of the major causes of fish injury, including collapse of the fins. This problem could be solved by using anaesthetic treatment.

Clove oil and eugenol were investigated for acute toxicity, which was determined using an aqueous dilution method at concentrations ranging from 0 to 35 mg/L. Based on a criterion of fish transportation within 2 days, induction times of anaesthesia, fish behavioural responses and mortality, and recovery times were monitored and recorded over a 48-h period. The 48-h LC<sub>50</sub> of clove oil and eugenol was 30.63 [with a 95 % confidence interval (CI) of 29.23–32.10 mg/ L] and 29.95 mg /L (with a 95 % CI of 28.50–31.48 mg/L), respectively. Concentrations of 10 and 15 mg/ L clove oil over a 48-h period induced a sedative effect, resulting in partial loss of reactivity and mobility while maintaining equilibrium. At these concentrations, the fish could recover behaviourally within 3–5 min. Recovery times decreased with lower doses and shorter exposure times. The higher the concentration of either clove oil or eugenol used, the lower the concentrations of total ammonia and un-ionized ammonia that were detected. This study revealed that clove oil and eugenol are highly effective anaesthetic agents as a transportation mixture for Siamese fighting fish.

## Materials and methods

### Test material 1

Name:	Clove oil ( <i>S. aromaticum</i> Linn.)
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma-Aldrich
Active substance content:	Eugenol 85%
Expiry date of lot/batch:	Not reported
Storage conditions:	To reduce the degree of photodegradation, both clove oil and eugenol stock solutions were kept in amber reagent bottles at an ambient laboratory temperature of approximately 19 °C.

### Test material 2

Name:	eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma-Aldrich
Active substance content:	Eugenol 85% (purified up to 99%)
Expiry date of lot/batch:	Not reported
Storage conditions:	To reduce the degree of photodegradation, both clove oil and eugenol stock solutions were kept in amber reagent bottles at an ambient laboratory temperature of approximately 19 °C.

### Test organism

Species:	Siamese fighting fish ( <i>B. splendens</i> )
Strain/clone:	Not reported
Age at study initiation:	Not reported
Weight/length at study initiation:	1.78 ± 0.3 g and 6.27 ± 0.16 cm
Source:	Commercial fish farm
Feeding during test:	Fish were starved for 24 h prior to the experiment.
Acclimation:	During acclimatization, water was changed daily. Fish were fed a commercial pelletized diet containing 40% protein, and live adult artemia (brine shrimp) twice a day to satiety. Fish were starved for 24 h prior to the experiment.

### Test conditions

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Hardness:	Not reported
Test temperature:	28 °C
pH:	7.24
Dissolved oxygen:	6.65 µL/L
Conductivity:	Not reported
Photoperiod:	Not reported
Light intensity:	Not reported
<i>Test system</i>	
Study type:	Acute toxicity (experiment 1) and anaesthetic effect induction time and recovery time (experiments 2 and 3)
Duration of study:	48 hours
Treatments:	25.00, 26.74, 28.60, 30.60, 32.72 and 35.00 mg eugenol/L or mg clove oil/L (nominal)
Analytical determination of test concentrations: No	
Negative control included:	No
Positive control included:	No
Parameters measured:	Mortality
Validity criteria:	Not reported

Clove oil had a eugenol content of 85 % w/w; eugenol was highly purified (99 % w/w). Either clove oil or eugenol was mixed with ethanol (95 %) in a ratio of 1:9 to facilitate the mixing process.

For the acute toxicity test, groups of 20 acclimated fish were exposed to different concentrations of either clove oil or eugenol. The stock solution of either clove oil or eugenol was mixed thoroughly with 10 L of fresh water to obtain test concentrations of 5, 10, 15, 20, 25, 30 and 35 mg eugenol/L or mg clove oil/L for the range-finding tests. Fresh solutions were prepared every other day and were protected from sun and heat to limit photodegradation and thermal degradation. From the range-finding test, it was found that both clove oil and eugenol yielded similar findings. The highest concentration of 25 mg eugenol/L resulted in 0 % mortality, whereas the lowest concentration of 35 mg eugenol/L led to 100 % mortality over a 48-hours period. Consequently, this concentration range was divided into a logarithmically spaced series for definitive testing: 25.00, 26.74, 28.60, 30.60, 32.72 and 35.00 mg eugenol/L or mg clove oil/L. Each low-density polyethylene (LDPE) bag (10 x 30 cm) contained one fish with 150 mL aerated fresh water and was tightened with a rubber band. Mortality was observed after 48 hours. The median lethal concentration (48-hours LC<sub>50</sub>) was estimated by probit analysis according to the method described by Litchfield and Wilcoxon (1949). All determinations were carried out in triplicate.

To address the anesthetic effects, 10 fishes were randomly selected from one of the 150-L tanks and transferred into individual LDPE bags which were filled with 150 mL aerated fresh water and tightened with a rubber band. Fish were visually monitored at each time interval; stage of anaesthesia was assessed by behavioural analysis. Based on a criterion of fish transportation within 2 days, induction times of anaesthesia, fish behavioural responses and mortality, and recovery times were monitored and recorded over a 48-h period.

### Results

Based on probit analysis, the 48-h LC<sub>50</sub> of eugenol was 29.95 mg/L (with a 95 % CI of 28.50–31.48 mg/L) and the 48-h LC<sub>50</sub> of clove oil was 30.63 (with a 95 % CI of 29.23–32.10 mg/L), all based on nominal concentrations. Higher anaesthetic concentrations significantly reduced the time for Siamese fighting fish to reach stage 4 anaesthesia (medullary collapse and death). For eugenol at a concentration of 5 mg/L, the time required to reach stage 1 (sedation) was 1 hour. The level of sedation remained at this stage through 24 h and then declined to stage 0 (normal) after 48 h. At concentrations of 10 and 15 mg eugenol/L, the induction time for eugenol at the same was 20 min. Recovery times for eugenol was 5 min, at concentrations of either 10 or 15 mg eugenol/L. It was found that eugenol afforded time to reach stage 2 (equilibrium loss) after 48 h at concentrations of 20 and 30 mg/L. The stage 2 was reached at 30 mg/L in 24 h for eugenol. The time required to reach stage 3 (reflex reactivity loss) and stage 4 (medullary collapse) anaesthesia steadily decreased with increasing concentrations of eugenol of 40, 50 and 100 mg eugenol/L. At concentrations of 10 and 15 mg/L eugenol afforded induction times of 19.50 and 16.10 min, and recovery times of 3.20 and 4.50 min, respectively. This study revealed that both clove oil and eugenol have shown to be highly effective anaesthetic agents as a transportation mixture for Siamese fighting fish.

### Assessment and conclusion

#### *Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

**Analytical verification of test concentrations was not reported.**

The use of a solvent control for the acute toxicity test is not clear.

**Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

48-hour LC<sub>50</sub> *B. splendens* (Siamese fighting fish) = 29.95 mg eugenol/L (95% confidence limits: 28.50–31.48 mg eugenol/L) based on nominal concentrations.

48-hour LC<sub>50</sub> *B. splendens* (Siamese fighting fish) = 30.63 mg clove oil/L (95% confidence limits: 29.23–32.10 mg clove oil/L) based on nominal concentrations.

<b>Data point:</b>	CA 9.6.3.4/03 [8.2.1]
<b>Report author</b>	Palmeira Barbosa de Oliveira, C., Henrique da Paixão Lemos, C., Oliveira Vidal, L.V., Couto, R.D., Peixoto Pereira, D.S., Copatti, C.E.
<b>Report year</b>	2019
<b>Report title</b>	Anaesthesia with eugenol in hybrid Amazon catfish ( <i>Pseudoplatystoma reticulatum</i> × <i>Leiarius marmoratus</i> ) handling: Biochemical and haematological responses
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 28 Aquaculture volume 501, Pages 255-259 (2019)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

**Abstract (copied from original literature)**

This study aimed to evaluate the effectiveness of eugenol for induction and anaesthesia recovery in hybrid Amazon catfish juveniles and to verify its effects after handling stress. Juveniles were exposed to different concentrations of eugenol: 0 (control), 10, 20, 30, 40, 50 and 60 µL/L (equivalent to 10.6, 21.2, 31.8, 42.4, 53.0 or 63.6 mg/L). The fish were divided into two groups: anaesthetised with 53.0 mg/L eugenol and non-anaesthetised. Biochemical and haematological variables showed different responses for eugenol in handling (0, 1 and 5 h). Eugenol was recommended for sedation and anaesthesia with 21.2 and 53.0 mg/L, respectively. In control fish, 25 and 50% mortality occurred at zero and one hour after handling stress. Plasma cortisol levels were higher in control fish than anaesthetised fish (0 h). Plasma AST and albumin levels were higher in control fish when compared to anaesthetised fish (0 and 1 h, respectively). In control fish, plasma cortisol, glucose, total protein and albumin levels were significantly lower at five hours after handling stress. Thrombocyte and neutrophil values were lower in control fish when compared to anaesthetised fish (1 and 5 h, respectively). The use of 53.0 mg/L eugenol is indicated as an anaesthetic for Amazon catfish and reduces post-handling stress by reducing mortality, improving biochemical responses and promoting increased immune system.

**Materials and methods**

*Test material 1*

Name: eugenol  
 Formulation type: Not relevant  
 Source and lot/batch no.: Biodinâmica, Ibitiporã, Brazil

Active substance content: Not reported  
 Expiry date of lot/batch: Not reported  
 Storage conditions: Not reported

### Test organism

Species: Juvenile fish Amazon hybrid catfish (female cachara *Pseudoplatystoma reticulatum* x male Amazonian silver catfish *Leiarius marmoratus*)  
 Strain/clone: Not reported  
 Age at study initiation: Juvenile  
 Weight/length/height at study initiation:  $81.02 \pm 3.56$  g;  $23.03 \pm 0.28$  cm  
 Source: fish farms in Brazil  
 Feeding during test: No  
 Acclimation: Acclimation period 15 days. The animals were provided a diet of commercial feed with 32.00% crude protein and 3500 kcal/kg digestible energy three times a day (8:00 a.m., 1:00 p.m. and 5:00 p.m.) until apparent satiety.

### Test conditions

Hardness:  $80 \pm 0.00$  mg/L calcium carbonate.  
 Test temperature:  $26.5 \pm 0.6$  °C  
 pH:  $7.5 \pm 0.02$   
 Dissolved oxygen:  $7 \pm 0.18$  mg O<sub>2</sub>/L  
 Salinity: Not reported  
 Conductivity: Not reported  
 Photoperiod: Not reported  
 Light intensity: Not reported

#### Test system

Study type: Acute toxicity test (anaesthesia effects)  
 Duration of study: 72 hours (exposure period maximum 30 minutes)  
 Treatments: 10, 20, 30, 40, 50 or 60 µL/ L (equivalent to 10.6, 21.2, 31.8, 42.4, 53.0 or 63.6 mg/L, because density of eugenol was 1.06 g/cm<sup>3</sup>).

tested (nominal)

Analytical determination of test concentrations: No.

Negative control included: Yes (water only and solvent (ethanol) controls)

Positive control included: No

Parameters measured: Sedation (partial loss of balance with response to stimuli); anaesthesia (total loss of balance without response to stimuli); mortality (the paper also includes effects on biochemical, plasma and haematological markers, but these are not discussed further in this summary as not considered relevant).

Validity criteria: Not reported

The time to anaesthesia induction and recovery were tested in 64 juveniles. The procedure involved transferring juveniles to aquaria containing 4 L of water and eugenol (Biodinâmica, Ibiporã, Brazil) at concentrations of 10, 20, 30, 40, 50 or 60 µL/L (equivalent to 10.6, 21.2, 31.8, 42.4, 53.0 or 63.6 mg/L, because density of eugenol was 1.06 g/cm<sup>3</sup>). The concentrations were diluted 1:10 with ethanol. An ethanol group was transferred to aquaria that contained only ethanol (540 µL/L) at a concentration that was equivalent to the dilution used for 60 µL/L (= 63.6 mg/L) eugenol. Control fish were submitted to the same handling process using water only. To evaluate the anaesthetic induction time, two fish were placed simultaneously in each aquarium. The sedation (partial loss of balance with response to stimuli) and anaesthesia (total loss of balance without response to stimuli) were evaluated up to 30 minutes and each fish was used only once. Then, the juveniles were transferred to an anaesthetic-free aquarium (4 L) to measure the post-anaesthetic recovery time (swimming, equilibrium and behaviours similar to the fish kept in maintenance tanks). Survival was monitored up to 72 hours after anaesthetic induction.

## Results

Following a maximum exposure period of 30 minutes to controls or 10.6 mg eugenol/L, there were no anaesthetic effects on juvenile Amazon catfish (*Pseudoplatystoma reticulatum* × *Leiarius marmoratus*). Above this concentration (at 21.2 to 63.6 mg/L), eugenol caused sedation and anaesthesia in Amazon catfish, where 21.2 mg/L was the minimum effective concentration able to cause sedation. The lowest sedation/anaesthesia times were observed in juveniles submitted to 53.0 and 63.6 mg/L of eugenol ( $84.12 \pm 4.2$  and  $80.87 \pm 9.3$ , respectively, for sedation;  $169.00 \pm 29.0$  and  $155.00 \pm 33.0$  s, respectively, for anaesthesia), where 53.0 mg/L was the minimum effective concentration able to cause anaesthesia. The results of regression showed that higher concentrations of eugenol resulted in a shorter time being required for sedation and anaesthesia in fish.

Juveniles exposed to all concentrations of eugenol (nominally 10.6 to 63.6 mg/L) for up to 30 minutes recovered completely and there was no mortality during the experiment or after 72 hours of the initial exposure. Anaesthetic recovery time ranged from  $228.00 \pm 14.23$  to  $297.00 \pm 18.43$  s. There was no correlation of anaesthetic recovery time in relation to the concentrations tested.

## Assessment and conclusion

### Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02CA 9.4.2/02.

Proposed category: 3 not reliable.

Analytical verification of test concentrations was not reported. Purity of test item also not known.

### **Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

Juvenile Amazon catfish (*Pseudoplatystoma reticulatum* × *Leiarius marmoratus*): No adverse effects were observed on mortality after 72 hours following a maximum 30 minute exposure to concentrations up to 63.6 mg eugenol/L (based on nominal concentrations).

NOEC value for sedation (partial loss of balance with response to stimuli) = 10.6 mg eugenol/L (nominal); LOEC value for sedation (partial loss of balance with response to stimuli) = 21.2 mg eugenol/L (nominal). The lowest sedation times were observed in juveniles submitted to 53.0 and 63.6 mg eugenol/L ( $84.12 \pm 4.2$  and  $80.87 \pm 9.3$  seconds, respectively).

NOEC value for anaesthesia (total loss of balance without response to stimuli) = 42.4 mg eugenol/L (nominal); LOEC value for anaesthesia (total loss of balance without response to stimuli) = 53.0 mg eugenol/L (nominal). The lowest anaesthesia times were observed in juveniles submitted to 53.0 and 63.6 mg eugenol/L ( $169.00 \pm 29.0$  and  $155.00 \pm 33.0$  seconds, respectively).

<b>Data point:</b>	CA 9.6.3.4/04 [8.2.1]
<b>Report author</b>	Tarkhani, R., Imani, A., Jamal, H., Moghanlou, K.S.
<b>Report year</b>	2017
<b>Report title</b>	Anaesthetic efficacy of eugenol on Flowerhorn ( <i>Amphilophus labiatus</i> x <i>Amphilophus trimaculatus</i> )
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 31 Aquaculture Research volume 48, Pages 3207-3215
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

### Abstract (copied from original literature)

Anaesthetic efficacy of eugenol was investigated on Flowerhorn (*Amphilophus labiatus* x *Amphilophus trimaculatus*). A total of 104 fish with average weights of  $12 \pm 2.5$ ,  $28 \pm 5$  and  $53 \pm 5.1$  g were subjected to 25–200 mg eugenol/L and behavioural responses as well as induction and recovery times were recorded. Induction and recovery times were significantly affected by eugenol concentration as well as fish weight ( $P < 0.05$ ).

Generally, 49.9–127.3 s after exposure to 50–200 mg eugenol/L, fish reached stage 3 anaesthesia (suitable for general handling). Fish entered stage 4 anaesthesia (suitable for surgery and blood sampling) over 57.3– 140.4 s post exposure to such concentrations.

Recovery time was 91.7–312 s in all weight classes for all eugenol concentrations. Mortality (23%) was only observed in 12-g fish when were subjected to 200 mg eugenol/L. This study showed the behavioural response of Flowerhorn to anaesthesia and eugenol efficacy as an anaesthetic in this important ornamental species. The general quadratic equation revealed that concentrations of eugenol and fish size along with their interactive effects have significantly contributed to the model, with concentration recording the highest beta value in all models ( $\beta = -0.809, -0.818$  and  $-0.909, P = 0.000$ ). According to the results, minimum eugenol concentration to induce anaesthesia in less than 3 min was 50 mg eugenol/L.

### Materials and methods

#### Test material 1

Name:	Eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma, St. Louis, MO, USA
Active substance content:	99%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

#### Test organism

Species:	Flowerhorn ( <i>Amphilophus labiatus</i> x <i>Amphilophus trimaculatus</i> )
Strain/clone:	Not reported
Age at study initiation:	Not reported
Weight/length/height at study initiation:	12 ± 2.5 g (7 ± 2 cm), 28 ± 5 g (11 ± 2.2 cm) and 53 ± 5.1 g (15 ± 3.4 cm).
Source:	Local farm
Feeding during test:	No
Acclimation:	7 days, fed with commercial diet.

#### Test conditions

Hardness:	Not reported
Test temperature:	28 ± 1°C
pH:	8.0-8.4
Dissolved oxygen:	7 ± 1 mg O <sub>2</sub> /L
Conductivity:	Not reported
Photoperiod:	Not reported
Light intensity:	Not reported

#### Test system

Study type:	Acute toxicity test (anaesthesia effects)
Duration of study:	24 hours (exposure period was approximately only 3 – 15 minutes; see further details below)
Treatments:	25, 50, 100, 150, 200 mg eugenol/L (nominal)
Analytical determination of test concentrations:	No
Negative control included:	Yes (ethanol)
Positive control included:	No

Parameters measured: Behaviour (anaesthesia effects); Mortality  
Validity criteria: Not reported

Stock solution was prepared by mixing eugenol and ethanol with respective volumetric ratio of 1:2. Solutions were freshly prepared right before experimentation. Plastic 2-L containers with continuous aeration were used. Exposure of fish to ethanol did not bring about anaesthesia or any apparent modifications in fish behaviour including opercular ventilation rate, indicating that the concentration of ethanol used had no effects on the fish during the experiment. Fish were fasted for 24 hours before conducting experiment.

To evaluate the anaesthetic properties of eugenol in different weight classes, fish from each class were subjected to different nominal concentrations of eugenol including 25, 50, 100, 150, 200 mg/L (ten fish per weight class and eugenol concentration). After anaesthetic exposure, elapsed time to reach each anaesthetic stage was recorded and when reaching the stage 4, fish weight and length were quickly recorded prior to transfer to recovery tank (containing clean and well aerated water). Recovery time was recorded from transferring the fish to recovery container until equilibrium regained. Finally, fish were transferred to freshwater aquaria to monitor potential mortality over a 24-hour period. All statistical analyses (ANOVA) were performed using IBM SPSS Statistics for Windows, Version 20.0.

### Results

Following a maximum exposure period of 3 minutes 200 mg eugenol/L, eugenol caused deep anaesthesia in Flowerhorn. The lowest deep anaesthesia times were observed in 12 g Flowerhorn submitted to 50 and 200 mg/L of eugenol ( $49.9 \pm 2.1$  and  $98.6 \pm 2.7$  s, respectively), where 50 mg eugenol/L was the minimum effective concentration able to cause deep anaesthesia in 12-53 g Flowerhorn.

12 g Flowerhorn fish exposed to all concentrations of eugenol (nominally 200 mg/L) for up to 3 minutes showed 23% mortality during the experiment or after 24 hours of the initial exposure. Anaesthetic recovery time ranged from  $127.9 \pm 3.9$  to  $312.1 \pm 3.7$  s.

18 g Flowerhorn fish exposed to all concentrations of eugenol (nominally 200 mg/L) for up to 3 minutes recovered completely and there was no mortality during the experiment or after 24 hours of the initial exposure. Anaesthetic recovery time ranged from  $103.2 \pm 3.2$  to  $272.6 \pm 1.7$  s.

53 g Flowerhorn fish exposed to all concentrations of eugenol (nominally 200 mg/L) for up to 3 minutes recovered completely and there was no mortality during the experiment or after 24 hours of the initial exposure. Anaesthetic recovery time ranged from  $91.7 \pm 2.4$  to  $212.6 \pm 1.7$  s.

The results of regression showed that higher concentrations of eugenol resulted in a shorter time being required for sedation and anaesthesia in fish.

### Assessment and conclusion

#### Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported.

#### **Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

At nominal concentrations of 50-200 mg eugenol/L Flowerhorn (*Amphilophus labiatus* x *Amphilophus trimaculatus*) (fish) reached deep anesthesia (slow and irregular opercular movement) after approximately 3 minutes, and recovered after approximately 2 to 4 minutes.

28 to 53 g Flowerhorn (*Amphilophus labiatus* x *Amphilophus trimaculatus*): No adverse effects were observed on mortality after 24 hours following a maximum 3 minute exposure to concentrations up to 200 mg eugenol/L (based on nominal concentrations) during the experiment or after 24 hours of the initial exposure.

12 g Flowerhorn (*Amphilophus labiatus* x *Amphilophus trimaculatus*): No adverse effects were observed on mortality after 24 hours following a maximum 3 minute exposure to concentrations up to 150 mg eugenol/L (based on nominal concentrations) during the experiment or after 24 hours of the initial exposure.

<b>Data point:</b>	CA 9.6.3.4/05 [8.2.1]
<b>Report author</b>	Hoseini, S.M., Rajabiesterabadi, H., Tarkhani, R.
<b>Report year</b>	2015
<b>Report title</b>	Anaesthetic efficacy of eugenol on iridescent shark, <i>Pangasius hypophthalmus</i> (Sauvage, 1878) in different size classes
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 32 Aquaculture Research Volume 46, Pages 405-412 (2015)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

#### Abstract (copied from original literature)

Anaesthetic efficacy of eugenol was investigated on iridescent shark, *Pangasius hypophthalmus*. Fish (2, 5, 10 and 20 g) subjected to 20–200 mg eugenol/L and behavioural response as well as induction and recovery times were recorded. Induction and recovery times were significantly affected by eugenol concentration as well as fish weight ( $P < 0.05$ ).

Generally, 27–300 s after exposure to 20–200 mg eugenol/L, iridescent sharks reached stage 3 anaesthesia (suitable for general handling). Fish entered stage 4 anaesthesia (suitable for surgery and blood sampling) over 54–710 s exposure to such concentrations. Recovery time was 109–600 s in all weight classes as well as eugenol concentrations.

Mortality (44–100%) was only observed in 2 g fish when subjected to 110–170 mg eugenol/L. This study, for the first time, showed behavioural response of iridescent shark to anaesthesia as well as effectiveness of eugenol as anaesthetic in this important aquaculture-ornamental species. According to the models obtained in this study, minimum eugenol concentrations to induce anaesthesia over less than 3 min were 53.8–81.5 mg eugenol/L in 2–20 g fish. Likewise, maximum eugenol concentrations in which fish recovered over less than 5 min were 65.9–105.8 mg eugenol/L in 2–20 g fish.

#### Materials and methods

##### Test material

Name:	eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma, St. Luis, USA
Active substance content:	99%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

##### Test organism

Species:	<i>Pangasius hypophthalmus</i> , iridescent shark
Strain/clone:	Not reported
Age at study initiation:	30-80 days old
Weight/length/height at study initiation:	2.05-20.60 g/4.3-13.2 mm
Source:	Local farm
Feeding during test:	No

Acclimation: 7 days, they were fed with commercial diet

#### *Test conditions*

Hardness:  $140 \pm 2.7$  mg CaCO<sub>3</sub>/L  
Test temperature:  $25 \pm 1$  °C  
pH:  $7.7 \pm 0.1$   
Dissolved oxygen:  $6.3 \pm 0.2$  mg O<sub>2</sub>/L  
Salinity: Not reported  
Conductivity: Not reported  
Photoperiod: Not reported  
Light intensity: Not reported

#### *Test system*

Study type: Acute toxicity test (anaesthesia effects)  
Duration of study: 24 hours (exposure period was approximately only 3 – 5 minutes; see further details below)  
Treatments: 40, 60, 80, 110, 140, 170 mg eugenol/L (nominal)  
Analytical determination of test concentrations: No  
Negative control included: No  
Positive control included: No  
Parameters measured: Mortality  
Validity criteria: Not reported

Four experiment groups of fish of different weight classes (2, 5, 10 and 20 g) were exposed to nominally 40, 60, 80, 110, 140, 170 mg eugenol/L. From each weight class, nine fish were subjected to each eugenol concentration. Fish were netted gently from each tank and placed in the anaesthetic bath. After anaesthesia fish were transferred to freshwater aquariums to monitor recovery from anaesthesia and potential mortality over a 24-hour period. Statistical analyses Kolmogorov-Smirnov test and ANOVA were performed.

#### **Results**

All nominal concentrations tested (40, 60, 80, 110, 140, 170 mg eugenol/L) led to anaesthesia (no response to mechanical stimulation) of the iridescent sharks. The time to reach anaesthesia was approximately 3 minutes. Following this short exposure period, 100% of iridescent sharks of the 5 - 20 g group recovered. However, iridescent sharks of the 2 g group showed 44, 100 and 100% mortality when anaesthetized by 110, 140 and 170 mg eugenol/L respectively. The recovery time was 109–600 s in all weight classes as well as eugenol concentrations. Univariate analysis of variance showed that the significant ( $P < 0.0001$ ) and positive correlation between eugenol concentration and induction time for stage 3 and 4, in all weight classes. Likewise, significant ( $P < 0.0001$ ) and negative correlation was observed between recovery and eugenol concentration (Fig. 1). Induction time to stage 3 and 4 as well as recovery time were significantly ( $P < 0.0001$ ) correlated to fish weight. Recovery time was significantly ( $P < 0.0001$ ) correlated to time of induction to stage 4 in all weights. No mortality occurred for any treatment group in the following 1 days in untreated freshwater.

Whilst the additional experiments found significant effects on anaesthesia and recovery times when iridescent sharks were exposed at different body weights, in all cases the mean anaesthesia time was no less than approximately 3 minutes, and the mean recovery time was no more than approximately 5 minutes. 100% of iridescent sharks (group 5 – 20 g) recovered and survived the eugenol exposures.

#### **Assessment and conclusion**

##### *Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02K.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported, and no negative control included.

**Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

At nominal concentrations of 20 – 170 mg eugenol/L, *Pangasius hypophthalmus* (iridescent shark) reached anaesthesia after approximately to 3 minutes, and recovered after approximately 5 minutes. No mortality or other negative effects on *Pangasius hypophthalmus* (iridescent shark group 5-20 g) at 170 mg eugenol/L during the anesthetic exposure (approximately 3 minutes) or in the 24 hours afterwards.

Mortality on *Pangasius hypophthalmus* (iridescent shark group 2 g) during anaesthetic exposure (approximately 3 minutes) and in the 24 hours afterwards was: 44% mortality at 110 mg eugenol/L and 100% mortality at 140 and 170 mg eugenol/L.

<b>Data point:</b>	CA 9.6.3.4/06 [8.2.1]
<b>Report author</b>	Tarkhani, R., Imani, A., Jamali, H., Farsani, H. G.
<b>Report year</b>	2017
<b>Report title</b>	Anaesthetic efficacy of eugenol on various size classes of angelfish ( <i>Pterophyllum scalare</i> Schultze, 1823)
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 33 Aquaculture Research Volume 48, Pages 5623-5270 (2017)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

**Abstract (copied from original literature)**

Anaesthetic efficacy of eugenol was investigated on *Pterophyllum scalare*. A total of 130 fish with average weights of  $1.0 \pm 0.5$ ,  $5.0 \pm 1.0$  and  $10.0 \pm 1.0$  g were subjected to 1.25, 2.5, 4.0, 5.5 and 7.0 mg/L eugenol, and behavioural responses were observed. Induction and recovery times were significantly affected by the interactive effect of eugenol concentration and fish weight ( $p < .05$ ). Generally, 49.9–128 s after exposure to 1.25–7 mg/L eugenol, fish reached stage 3. Fish entered stage 4 over 55–135 s post exposure to such concentrations. Recovery time was 393.5– 597.7 s in all sizes. Any increase in eugenol concentration led to a significant decrease in the induction time with a subsequent increment of the recovery time. Concentrations of eugenol and fish size along with their interactive effects have significantly contributed to the regression models, with concentration recording the highest beta values for stages 1, 2, 3 and 4 (-0.903, -0.898, -0.976 and -0.864 respectively) and the product of size and anaesthetic concentration for full recovery in smaller fish (0.647) and eugenol concentration in larger ones (0.967). Recovery time was fitted to induction time to stage 4 via quadratic and linear regression models in smaller and larger fish respectively. Results revealed the minimal eugenol concentration to induce anaesthesia in various size classes of angelfish in less than 3 min was 1.25 mg/L. Our results showed eugenol as an effective and safe anaesthetic; however, it is not advisable for live fish transportation.

**Materials and methods***Test material 1*

Name:	eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma, St. Louis, MO, USA
Active substance content:	99%

Expiry date of lot/batch: Not reported  
Storage conditions: Not reported

### *Test organism*

Species: *Pterophyllum scalare*, Angelfish.  
Strain/clone: Not reported  
Age at study initiation: Not reported  
Weight/length/height at study initiation:  $1.0 \pm 0.5$ ,  $5.0 \pm 1.0$  and  $10.0 \pm 1.0$  g  
Source: local ornamental fish farm  
Feeding during test: No  
Acclimation: Feeding was given up 24 h before the experiment and continued a day after recovery from anaesthesia. Fish were fed at 1.5% of their body weight per day with a commercial diet.

### *Test conditions*

Hardness:  $140 \pm 12.7$  mg CaCO<sub>3</sub>/L  
Test temperature:  $25.1 \pm 1.4$  °C  
pH: 7.0-7.5  
Dissolved oxygen:  $6.3 \pm 0.2$  mg O<sub>2</sub>/L  
Conductivity: Not reported  
Photoperiod: Not reported  
Light intensity: Not reported

### *Test system*

Study type: Acute toxicity test (anaesthesia effects)  
Duration of study: 24 hours (exposure period was approximately only 1 – 3 minutes; see further details below)  
Treatments: 1.25, 2.5, 4.0, 5.5 and 7.5 mg eugenol/L (nominal)  
Analytical determination of test concentrations: No  
Negative control included: Yes (ethanol)  
Positive control included: No  
Parameters measured: Behaviour (anaesthesia effects); Mortality  
Validity criteria: Not reported

Nominally 1.25, 2.5, 4.0, 5.5 and 7.5 mg eugenol/L were freshly prepared right before experimentation. Fish (grouped according to three weight classes) were individually subjected to each anaesthetic solution (n = 7-10 per treatment and weight class). Plastic 2-L containers with continuous aeration were used. Time required to get different stages of anaesthesia was recorded according to fish behaviour. Time required getting complete equilibrium was recorded from transferring the fish to recovery container (60-L aquaria containing 40 L aerated fresh water). Finally, fish were transferred to freshwater aquaria to monitor potential mortality over a 24-h period. Levene's test and Kolmogorov–Smirnov were used to evaluate the homogeneity of variance of the dependent variables and normality of data set respectively. Two-way ANOVA was used to illustrate whether or not there were significant differences among different experimental groups. All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 20.0.

### **Results**

After 24 hours, no mortalities were observed in all experimental groups, and the fish were feeding well within 1 day after treatment. All size classes showed all anaesthetic stages at 1.25 mg/L eugenol. Results showed that 7.50 mg eugenol /L effectively induced stages 3 and 4 very rapidly in comparison to other concentrations. According to the results, those fish exposed to higher concentrations of the anaesthetic agent required longer time to fully recover and regain their equilibrium.

All nominal concentrations tested (1.25, 2.5, 4.0, 5.5 and 7.5 mg eugenol/L) led to anaesthesia (no response to mechanical stimulation) of the Angelfish. Following a maximum exposure period of 3 minutes 1.25 mg eugenol/L, eugenol caused deep anaesthesia in Angelfish. The lowest deep anaesthesia times were observed in 1, 2 and 10 g Angelfish submitted to 7.50 mg/L of eugenol ( $55.600 \pm 1.714$  s,  $68.380 \pm 0.565$  s and  $81.860 \pm 0.829$  s). Therefore, 7.5 mg eugenol/L was the minimum effective concentration able to cause deep anaesthesia in Angelfish with different body weight. Following this short exposure period, 100% of the 1, 2 and 10 g Angelfish s recovered, with time to recovery of 434.90, 465.63 and 597.71 second for the groups exposed to 7.5 mg eugenol/L (nominal), respectively. No mortality occurred for any treatment group in the following 1 days in untreated water.

#### Assessment and conclusion

##### Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported.

There is a discrepancy in the paper and therefore unclear if 7.0 or 7.5 was tested.

#### **Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

At nominal concentrations of 1.25 – 7.5 mg eugenol/L, *Pterophyllum scalare* (angelfish) reached anaesthesia after approximately to 3 minutes, and recovered after approximately to 10 minutes. No mortality or other negative effects on the angelfish occurred in any of the eugenol treatments, up to 7.50 mg eugenol/L, during anaesthetic exposure (approximately 3 minutes) or in the 24 hours afterwards.

<b>Data point:</b>	CA 9.6.3.4/07 [8.2.1]
<b>Report author</b>	Park, I. S., Park, S. J., Gil, H. W., Nam, Y. K., Kim, D.S.
<b>Report year</b>	2011
<b>Report title</b>	Anesthetic effects of clove oil and lidocaine-HCl on marine medaka ( <i>Oryzias dancena</i> )
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 36 Lab Animal Volume 40, Issue 2, Pages 45-51 (2011)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

#### **Abstract (copied from original literature)**

Fish may be anesthetized for various experimental and practical purposes, primarily to immobilize them in order to facilitate handling. Marine medaka (*Oryzias dancena*) is a teleost fish used in marine ecotoxicology studies. Despite the importance of anesthesia in handling experimental fish, the effects of anesthesia in marine medaka have not yet been investigated. In this study, the authors evaluated the anesthetic effects (time required for anesthesia to take effect and recovery time) of two anesthetic agents, clove oil and lidocaine-HCl, on marine medaka. They anesthetized fish at different water temperatures (23 °C, 26 °C and 29 °C) and using different concentrations of clove oil (50, 75, 100, 125, 150 and 175 mg/L) or lidocaine-HCl (300, 400, 500, 600, 700 and 800 mg/L). The time required for anesthesia to take effect decreased significantly as both anesthetic concentration and water temperature increased for both clove oil and lidocaine-HCl. To anesthetize marine medaka within approximately 1 min, the optimal concentrations for clove oil were 125 ppm at 23 °C, 100 ppm at

26 °C and 75 ppm at 29 °C and for lidocaine–HCl were 800 ppm at 23 °C and 700 ppm at both 26 °C and 29 °C. The authors also compared anesthetic effects in marine medaka of different sizes. Both anesthetic exposure time and recovery time were significantly shorter for smaller fish than for larger fish. These results provide a useful foundation for the laboratory handling of marine medaka.

## Materials and methods

### *Test material*

Name:	Clove oil
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma, St. Louis, MO
Active substance content:	eugenol 85%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

### *Test organism*

Species:	Japanese medaka, <i>Oryzias latipes</i>
Strain/clone:	Not reported
Age at study initiation:	adult
Weight/length/height at study initiation:	Mean body length and body weight ( $\pm$ s.d.) were $30.2 \pm 2.21$ mm and $324.3 \pm 58.60$ mg, respectively, for the large fish ( $n = 20$ ) and $11.0 \pm 1.31$ mm and $21.1 \pm 5.10$ mg, respectively, for the small fish ( $n = 20$ ).
Source:	Institute of Marine Living Modified Organisms, Pukyong National University, Korea
Feeding during test:	No
Acclimation:	The fish were reared and bred in the Fishery Genetics and Breeding Science Laboratory of Korea Maritime University. The fish were allowed to adapt to a 400-l glass tube (breeding tube) maintained at one of three temperatures (23 °C, 26 °C or 29 °C) using a water heater (Young-II, Daegu, Korea). Fish were fed brine shrimp ( <i>Artemia salina</i> nauplii; Inve, Inve Premium, Salt Lake City, UT) once per day and commercial artificial diet. All fish were starved for 24 h before the experiment.

### *Test conditions*

Hardness:	Not reported
Test temperature:	23, 26 and 29 °C.
pH:	Not reported
Dissolved oxygen:	Not reported
Salinity:	Not reported
Conductivity:	Not reported
Photoperiod:	13 hours light: 11 hours dark
Light intensity:	not reported

*Test system*

Study type:	Acute toxicity test (anaesthesia effects)
Duration of study:	approximately 15 minutes
Treatments:	50, 75, 100, 125, 150 and 175 mg clove oil/L (nominal)
Analytical determination of test concentrations:	No
Negative control included:	No
Positive control included:	Yes
Parameters measured:	Mortality/behaviour
Validity criteria:	Not reported

Adult fish were exposed to the anesthetic effect of eugenol at six different nominal concentrations of clove oil: 50, 75, 100, 125, 150 and 175 mg clove oil/L. The stock solution of clove oil was dissolved in 95% methanol (Sigma) at a ratio of 1:10. To neutralize the anesthetic solution and to amplify its effect<sup>3</sup>, 11, 17, we diluted it in sodium bicarbonate solution (Sigma) to a final concentration of 1,000 ppm.

The time required for anesthesia to take effect was measured from the point when each fish was placed in the anesthetic tube to the time when the fish reached stage A6 (Perfect sedation; only opercular movement), in which the fish was perfectly sedate with minimum opercular movements. Recovery time was measured from the point when each fish was placed in the recovery tube to the time when the fish reached stage R6 (Normal swimming; responsiveness to visual stimuli), in which normal swimming and responsiveness to visual stimulation had recommenced.

Differences between groups were analyzed by ANOVA using the SPSS statistics package (SPSS 9.0, SPSS Inc., Chicago, IL), and multiple comparisons were performed using Duncan's multiple range test.

**Results**

Following a maximum exposure period of 3 minutes to nominally 50 mg clove oil/L on fish, clove oil caused sedation and anaesthesia in Japanese medaka (*Oryzias latipes*). Above this concentration (at 75 to 175 mg/L), clove oil caused sedation and anaesthesia in Japanese medaka (*Oryzias latipes*), where 50.0 mg clove oil/L was the minimum effective concentration able to cause sedation. The lowest sedation/anaesthesia times were observed in fish exposed to 175 mg clove oil/L;  $42 \pm 9.1$  seconds and  $28 \pm 3.2$  seconds, at 23 °C and 29 °C respectively. At each anesthetic concentration, higher water temperatures resulted in shorter periods of time required for anesthesia to take effect.

Japanese medaka (*Oryzias latipes*) exposed to all concentrations of clove oil (nominally 50 to 175 mg/L) for up to 3 minutes recovered completely and there was no mortality during the experiment. Anaesthetic recovery time ranged from  $102.0 \pm 5.5$  to  $190.0 \pm 26.2$  s. The ratio of the recovery time to the anesthetic exposure time gradually increased as the clove oil concentration increased at each water temperature. However, the ratio of recovery time to exposure time showed no clear correlation with water temperature.

**Assessment and conclusion***Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported. Dissolved oxygen was not reported.

**Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

Japanese medaka, *Oryzias latipes*: No adverse effects were observed on mortality following a maximum 3 minute exposure to concentrations up to 175 mg clove oil/L (based on nominal concentrations).

LOEC value for anaesthesia (perfect sedation; only opercular movement) = 50.0 mg clove oil/L (nominal). The lowest anaesthesia times were observed in fish submitted to 175 mg clove oil/L at 29 °C ( $28.0 \pm 3.2$  seconds).

<b>Data point:</b>	CA 9.6.3.4/08 [8.2.1]
<b>Report author</b>	Park, I. S., Lee, T.H., Lim, S. G.
<b>Report year</b>	2018
<b>Report title</b>	Anesthetic efficacy and physiological responses of clove oil on juvenile and adult red spotted grouper, <i>Epinephelus akarra</i>
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 38 Fisheries and Aquatic Sciences, 21:25 (2018)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

#### Abstract (copied from original literature)

The main objective of this study was to provide anesthetic criteria of clove oil for an effective manipulation and transportation of red spotted grouper, *Epinephelus akaara*. When anesthesia temperature (20, 24, and 28 °C) and concentration of clove oil (25, 50, and 75 ppm) were increased, the anesthesia and recovery time decreased and tended to be similar to each other between juvenile and adult. Also, as the temperature and concentration increased, the ratio of exposure time and recovery time between juvenile and adult were decreased. When plasma cortisol concentrations were compared for 48 h after anesthesia with 50 ppm of clove oil, both the juvenile and adult fish grew up to 12 h; however, thereafter decreased and there was no significant difference from control at 48 h.

#### Materials and methods

##### Test material

Name:	Clove oil
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma, St. Louis, MO
Active substance content:	eugenol 82-87%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

##### Test organism

Species:	red spotted grouper, <i>Epinephelus akarra</i>
Strain/clone:	Not reported
Age at study initiation:	juvenile and adults
Weight/length/height at study initiation:	standard length $9.1 \pm 1.78$ cm, body weight $14.3 \pm 4.21$ g; mean $\pm$ SD) and ten adult specimens ( $35.1 \pm 5.92$ cm, $1044.5 \pm 149.63$ g)
Source:	
Feeding during test:	No
Acclimation:	fish samples were acclimated for 1 week at different water temperatures (20, 24, and 28 °C) with filtration and aeration. All fish samples were starved for 24 h prior to experiments

##### Test conditions

Hardness:	Not reported
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Test temperature:	20, 24 and 28 °C.
pH:	7.5 ± 0.07
Dissolved oxygen:	7.1 ± 0.35
Salinity:	31.2 ± 0.17
Conductivity:	Not reported
Photoperiod:	13 hours light: 11 hours dark
Light intensity:	not reported

#### *Test system*

Study type:	Acute toxicity test (anaesthesia effects)
Duration of study:	approximately 15 minutes
Treatments:	25, 50 and 75 mg clove oil/L (nominal)
Analytical determination of test concentrations:	No
Negative control included:	No
Positive control included:	Yes
Parameters measured:	Mortality/behaviour
Validity criteria:	Not reported

Red spotted grouper fish were exposed to the anesthetic effect of eugenol at three different nominal concentrations of clove oil: 25, 50 and 75 mg clove oil/L. The stock solution of clove oil was dissolved in 95% ethanol (Sigma) at a ratio of 1:10.

The time required for anesthesia to take effect was measured from the point when each fish was placed in the anesthetic tube to the time when the fish reached stage A6 (Perfect sedation; only opercular movement), in which the fish was perfectly sedate with minimum opercular movements. Recovery time was measured from the point when each fish was placed in the recovery tube to the time when the fish reached stage R6 (Normal swimming; responsiveness to visual stimuli), in which normal swimming and responsiveness to visual stimulation had recommenced.

Differences between groups were analyzed by ANOVA using the SPSS statistics package (SPSS 9.0, SPSS Inc., USA), and multiple comparisons were performed using Duncan's multiple range test.

#### **Results**

Following a maximum exposure period of 5 minutes to nominally 25 mg clove oil/L on fish, clove oil caused sedation and anaesthesia in red spotted grouper, *Epinephelus akaara*. Above this concentration (at 50 to 75 mg/L), clove oil caused sedation and anaesthesia in red spotted grouper, *Epinephelus akaara*, where 25.0 mg clove oil/L was the minimum effective concentration able to cause sedation. The lowest sedation/anaesthesia times were observed in juvenile and adult fish exposed to 75 mg clove oil/L; 59 ± 7.9 seconds and 71 ± 9.9 seconds, at 28 °C. At each anesthetic concentration, higher water temperatures resulted in shorter periods of time required for anesthesia to take effect.

Red spotted grouper, *Epinephelus akaara*, exposed to all concentrations of clove oil (nominally 25 to 75 mg/L) for up to 5 minutes recovered completely and there was no mortality during the experiment. Anaesthetic recovery time ranged from 229 ± 12.1 to 291.0 ± 23.5 s for juveniles and 83 ± 9.1 to 280 ± 21.7 for adults. The ratio of the recovery time to the anesthetic exposure time gradually increased as the clove oil concentration increased at each water temperature.

#### **Assessment and conclusion**

##### *Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported.

#### **Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

Red spotted grouper, *Epinephelus akaara*: No adverse effects were observed on mortality following a maximum 3 minute exposure to concentrations up to 75 mg clove oil/L (based on nominal concentrations).

LOEC value for anaesthesia (perfect sedation; only opercular movement) = 25.0 mg clove oil/L (nominal). The lowest anaesthesia times were observed in juvenile fish submitted to 75 mg clove oil/L at 28 °C (59 ± 7.9 seconds).

<b>Data point:</b>	CA 9.6.3.4/09 [8.2.1]
<b>Report author</b>	Palić, D., Herolt, D.M., Andreasen, C.B., Menzel, B.W., Roth , J.A.
<b>Report year</b>	2006
<b>Report title</b>	Anesthetic efficacy of tricaine methanesulfonate, metomidate and eugenol: Effects on plasma cortisol concentration and neutrophil function in fathead minnows ( <i>Pimephales promelas</i> Rafinesque, 1820)
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 40 Aquaculture Volume 254, Pages 675–685 (2006)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

#### Abstract (copied from original literature)

Anesthetic efficacy, plasma cortisol concentration, and two parameters of neutrophil function (oxidative burst and degranulation of primary granules) were compared among three anesthetics in the fathead minnow: tricaine methanesulfonate (MS 222), metomidate hydrochloride (MTMD), and eugenol (EUG). The optimum anesthetic concentration was determined as: MS 222 75 mg/L, EUG 30 mg/L and MTMD 4 mg/L. Handling and crowding stress was induced in fish with (SA) and without (S) anesthetic. Plasma cortisol concentration was measured at 0, 30, 90, and 240 min after stress and found to increase at 30 min post-stress in S and SAMS 222 groups, but not in SA MTMD and SA EUG groups. To test the effects of different anesthetics on neutrophil function, fish were divided into a baseline control group, a group exposed to handling and crowding stress (S) and a stressed anesthetized group (SA). Fish were assayed for neutrophil function before and after stress (24 h, 72 h and 7 days). The degranulation of neutrophil primary granules was measured as exocytosis of myeloperoxidase (MPO) using 3, 3', 5, 5'-tetramethylbenzidine as a substrate. Degranulation of primary granules was decreased to 60–75% of non-stressed control in stressed and fish treated with MS 222, and was not affected when MTMD and EUG were used. The degranulation of primary granules proved to be a useful assay for measuring the effects of stress on neutrophil function in fish. Eugenol and metomidate prevented stress-induced decrease of neutrophil function while MS 222 did not.

#### Materials and methods

##### *Test material 1*

Name:	eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma, St. Louis, MO, USA
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

##### *Test organism*

Species:	<i>Pimephales promelas</i> , fathead minnows
Strain/clone:	Not reported
Age at study initiation:	Adult

Weight/length/height at study initiation: 3 g  
Source: Department of Natural Resource Ecology and Management,  
Iowa State University, Ames, Iowa, USA.  
Feeding during test: No  
Acclimation: Acclimation period not reported, fed daily with dried flake  
food

#### *Test conditions*

Hardness: Not reported  
Test temperature:  $20 \pm 1^\circ\text{C}$   
pH:  $8.0 \pm 0.2$   
Dissolved oxygen:  $7 \pm 1 \text{ mg O}_2/\text{L}$   
Conductivity: Not reported  
Photoperiod: Not reported  
Light intensity: Not reported

#### *Test system*

Study type: Acute toxicity test (anaesthesia effects)  
Duration of study: 24 hours (exposure period was only 3 – 20 minutes; see further details  
below)  
Treatments: 10, 20, 30, 40, 80 mg eugenol/L (nominal)  
Analytical determination of test concentrations: No  
Negative control included: No  
Positive control included: No  
Parameters measured: Anaesthesia effects (rapid immobility at stage 3 anaesthesia defined as total  
loss of equilibrium and cessation of locomotion, without medullary collapse  
and with rapid recovery); Mortality.  
Molecular biomarkers (including plasma cortisol concentrations and  
neutrophil function) were also measured, but are not discussed further in  
this summary as not considered relevant.  
Validity criteria: Not reported

10 fish (in two beakers of five fish each) were exposed to eugenol for 20 minutes and then moved to 4 L beaker with 3 L aerated tank water without eugenol. The nominal concentrations of eugenol were 10, 20, 30, 40, 80 mg eugenol/L. The percentage of fish in stage 3 anaesthesia after 3 minutes of induction, average recovery time for individual fish (after 20 minutes exposure), and percent of survival at the time of recovery were determined for each concentration. Fish were moved to the stock tank where food was introduced the next morning and behaviour was monitored over the next week.

### **Results**

Anesthesia with eugenol exhibited a narrow margin of safety. With concentrations of eugenol 80 mg/L the percentage of survival is 0% survival. Optimal concentrations of the anesthetics are determined to be 30 mg eugenol/L. At this dose, all fish resumed eating 24 h after treatment and no mortalities occurred during the monitoring period.

All nominal concentrations tested 10, 20, 30, 40, 80 mg eugenol/L led to anaesthesia stage 3 (defined as total loss of equilibrium and cessation of locomotion) of the fathead minnows. The time to reach anaesthesia was 3-6 minutes when exposed to 10, 20, 30, 40, 80 mg eugenol/L (nominal).

Following a short exposure period of 20 minutes, 100% of fathead minnows recovered exposed to 10, 20 and 30 mg eugenol/L, with a time to recovery approximately of 3 to 4 minutes respectively.

When fathead minnows were exposed during 20 minutes to 40 mg eugenol/L, 40% of mortality was observed with a time to recovery approximately of 6 minutes. No survival of fathead minnows exposed to 80 mg eugenol/L during 20 minutes.

### **Assessment and conclusion**

#### *Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations and test item purity were not reported.

**Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

Fathead minnows no mortality up to nominally 30 mg eugenol/ L after 24 hours.

At nominal concentrations of 10 – 80 mg eugenol/L, fathead minnows (*Pimephales promelas*) reached anaesthesia after approximately 3 to 6 minutes, and recovered after approximately 3 to 6 minutes. No mortality or other negative effects on the fish occurred in any of the eugenol treatments, up to 30 mg eugenol/L, during anaesthetic exposure (approximately 3 to 6 minutes) or in the 24 hours afterwards.

<b>Data point:</b>	CA 9.6.3.4/10 [8.2.1]
<b>Report author</b>	Correia, A.M., Pedrazzani, A.S., Mendonça, R.C., Massucatto, A., Ozorio, R.A. and Tsuzuki, M.Y.
<b>Report year</b>	2018
<b>Report title</b>	Basil, tea tree and clove essential oils as analgesics and anaesthetics in <i>Amphiprion clarkii</i> (Bennett, 1830)
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 53 Brazilian Journal of Biology, Volume 78, no. 3, Pages 436-442 (2018)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

**Abstract (copied from original literature)**

In this study were evaluated the anaesthesia and analgesic effects of clove *Eugenia caryophyllata*, tea tree *Melaleuca alternifolia* and basil *Ocimum basilicum* essential oils (EO) during handling of yellowtail clownfish *Amphiprion clarkii*. Juveniles ( $3.70 \pm 0.75$  cm and  $1.03 \pm 0.50$  g; mean  $\pm$  standard deviation) were submitted to concentrations of 40, 50, 60, 70 and 80  $\mu\text{L/L}$  of clove, 150, 200, 250, 300 and 350  $\mu\text{L/L}$  of basil and 200, 300, 400, 500 and 600  $\mu\text{L/L}$  of tea tree oils ( $n=10/\text{concentration}$ ), previously defined in pilot tests. Individually and only once, fish from each treatment were placed in a glass recipient containing 1 L of seawater at a temperature of 25°C, salinity of 35 g/L and the specific concentration of diluted EO (stock solution). Control (only seawater) and blank (seawater and ethanol at the highest concentration used to dilute the oils) treatments were also conducted. After reaching the stage of surgical anaesthesia, fish were submitted to biometry and a sensibility test. After that, they were transferred to clean seawater for anaesthesia recovery. The times of induction needed to reach each anaesthesia stage and anaesthesia recovery were recorded. Animals were observed for 72 hours after the procedures. All the EO provoked anaesthesia and analgesic effects in *A. clarkii*, but basil oil is not recommended because it caused involuntary muscle contractions and mortality in 100% and 12% of fish, respectively. The lower concentrations that promote suitable induction and recovery times are 50  $\mu\text{L/L}$  of clove oil and 500  $\mu\text{L/L}$  of tea tree oil. However, due to its complementary high analgesic efficiency, clove oil is recommended as the ideal anaesthetic for *A. clarkii*.

**Materials and methods**

*Test material*

Name: clove oil, extracted from *Eugenia caryophyllata* (eugenol = 86%)  
Formulation type: Not relevant

Source and lot/batch no.: composition of the product was informed by the producer company through technical reports

Active substance content: 86% of eugenol

Expiry date of lot/batch: Not reported

Storage conditions: Amber glass flasks, protected from humidity, light and heat sources.

#### *Test organism*

Species: *Amphiprion clarkia* (yellowtail clownfish)

Strain/clone: Not reported

Age at study initiation: juveniles

Weight/length/height at study initiation:  $1.03 \pm 0.50$  g in weight and  $3.70 \pm 0.75$  cm in total length (mean  $\pm$  standard deviation)

Source: cultivated at a density of 0.5 fish/L in an open system at  $25.5 \pm 1.5^\circ\text{C}$ , constant aeration, and salinity of  $35 \pm 1$  g/L.

Feeding during test: No feeding during exposure but they were fed 12 hours after the experiment (recovery phase) with a commercial marine ornamental fish diet (Inve, Belgium).

Acclimation: Fish were fed until apparent satiety twice a day with a commercial marine ornamental fish diet (Inve, Belgium), and deprived of food for 24 hours before the experiments.

#### *Test conditions*

Hardness: not reported

Test temperature:  $25.5 \pm 1.5^\circ\text{C}$

pH: not reported

Dissolved oxygen: not reported

Salinity:  $35 \pm 1$  g/L

Conductivity: not reported

Photoperiod: not reported

Light intensity: not reported

#### *Test system*

Study type: Acute toxicity test (anaesthesia and analgesic effects)

Duration of study: 72 hours (exposure period was approximately only 1 – 2 minutes; see further details below)

Treatments: Stock solution was prepared by diluting the essential oil in ethyl alcohol 92.8% at a proportion of 1:10.  
5 concentrations: 40, 50, 60, 70 and 80  $\mu\text{L}$  clove oil/L (nominal), 10 fish /concentration

Analytical determination of test concentrations: No

Negative control included: Yes, A blank group, in which only the highest ethanol concentration used to dilute the essential oils (540  $\mu\text{L}/\text{L}$ ) was added to seawater and a control group, without substances added to seawater, were also evaluated.

Positive control included: No

Parameters measured: Individually and only once, fish from each treatment were placed in a glass recipient containing 1 L of seawater. Fish were monitored visually and the times needed to reach anaesthesia stages were timed and recorded. Immediately after reaching anaesthesia stage IV (surgical anaesthesia (i.e.

reduction of opercular beatment, absence of natatory motion), the fish were removed from the glass recipient, lightly dried with paper towels, weighed and measured (total length, standard length and height), simulating routine biometry. To evaluate recovery, fish were immediately placed in another glass recipient containing 1 L of clean seawater with constant aeration. Animals were considered to be recovered when they responded to visual stimuli (movement of an object close to their head) and reached a horizontal swimming position, indicating an apparent return to equilibrium. The recovery time was measured and recorded. After recovery, fish were transferred to 17 net cages (one for each treatment) immersed in an open seawater system with similar characteristics as found before the experiment. They were fed 12 hours after the experiment. Mortality and possible atypical behaviour, such as lack of interest in food (ingestion) and absence of or difficulty in swimming were recorded at 24, 48 and 72 hours after the experiment.

Validity criteria:

Not reported

### Results

All the concentrations of clove oil (containing 86% eugenol) promoted analgesia and anaesthesia in the animals. The time to reach anaesthesia (stage IV) was approximately 50 to 160 seconds across all concentrations tested (40 - 80  $\mu\text{L}$  clove oil/L) (based on Figure 1A of paper). There was a significant difference between the concentrations of clove oil in relation to the time of anaesthetic induction (stage IV), with shorter times observed at higher concentrations. Animals submitted to clove oil had the lowest median times using 60, 70 and 80  $\mu\text{L}/\text{L}$  in relation to the lowest concentration (40  $\mu\text{L}/\text{L}$ ;  $p = 0.0000$ ). The time to recovery was approximately 50 to 350 seconds across all concentrations tested (40 - 80  $\mu\text{L}$  clove oil/L) (based on Figure 1B of paper). The concentrations of 40 and 50  $\mu\text{L}/\text{L}$  of clove presented faster anaesthesia recovery compared to the concentrations of 60 and 80  $\mu\text{L}/\text{L}$ . It was observed that all of the clove oil concentrations provoked an analgesic (pain relief) effect in 100% of the animals.

No mortality or other negative effects on the fish occurred in any of the clove oil treatments during anaesthetic exposure (approximately 50 to 160 seconds) or in the 72 hours after it.

### Assessment and conclusion

#### Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported.

### **Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

At nominal concentrations of 40 – 80  $\mu\text{L}$  clove oil/L (clove oil containing 86% eugenol), *Amphiprion clarkia* (yellowtail clownfish) reached anaesthesia (stage IV) after approximately 50 to 160 seconds, and recovered after approximately 50 to 350 seconds. No mortality or other negative effects on the fish occurred in any of the clove oil treatments, up to 80  $\mu\text{L}$  clove oil/L, during anaesthetic exposure (approximately 50 to 160 seconds) or in the 72 hours after it.

<b>Data point:</b>	CA 9.6.3.4/11 [8.2.1; 8.2.2.1]
<b>Report author</b>	Mácová, S.; Doleželová, P.; Pištěková, V.; Svobodová, Z.; Bedáňová, I.; Voslářová, E.
<b>Report year</b>	2008
<b>Report title</b>	Comparison of acute toxicity of 2-phenoxyethanol and clove oil to juvenile and embryonic stages of <i>Danio rerio</i>
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 96 Neuroendocrinology Letters Volume 29, Issue 5, Pages 680-684 (2008)
<b>Guidelines followed in study</b>	OECD 203 OECD 212
<b>Deviations from current test guideline</b>	Yes – analytical verification of test concentrations not reported, so validity criteria not met
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

#### Abstract (copied from original literature)

**OBJECTIVES:** Anaesthetics are used in aquaculture to prevent stress and mechanical damage to fish during handling or the treatment of fish in breeding, blood sampling and other veterinary interventions. Clove oil and 2-phenoxyethanol are used in the Czech Republic in a water bath for the short-term immobilization of the fish.

**DESIGN:** Acute toxicity tests were performed on aquarium fish *Danio rerio*, which is considered to be one of the model organisms most commonly used in toxicity testing. The semi-static method according to OECD No. 203 (Fish acute toxicity test) was used for testing juvenile fish. Embryo toxicity tests were performed in zebrafish embryos (*D. rerio*) in compliance with the OECD No. 212 methodology (Fish, short-term toxicity test on embryo and sac-fry stages). The results obtained (the number of dead individuals at particular test concentrations) were subjected to a probit analysis using the EKO-TOX 5.2 programme in order to determine LC<sub>50</sub> clove oil and 2-phenoxyethanol values. The statistical significance of the difference between LC<sub>50</sub> values in juvenile and embryonic stages of *D. rerio* was tested using the Mann-Whitney non-parametric test implemented in the Unistat 5.1 programme.

**RESULTS:** The LC<sub>50</sub> clove oil mean value was  $18.8 \pm 5.52$  mg.L<sup>-1</sup> in juvenile *D. rerio*, and  $15.64 \pm 3.30$  mg.L<sup>-1</sup> in embryonic stages of *D. rerio*. The LC<sub>50</sub> 2-phenoxyethanol mean value was  $338.22 \pm 15.22$  mg.L<sup>-1</sup> in juvenile *D. rerio*, whereas in embryonic stages of *D. rerio* it was  $486.35 \pm 25.53$  mg.L<sup>-1</sup>.

**CONCLUSIONS:** The study proved statistically significantly higher ( $p < 0.01$ ) sensitivity in juvenile fish to 2-phenoxyethanol compared to the embryonic stages. Acute toxicity values of clove oil for juvenile and embryonic stages were comparable.

#### Materials and methods

##### Test material 1

Name:	Clove oil
Formulation type:	Not relevant
Source and lot/batch no.:	Not reported
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

##### Test organism

Species:	<i>Danio rerio</i> (zebrafish)
Strain/clone:	Not reported

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Age at study initiation:	2- 3 months old (juvenile test), embryos within 8 hours after fertilization (embryonic test)
Weight/length at study initiation:	0.3±0.1 g/ 30±5 mm (juvenile test)
Source:	Not reported
Feeding during test:	Not reported
Acclimation:	Not reported

#### *Test conditions*

Hardness:	Not reported
Test temperature:	24±1°C (juvenile test); 24.5 – 25.5 °C (embryonic test)
pH:	7.89-8.62 (juvenile test)
Dissolved oxygen:	80-94% (juvenile test)
Conductivity:	Not reported
Photoperiod:	Not reported
Light intensity:	Not reported

#### *Test system*

Study type:	1) 96-hour acute toxicity test on juvenile fish (a semi-static exposure with solution replacement at 48 hours); 2) 144 – 168 hour fish short-term toxicity test on embryo and sac-fry stages (water bath replaced every 24 hours)
Duration of study:	1) Acute test - 96 hours 2) Embryonic test - tests were terminated after hatching and the absorption of the yolk sack in all individuals in the control dish (144–168 hours (i.e. 6-7 days) after placement onto the dish)
Treatments:	5 tested concentrations consisting of an approximate geometric progression (actual concentrations not reported)
Negative control included:	Yes
Positive control included:	No
Parameters measured:	Mortality
Validity criteria:	<20% mortality in control

The aim of the present study was to compare the acute toxicity of clove oil to embryonic and juvenile stages of zebrafish (*Danio rerio*). For the juvenile stage test, ten fish were used. For the embryonic stage test twenty fertilized eggs in a Petri dish were tested at each concentration and in one control. The eggs were placed in Petri dishes within 8 hours at the latest after fertilization. The tests were terminated after hatching and the absorption of the yolk sack in all individuals in the control dish (144–168 h after placement onto the dish). In both tests there was less than 20% mortality in the controls.

#### **Results**

The 96-hour acute LC<sub>50</sub> clove oil mean value was 18.8 ± 5.52 mg clove oil/L in juvenile *D. rerio*, and the 168-hour short-term embryonic LC<sub>50</sub> mean value was 15.64 ± 3.30 mg clove oil/L in embryonic stages of *D. rerio*. Both LC<sub>50</sub> values are based on nominal concentrations

#### **Assessment and conclusion**

##### *Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported, and eugenol content of the clove oil batch tested was also not reported.

**Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

*Danio rerio* 96-hour LC<sub>50</sub> juvenile = 18.8 ± 5.52 mg clove oil/L, based on nominal concentrations

*Danio rerio* 168-hour LC<sub>50</sub> embryo = 15.64 ± 3.30 mg clove oil/L, based on nominal concentrations

<b>Data point:</b>	CA 9.6.3.4/12 [8.2.1]
<b>Report author</b>	Vidal, L.V.O., Furuya, W.M., Graciano, T.S., Schamber, C.R., Dos Santos, L.D., Soares, C.M.
<b>Report year</b>	2007
<b>Report title</b>	Eugenol concentrations for deep anesthesia and acute toxicity in piavuçu juveniles ( <i>Leporinus macrocephalus</i> )
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 102 Acta Sci. Biol. Sci., Volume 29, Issue 4, Pages 357-362 (2007)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

**Abstract (copied from original literature)**

Eugenol concentrations for deep anesthesia and acute toxicity in piavuçu (*Leporinus macrocephalus*) juveniles. Three works were undertaken to evaluate the anesthetic capacity of eugenol in “piavuçu” juveniles; the influence of different concentrations on the anaesthetic effect, the acute toxicity (LD<sub>50</sub>) and the Time for Lethal Dose (TLD) for the species. Seventy-two fish (1.77 + 0.69 g) were submitted to eight different eugenol concentrations (25; 37.5; 50; 62.5; 75; 100; 125 and 150 mg L<sup>-1</sup>); 150 fish were submitted to five concentrations (25; 37.5; 50; 62.5; 75 mg L<sup>-1</sup>) for ten minutes; and 150 fish were submitted to 37.5 mg/L for five time intervals (300; 600; 900; 1200; 1500 seconds). Eugenol concentration had strong influence on the anesthetic effect for the species; LD for 600 seconds of exposure were LD<sub>01</sub> 25.21 mg L<sup>-1</sup>, LD<sub>50</sub> 45.13 mg L<sup>-1</sup> and LD<sub>99</sub> 65.05 mg L<sup>-1</sup>. The TLD were TLD<sub>01</sub> 322.8 seconds, TLD<sub>50</sub> 854.9 seconds and TLD<sub>99</sub> 1386.9 seconds. For fast and safe anesthesia of piavuçu juveniles, 37.5 mg L<sup>-1</sup> of eugenol is recommended.

**Materials and methods***Test material 1*

Name:	Eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	from Biodinâmica (trade mark)
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

*Test organism*

Species:	Piavuçu ( <i>Leporinus macrocephalus</i> )
Strain/clone:	Not reported
Age at study initiation:	juveniles
Weight at study initiation:	1.77 + 0,69 g

Source: Not reported  
 Feeding during test: Not reported  
 Acclimation: yes, temperature between 23 a 25 °C for 2 weeks prior to test

#### *Test conditions*

Hardness: Not reported  
 Test temperature: 25 ± 1°C  
 pH: Not reported  
 Dissolved oxygen: 6 + 0,5 mg/L  
 Conductivity: Not reported  
 Photoperiod: Not reported  
 Light intensity: Not reported

#### *Test system*

Study type: Acute toxicity test  
 Duration of study: 600 seconds  
 Treatments: 25, 37.5, 50, 62.5, 75, 100, 125 and 150 mg eugenol/L (nominal) to assess time to observe effects of anaesthesia and recovery;  
 25, 37.5, 50, 62.5 and 75 mg eugenol/L (nominal) to assess mortality after 600 seconds (10 minutes).

Analytical determination of test concentrations: No  
 Negative control included: Yes  
 Positive control included: No  
 Parameters measured: Mortality, effects of anaesthesia  
 Validity criteria: Not reported

#### **Results**

During the tests, at all treatment concentrations (nominally 25 – 150 mg eugenol/L), the fish presented a reaction of hyperactivity on initial contact with eugenol, demonstrated through fast movement in the aquarium, which reduced when the anaesthetic effect set in. At all concentrations, the fish assumed the behavioural pattern observed on anaesthetic induction. At a nominal concentration of 25 mg eugenol/L, some fish still presented reactions to external stimuli when they were removed from the water. This indicated that they would reach only anaesthetic stage III (narcosis), yet at the following concentrations, all reached anaesthetic stage IV (deep anaesthesia). Time to induce anaesthesia was approximately 15 to 110 seconds across all concentrations (with shorter times at higher concentrations). Recovery times were approximately 100 to 250 seconds across all concentrations (with short recovery times at lower concentrations).

The estimated LC<sub>50</sub> value after 600 seconds exposure was reported to be 45.13 mg eugenol/L (based on nominal concentrations).

#### **Assessment and conclusion**

##### *Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable since the analytical verification of test concentrations was not reported. Purity of test item also not reported.

#### **Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

*Leporinus macrocephalus* (piavuçu fish). Time to induce anaesthesia was approximately 15 to 110 seconds across all concentrations tested (nominally 25 – 150 mg eugenol/L), with shorter times at higher concentrations. Recovery times were approximately 100 to 250 seconds across all concentrations (with short recovery times at lower concentrations).

*Leporinus macrocephalus* juvenile fish 600-second (10-minute) LC<sub>50</sub> = 45.13 mg eugenol/L based on nominal concentrations

<b>Data point:</b>	CA 9.6.3.4/13 [8.2.1]
<b>Report author</b>	He, R., Lei, B., Su, Y., Wang, A., Cui, K., Shi, X., Chen, X.
<b>Report year</b>	2020
<b>Report title</b>	Effectiveness of eugenol as an anesthetic for adult spotted sea bass ( <i>Lateolabrax maculatus</i> )
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 134 Aquaculture volume 523, 735180, (2020)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (see details in summary below) – critically, no analytical verification of test concentrations, and acute toxicity tests only 10 minutes or 24 hours
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

#### Abstract (copied from original literature)

Spotted sea bass, *Lateolabrax maculatus*, is one of the most commercially important marine fish in China. It is very easy to suffer from stress and physical damage due to its aggressive nature and dorsal spines, which results in severe mortalities during handling and transportation. Therefore, most spotted sea bass are sold as fresh cooled products rather than live fish in domestic market, thus limiting its market value and market scale as well as the industry development. Anesthetics could be useful to reduce the physical stress during handling and transportation. However, few studies have been done to investigate the application of anesthetics in spotted sea bass. In the present study, the anesthetic efficacy of eugenol (2-methoxy-4-prop-2-enyl-phenol) in adult spotted sea bass was extensively investigated. Firstly, the acute toxicity of eugenol was measured. The estimated 10-min LC<sub>50</sub> and 24-h LC<sub>50</sub> were 98.13 mg/L and 19.73 mg/L. Secondly, the needed time periods for induction and recovery from anesthesia were measured and compared to clove oil and MS-222. Based on the time criteria of ideal induction (less than 3 min) and recovery (less than 10 min), the lowest effective concentrations for spotted sea bass were 60 mg/L for eugenol, 120 mg/L for clove oil and 140 mg/L for MS-222 at around 20 °C. In addition, the anesthetic efficacies of different concentrations of eugenol at water temperatures of 20 and 30 °C were compared. Results showed that longer induction time to deep anesthesia and shorter recovery time were observed when fish were exposed to 20 and 30 mg/L eugenol at 30 °C compared to that at 20 °C. On the contrary, shorter time to induce deep anesthesia and longer time to recovery were found when fish were exposed to 40 and 50 mg/L eugenol at 30 °C compared to that at 20 °C. Lastly, water quality and survival of spotted sea bass subjected to different concentrations of eugenol during simulated transportation were evaluated. Current study indicated that the addition of eugenol did not show significant improvements compared to the control group. In conclusion, eugenol was an effective anesthetic for inducing anesthesia on spotted sea bass, but the addition of it did not improve the transportation of spotted sea bass.

#### Materials and methods

##### Test material 1

Name:	eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Shanghai Yuanye Bio-Technology Co., Ltd., China
Active substance content:	98.5%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

##### Test material 2

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Name:	Clove oil
Formulation type:	Not relevant
Source and lot/batch no.:	Shanghai Yuanye Bio-Technology Co., Ltd., China
Active substance content:	eugenol 85%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

*Test organism*

Species:	<i>Lateolabrax maculatus</i> , spotted sea bass
Strain/clone:	Not reported
Age at study initiation:	Not reported
Weight/length/height at study initiation:	546.42 ± 14.27 g / 37.34 ± 0.55 cm
Source:	Estuarine Fishery Research Institute of Doumen District (Zhuhai, Guangdong province, China).
Feeding during test:	No
Acclimation:	Acclimatized in 3000 L indoor circular fiber glass tanks with a constant flow of aerated brackish water (5 g/L) for two weeks under natural photoperiod. Fish were fed a commercial pellet feed (Marine fish compound feed, Zhuhai haiwei feed Co., Ltd., China) twice a day to satiation and fasted for 24 h prior to the experiments

*Test conditions*

Hardness:	Not reported
Test temperature:	27 ± 1.0 °C
pH:	7.5 ± 0.3
Dissolved oxygen:	6.5 ± 0.5 mg O <sub>2</sub> /L
Salinity:	5.0 ± 1.0 g/L
Conductivity:	Not reported
Photoperiod:	Natural photoperiod
Light intensity:	Not reported
<i>Test system 1</i>	
Study type:	Acute toxicity test
Duration of study:	Test 1: 10 minutes; Test 2: 24 hours
Treatments:	Test 1: 0, 5, 10, 15, 20 and 25 ; test 2:15, 17.5, 20, 22.5 and 25 mg eugenol/L (nominal)
Analytical determination of test concentrations:	No
Negative control included:	Yes (ethanol)
Positive control included:	No
Parameters measured:	Mortality
Validity criteria:	Not reported

Groups of 10 acclimated fish were exposed to different nominal concentrations of eugenol; 15, 17.5, 20, 22.5 and 25 mg eugenol/L for 24-hours. Mortalities were recorded after 24 hours. All determinations were carried out in triplicate. The median lethal concentrations (24-h LC<sub>50</sub>) were estimated by probit analysis according to the method described by Litchfield and Wilcoxon (1949).

*Test system 2*

Study type:	Acute toxicity test (anaesthesia effects)
Duration of study:	7 days (exposure period was approximately only 15 minutes; see further details below)

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Treatments:	0, 5, 10, 15, 20 and 25 mg eugenol/L (nominal)
Analytical determination of test concentrations:	No
Negative control included:	Yes (ethanol)
Positive control included:	No
Parameters measured:	Mortality
Validity criteria:	Not reported

The following concentrations of anesthetics were evaluated: 20, 30, 40, 50 and 60 mg/L for eugenol and 100, 110, 120, 130 and 140 mg/L for clove oil. Ten fish were individually exposed to each anesthetic concentration for a period of 15 min, monitored for behavioral responses, then removed from anesthetic solution and placed in free anesthetic water to monitor recovery. Following recovery, fish were returned to tanks and monitored for either mortality or abnormality for 7 days. The efficacy criteria of anesthetic used were the acquisition of deep anesthesia within 3 min, fish recovery within 10 min, and survival of a 15 min exposure trial.

### Results

In the 24-hour acute toxicity test with *Lateolabrax maculatus*, spotted sea bass the lowest nominal concentration of 15 mg eugenol/L resulted in 0% mortality and the highest nominal concentration of 25 mg eugenol/L led to 100% mortality. Based on probit analysis, the 24-h LC<sub>50</sub> of eugenol was 19.37 mg/L (with a 95% CI of 15.68–21.33 mg/L), based on nominal concentrations.

In the 10-minutes acute toxicity test with *Lateolabrax maculatus*, spotted sea bass the lowest nominal concentration of 60 mg eugenol/L resulted in 0% mortality and the highest nominal concentration of 110 mg eugenol/L led to 100% mortality. Based on probit analysis, the 24-h LC<sub>50</sub> of eugenol was 98.13 mg/L (with a 95% CI of 91.74–102.06 mg/L), based on nominal concentrations.

All nominal concentrations tested (20, 30, 40, 50 and 60 mg eugenol/L) led to anaesthesia (loss of reflex activity or failure to respond to strong external stimuli) of the fish. The time to reach anaesthesia was 423, 272, 257, 207 and 182 seconds when exposed to 20, 30, 40, 50 and 60 mg eugenol/L (nominal), respectively. Following this short exposure period, 100% of fish recovered, with a mean time to recovery of 166, 261, 300, 506 and 584 s for the groups exposed to 20, 30, 40, 50 and 60 mg eugenol/L (nominal), respectively. No mortality occurred for any treatment group in the following 7 days post recovery from anaesthesia.

All nominal concentrations tested (100, 110, 120, 130 and 140 mg clove oil/L) led to anaesthesia (loss of reflex activity or failure to respond to strong external stimuli) of the fish. The time to reach anaesthesia was 257, 186, 175, 155 and 94 seconds when exposed to 20, 30, 40, 50 and 60 mg eugenol/L (nominal), respectively. Following this short exposure period, 100% of fish recovered, with a mean time to recovery of 377, 388, 454, 569 and 977 s for the groups exposed to 20, 30, 40, 50 and 60 mg eugenol/L (nominal), respectively. No mortality occurred for any treatment group in the following 7 days post recovery from anaesthesia.

### Assessment and conclusion

#### Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported.

### **Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

*Lateolabrax maculatus* (spotted sea bass) 24-hour LC<sub>50</sub> = 13.97 mg eugenol/L (95% confidence limits: 15.68–21.33 mg eugenol/L) based on nominal concentrations.

At nominal concentrations of 20 – 60 mg eugenol/L, *Lateolabrax maculatus* (spotted sea bass) reached anaesthesia after approximately 15 minutes, and recovered after approximately 2 to 9 minutes. No mortality or other negative effects on the spotted sea bass occurred in any of the eugenol treatments, up to 60 mg eugenol/L, during anaesthetic exposure (approximately 15 minutes) or in the 7 days afterwards.

At nominal concentrations of 100 – 140 mg clove oil/L, *Lateolabrax maculatus* (spotted sea bass) reached anaesthesia after approximately 15 minutes, and recovered after approximately 6 to 15 minutes. No mortality or other negative effects on the spotted sea bass occurred in any of the eugenol treatments, up to 60 mg eugenol/L, during anaesthetic exposure (approximately 15 minutes) or in the 7 days afterwards.

<b>Data point:</b>	CA 9.6.3.4/14 [8.2.1]
<b>Report author</b>	de Souza Romaneli, R., Zuffo Boaratti, A., Tellechea Rodrigues, A., Monge de Almeida Queiroz D., Ullah Khan, K., Torres Nascimento, T. M., and Kochenborger Fernandes, J. B.
<b>Report year</b>	2018
<b>Report title</b>	Efficacy of Benzocaine, Eugenol, and Menthol as Anesthetics for Freshwater Angelfish
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 144 Journal of Aquatic Animal Health Volume 30, Pages 210–216 (2018)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

#### Abstract (copied from original literature)

For the production and commercialization of ornamental fish species, it is indispensable to collect biometric data that facilitate the selection of animals for trade and genetic improvement of the stock. However, during the handling process, fish receive more stress if proper anesthetics are not used. Thus, application of appropriate anesthetics is an important tool for minimizing stress in animals. The objective of this study was to determine the effective concentrations of benzocaine, eugenol, and menthol for achieving anesthesia in Freshwater Angelfish *Pterophyllum scalare* and to develop induction and recovery response curves for different concentrations of these anesthetics. In total, 75 fish were exposed to five concentrations of the three anesthetics in a completely randomized design: benzocaine at 60, 85, 110, 135, and 160 mg/L; eugenol at 40, 80, 120, 160, and 200 mg/L; and menthol at 50, 75, 150, 200, and 250 mg/L. Each concentration (5 fish/concentration) consisted of five replicates, with each replicate represented by a single fish. The results indicated that the tested substances met the criteria of anesthetic efficiency. The effective concentrations of benzocaine, eugenol, and menthol for the anesthesia of Freshwater Angelfish were identified as 89.25, 90.6, and 92.1 mg/L, respectively.

#### Materials and methods

##### Test material 1

Name:	Eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Biodynamics Chemicals and Pharmaceuticals Ltd., Brazil
Active substance content:	99%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

##### Test organism

Species:	<i>Pterophyllum scalare</i> , Angelfish
Strain/clone:	Not reported
Age at study initiation:	juvenile
Weight/length/height at study initiation:	16.45 ± 1.75 g
Source:	Ornamental Fish Laboratory, Aquaculture Center, Sao Paulo State University, Sao Paulo, Brazil

Feeding during test: Not reported  
 Acclimation: Not reported acclimated for 1 week in semi-static conditions.

### *Test conditions*

Hardness: Not reported  
 Test temperature:  $27.05 \pm 0.64$  °C  
 pH:  $6.91 \pm 0.42$   
 Dissolved oxygen:  $6.77 \pm 0.21$  mg O<sub>2</sub>/L  
 Conductivity: Not reported  
 Photoperiod: Not reported  
 Light intensity: Not reported

### *Test system*

Study type: Acute toxicity test (anaesthesia effects)

Duration of study: 7 days (exposure period was approximately only 5 minutes; see further details below)

Treatments: 40, 80, 120, 160, and 200 mg eugenol/L (nominal)

Analytical determination of test concentrations: No

Negative control included: Yes (ethanol)

Positive control included: Yes (benzocaine)

Parameters measured: Anaesthesia (as opercular movements, equilibrium, and absence of tactile movements); Mortality

Validity criteria: Not reported

A total of 75 fishes were used in the present study. After the acclimation period, five aquaria were assigned to each concentration of eugenol for the assessment of anaesthesia. The animals were exposed to five nominal concentrations of 40, 80, 120, 160, and 200 mg eugenol/L. The anesthetized fish were then transferred individually to another five anaesthetic-free aquaria assigned to recovery times.

## **Results**

All nominal concentrations tested (40, 80, 120, 160 and 200 mg eugenol/L) led to anaesthesia (no response to mechanical stimulation) of the fish. Nominal concentrations of 120, 160, and 200 mg eugenol/L resulted in induction times less than 3 minutes, and induction times decreased with increasing anaesthetic concentration. For the lowest concentration of 40 mg eugenol/L the induction time was approximately 350 seconds. However, for the recovery time, no change was observed between the treatments; average recovery time across treatments was  $3.5 \pm 0.71$  minutes. The effective concentration of eugenol for anaesthesia induction in Freshwater Angelfish was 90.6 mg/L, with a time of 79 seconds, which was the shortest time observed among the three anaesthetics tested. The exposure of Freshwater Angelfish to effective concentrations of the anaesthetics has demonstrated efficient anaesthesia induction in 3 min and a recovery time of 5 min without causing any mortality or pathological signs.

## **Assessment and conclusion**

### *Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported.

**Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

At nominal concentrations of 40 – 200 mg eugenol/L, *Pterophyllum scalare* (fish) reached anesthesia approximately 5 minutes, and recovered after approximately 4 minutes. No mortality up to 200 mg eugenol/L (nominal) for 3 minutes of anesthesia or in the 7 days afterwards

<b>Data point:</b>	CA 9.6.3.4/15 [8.2.1]
<b>Report author</b>	Kristan, J., Stara, A., Polgesek, M., Drasovean, A., Kolarova, J., Priborsky, J., Blecha, M., Svacina, P., Policar, T., Velisek, J.
<b>Report year</b>	2014
<b>Report title</b>	Efficacy of different anaesthetics for pikeperch ( <i>Sander lucioperca</i> L.) in relation to water temperature
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 147 Neuroendocrinology Letters Volume 35, (supplement 2), Pages 81-85 (2014)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

**Abstract (copied from original literature)**

The objectives of the study were to compare the different doses of clove oil, Propiscin, and tricaine methane sulphonate (MS 222) in relation to water temperature in pikeperch aquaculture.

For assessment of this experiment 168 fish ( $10.77 \pm 0.59$  cm total body length and  $7.88 \pm 1.74$  g body weight) were used. Three different anaesthetic treatments (Propiscin, clove oil and MS 222) were used. Three doses of each anaesthetic treatment (Propiscin: 0.5; 1; 1.5 ml/L, clove oil: 15; 30; 60 mg/L, MS 222: 50; 100; 150 mg/L) were compared at three different temperatures 9.5; 15.5 and 23 °C.

In comparison of these doses of anaesthetic in different temperature, the significantly shortest time to attain phase A7 (total complete anaesthesia) was observed for Propiscin (1.5 ml/L)  $0:3 \pm 0:04$  min (23 °C) to  $0:33 \pm 0:25$  min (9.5 °C) compared to MS 222 (150 mg/L)  $1:04 \pm 0:21$  min (23 °C) to  $1:54 \pm 0:32$  min (9.5 °C) and clove oil (60 mg/L)  $1:05 \pm 0:17$  min (23 °C) to  $3:05 \pm 0:31$  min (9.5 °C).

On the other hand, the longest time of anaesthesia recovery was attained using Propiscin (1.5 ml/L)  $10:35 \pm 1:40$  min (23 °C) to  $32:30 \pm 1:10$  min (9.5 °C) compared to clove oil (60 mg/L)  $2:39 \pm 0:50$  min (23 °C) to  $9:36 \pm 2:34$  min (60 mg/L, 9.5 °C) and MS 222 (150 mg/L)  $2:26 \pm 1:27$  min (23 °C) to  $4:59 \pm 0:39$  min (9.5 °C).

The results from this study showed that the optimal and sufficient doses in all tested temperatures for pikeperch are 30 mg/L–1 of clove oil, 100 mg/L of MS 222 and 0.5 ml/L of Propiscin.

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**Materials and methods***Test material 1*

Name:	Clove oil
Formulation type:	Not relevant
Source and lot/batch no.:	Kulich Company
Active substance content:	eugenol 78%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

*Test organism*

Species:	<i>Sander lucioperca</i> L., pikeperch
Strain/clone:	Not reported
Age at study initiation:	Not reported
Weight/length/height at study initiation:	7.88 ± 1.74 g / 10.77 ± 0.59 cm
Source:	University of South Bohemia, Faculty of Fisheries and Protection of Waters.
Feeding during test:	No
Acclimation:	Fish were fed with food (Inicio Plus, BioMar). All fish were starved for 24 h before experiments were conducted.

*Test conditions*

Hardness:	Not reported
Test temperature:	9.5, 15.5 and 23 °C
pH:	7.5
Dissolved oxygen:	> 90%
Conductivity:	Not reported
Photoperiod:	Not reported
Light intensity:	Not reported

*Test system*

Study type:	Acute toxicity test (anaesthesia effects)
Duration of study:	maximum 15 minute exposure
Treatments:	Unclear – reported as 15, 30 and 60 mg clove oil/L (nominal) and 0.015, 0.03 and 0.06 mg clove oil/L (nominal) in different parts of the paper
Analytical determination of test concentrations:	No
Negative control included:	No
Positive control included:	No
Parameters measured:	Behaviour (anaesthesia effects)
Validity criteria:	Not reported

Eight fish were used for each temperature and each concentration of clove oil. Exposure to clove oil was chosen in advance to be maximum 15 minutes. Then, the fish were placed to clean aerated water of the same temperature and recovery time was observed. All experiments were performed in triplicate. Statistical analysis was performed using program Statistica 9.0 for Windows.

**Results**

At the lowest nominal test concentration (15 or 0.015 mg clove oil/L (unclear from paper)), anesthesia was not observed after 15 minutes of exposure at 9.5 °C, but time to anesthesia was approximately 8-10 minutes at 15.5 and 23 °C. Recovery time was approximately 3 minutes at both temperatures.

At the middle nominal test concentration (30 or 0.030 mg clove oil/L (unclear from paper)), time to anesthesia was approximately 2-3 minutes at all temperatures tested, with recovery times of approximately 2-9 minutes.

At the highest nominal test concentration (60 or 0.060 mg clove oil/L (unclear from paper)), time to anesthesia was approximately 1-3 minutes at all temperatures tested, with recovery times of approximately 2-7 minutes.

#### Assessment and conclusion

##### Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Concentrations tested are unclear – reported as 15, 30 and 60 mg clove oil/L (nominal) and 0.015, 0.03 and 0.06 mg clove oil/L (nominal) in different parts of the paper. Analytical verification of test concentrations was not reported.

#### **Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

No results concluded here as concentrations tested are unclear (see above).

<b>Data point:</b>	CA 9.6.3.4/16 [8.2.1]
<b>Report author</b>	Charoendat, U., Areechon, N., Srisapoome, P., Chantasart, D.
<b>Report year</b>	2009
<b>Report title</b>	Efficacy of Synthetic Eugenol as an Anesthetic for Nile Tilapia ( <i>Oreochromis niloticus</i> Linn)
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 153 Kasetsart J. (Nat. Sci.), Volume 43, Pages 132 - 140 (2009)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

#### Abstract (copied from original literature)

The efficacy of synthetic eugenol as an anaesthetic for Nile tilapia (*Oreochromis niloticus* Linn.) fry was investigated. An acute toxicity test and the efficacy of synthetic eugenol as an anaesthetic were studied and compared with clove oil-derived eugenol and MS-222 under similar conditions. The acute toxicity test indicated that the 24-hr LC<sub>50</sub> value of synthetic eugenol, clove oil-derived eugenol and MS-222 was 16.98, 16.95 and 72.5 ppm, respectively. The efficacy test, involving 20 min exposure to various concentrations of the three anaesthetics indicated that synthetic and clove oil-derived eugenol caused sedation at 5 ppm. A dose of 20 ppm of synthetic eugenol caused the loss of reflex reactivity (stage 5 of anaesthesia) with the induction time (3.40 min) slightly over the limit of 3 min, while it took 2.86 min for clove oil-derived eugenol. A higher dose of MS-222 was required than for the two other anaesthetics, with 30 ppm necessary to induce the sedation stage and 120 ppm to induce stage 5 anaesthesia within 2.16 min. However, this concentration caused 50% mortality after 20 min of exposure. The recovery time from anaesthesia for fish exposed to each anaesthetic was prolonged according to the higher dose exposure of each anaesthetic. The results of this experiment clearly indicated that synthetic eugenol could be an effective anaesthetic for handling and transport purposes of this species judging from the concentration for the induction of various stages of anaesthesia, recovery time and safety for tilapia fry.

#### Materials and methods

##### Test material 1

Name: Eugenol  
 Formulation type: Not relevant  
 Source and lot/batch no.: Not reported (obtained from the Faculty of Pharmacy, Mahidol University)  
 Active substance content: 100% (by weight)

Expiry date of lot/batch: Not reported  
Storage conditions: Not reported

#### *Test material 2*

Name: Clove oil  
Formulation type: Not relevant  
Source and lot/batch no.: Not reported (obtained from the Faculty of Pharmacy, Mahidol University)  
Active substance content: 99% eugenol (by weight)  
Expiry date of lot/batch: Not reported  
Storage conditions: Not reported

#### *Test organism*

Species: Nile tilapia (*Oreochromis niloticus* Linn.)  
Strain/clone: Not reported  
Age at study initiation: Not reported  
Weight/ length at study initiation: 3.0±0.09 g (mean, SE), 2.63±0.25 cm  
Source: Not reported  
Feeding during test: Not reported  
Acclimation: fed with commercial pellet feed for three days in a 1,000 litre fiberglass tank

#### *Test conditions*

Hardness: Not reported  
Test temperature: 26.7±1.67 °C  
pH: Not reported  
Dissolved oxygen: > 85% saturation  
Conductivity: Not reported  
Photoperiod: Not reported  
Light intensity: Not reported

#### *Test system*

Study type: Acute toxicity test  
Duration of study: 24 hours  
Treatments: 0, 5, 10, 15, 20, 25 and 30 ppm (nominal)  
Analytical determination of test concentrations: No  
Negative control included: Yes  
Positive control included: No  
Parameters measured: Mortality and behaviour  
Validity criteria: Not reported

### **Results**

Nile tilapia (*Oreochromis niloticus*) LC<sub>50</sub> value following 24 hours exposure was reported to be 16.98 mg eugenol/L and 16.95 mg clove-oil derived eugenol/L (based on nominal concentrations).

### **Assessment and conclusion**

#### *Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable.

The test is considered not reliable because no analytical verification of the test concentrations has been reported.

**Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

*Oreochromis niloticus* (Nile tilapia fish) 24-hour LC<sub>50</sub> = 16.98 mg eugenol/L (95% confidence limits: 16.35 – 17.60 mg/L) based on nominal concentrations

*Oreochromis niloticus* (Nile tilapia fish) 24-hour LC<sub>50</sub> = 16.95 mg synthetic clove oil derived eugenol/L (95% confidence limits: 16.25 – 17.65 mg/L) based on nominal concentrations

<b>Data point:</b>	CA 9.6.3.4/17 [8.2.1]
<b>Report author</b>	Ribeiro, P.A.P.; Miranda-Filho, K.C.; De Melo, D.C.; Luz, R.K.
<b>Report year</b>	2015
<b>Report title</b>	Efficiency of eugenol as anesthetic for the early life stages of Nile tilapia ( <i>Oreochromis niloticus</i> )
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 156 Annals of the Brazilian Academy of Sciences, Volume 87, Pages 529-535 (2015)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

**Abstract (copied from original literature)**

In aquaculture, activities with anaesthetic compounds are usually used in order to ensure the welfare of farmed fish, allowing handling out of water with decreased trauma by stress. Presently, there is no information about anaesthetic action of eugenol in early life stages of Nile tilapia (*Oreochromis niloticus*). The objective of this study was to evaluate different concentrations of eugenol for larvae and juveniles of Nile tilapia. Sixty animals were used for each group of weight, group I = 0.02 g; group II = 0.08 g; group III = 0.22 g; group IV = 2.62 g; and group V = 11.64 g. The eugenol concentrations tested were 50, 75, 100, 125, 150 and 175 mg/L. No mortality was reported during the tests with eugenol. Tilapia larvae with 0.02 g and juveniles around 11.64 g can be anesthetized with eugenol concentrations between 150 and 175 mg/L, since they determine the shortest sedation time (23 and 72 seconds, for the group of lowest and highest weights, respectively).

**Materials and methods***Test material*

Name:	Eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Not reported
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

*Test organism*

Species:	Nile tilapia ( <i>Oreochromis niloticus</i> )
Strain/clone:	Not reported

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Age at study initiation:	larvae and juveniles
Weight at study initiation:	group I = $0.02 \pm 0.001$ g; group II = $0.08 \pm 0.007$ g; group III = $0.22 \pm 0.04$ g; group IV = $2.62 \pm 0.33$ g; group V = $11.64 \pm 0.80$ g
Source:	Aquaculture Laboratory of the Federal University of Minas Gerais, Veterinary School, Brazil
Feeding during test:	No.
Acclimation:	Tilapia larvae were maintained in 30 L tanks in a recirculating water system, with a temperature of $27.0 \pm 0.5^\circ\text{C}$ , dissolved oxygen > 4 mg/L, 10 h photoperiod. During the first 30 days, fish were fed five times a day (8:00, 10:00 and 12:00 a.m.; 2:00, and 4:00 p.m.) with commercial tilapia diet containing 50% crude protein (Fri-Ribe®). Juveniles were fed four times a day (8:00 and 11:00 a.m.; 2:00 and 5:00 p.m.) with commercial tilapia diet containing 40% crude protein (Fri-Ribe®).

### *Test conditions*

Hardness:	Not reported
Test temperature:	$26.17 \pm 0.37$ °C
pH:	$7.83 \pm 0.38$
Dissolved oxygen:	$5.78 \pm 0.51$
Conductivity:	Not reported
Photoperiod:	Not reported
Light intensity:	Not reported

### *Test system*

Study type:	Acute toxicity test
Duration of study:	exposure ranged from 20 to 140 seconds, recovery for 24 hours
Treatments:	50, 75, 100, 125, 150 and 175 mg eugenol/L (nominal)
Analytical determination of test concentrations:	No
Negative control included:	No
Positive control included:	No
Parameters measured:	Mortality
Validity criteria:	Not reported

The aim of the study was to evaluate different concentrations of eugenol as anaesthetics for larvae and juveniles of Nile tilapia. Sixty animals were used in each group of weight (10 replicates (individually animals) for each concentration (6)).

The results were analyzed using SAS-Statistical Analysis System software (SAS 2002).

### **Results**

Concentrations of eugenol up to 175 mg eugenol/L caused no mortality to larvae and juveniles of Nile tilapia (based on nominal concentrations).

### **Assessment and conclusion**

#### *Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

The study is considered not reliable since the analytical verification of test concentrations was not reported.

**Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

*Oreochromis niloticus*: Concentrations of eugenol up to 175 mg eugenol/L caused not mortality to larvae and juveniles of Nile tilapia (based on nominal concentrations) after an exposure of about 80 seconds.

<b>Data point:</b>	CA 9.6.3.4/18 [8.2.1]
<b>Report author</b>	Roubach, R. Carvalho Gomes, L., Leao Fonseca, F. A., Val, A. L.
<b>Report year</b>	2005
<b>Report title</b>	Eugenol as an efficacious anaesthetic for tambaqui, <i>Colossoma macropomum</i> (Cuvier)
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 165 Aquaculture Research volume 36, Pages 1056- 1061 (2005)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (see details in summary below) – critically, no analytical verification of test concentrations, and acute toxicity tests only 10 minutes
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

**Abstract (copied from original literature)**

Anaesthetics are important in fish culture to reduce handling stress and mortality. Eugenol is a promising anaesthetic because of its low cost, efficacy, safety margin for fish and lack of toxicity to humans. The goal of this study was to establish a protocol using eugenol as a fish anaesthetic for tambaqui *Colossoma macropomum* (Cuvier), and provide information for regulating authorities on establishing safety dosage protocols for its use. Juvenile and sub-adult tambaqui were first individually exposed to doses of 35, 50, 65, 85, 100 or 135 mg/L eugenol for 10 min. A second experiment examined the effect of the duration of exposure to eugenol on the time required for recovery and survival of tambaqui. A eugenol dose of 65 mg/L was adequate to induce fish of both sizes into a surgical anaesthetic state, and recovery time was similar for dosages up to 100 mg/L. Exposure to the ideal dose (65 mg/L) for up to 30 min did not cause fish mortality. Fish blood glucose values were similar for all the tested eugenol doses as well as with the benzocaine control. The results show that eugenol is an efficient and safe anaesthetic for tambaqui.

**Materials and methods***Test material*

Name:	eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Biomedicinals, Manaus, Brazil
Active substance content:	90 %
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

*Test organism*

Species: *Colossoma macropomum*, tambaqui

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Strain/clone:	Not relevant
Age at study initiation:	juveniles and sub-adult
Weight/length/height at study initiation:	juvenile $56.6 \pm 7.7$ g / sub adult $1100 \pm 90.7$ g
Source:	juveniles from Balbina hatchery, sub adult from San Diego fish farm (both in Amazonas, Brazil)
Feeding during test:	No
Acclimation:	Feed was withheld for 24 h before the experiments.

*Test conditions*

Hardness:	Not reported
Test temperature:	26 - 27 °C (for test 1 only).
pH:	6.5 (only acclimation conditions reported)
Dissolved oxygen:	> 5 mg O <sub>2</sub> / L (only acclimation conditions reported)
Conductivity:	Not reported
Photoperiod:	Not reported
Light intensity:	Not reported

*Test conditions* (after recovery juveniles were transferred to continuous aerated aquaria)

Hardness:	Not reported
Test temperature:	27 °C
pH:	6.9
Dissolved oxygen:	> 5 mg O <sub>2</sub> / L
Conductivity:	Not reported
Photoperiod:	Not reported
Light intensity:	Not reported

*Test conditions* (after recovery sub-adults were transferred to a 25 m<sup>3</sup> pond)

Hardness:	Not reported
Test temperature:	28 °C
pH:	6.5
Dissolved oxygen:	> 5 mg O <sub>2</sub> / L
Conductivity:	Not reported
Photoperiod:	Not reported
Light intensity:	Not reported

*Test system*

Study type:	Acute toxicity test (anaesthesia effect)
Duration of study:	Test 1: 10 minutes of exposure; Test 2: 10, 20 and 30 min of exposure
Treatments:	Test 1: 35, 50, 65, 85 100 and 135 mg eugenol/L (nominal); Test 2: 65 mg eugenol/L (nominal)
Analytical determination of test concentrations:	No
Negative control included:	No
Positive control included:	Yes, benzocaine known for anaesthetic effects, not for toxicity effects
Parameters measured:	Mortality after 96 hours
Validity criteria:	Not reported

10 Tambaqui juveniles and 5 sub-adults were exposed to various concentrations of eugenol, 35, 50, 65, 85 100 and 135 mg eugenol/L for 10 minutes.

The second series of experiments examined the effect of duration of exposure to eugenol on the time required for recovery and survival of tambaqui. Five juvenile and sub-adults were individually exposed to 65 mg eugenol/L for periods of 10, 20 and 30 min, and then removed from the anaesthetic solution.

The anaesthesia time was defined as there was no response to mechanical stimulation to fish. After anaesthesia, the fish were immediately moved to fresh aquaria water or a fresh pond for recovery. The recovery time was considered when fish recovery of equilibrium and swimming actively.

### Results

All nominal concentrations tested in test 1 (35, 50, 65, 85 100 and 135 mg eugenol/L) led to anaesthesia (no response to mechanical stimulation) during an exposure period of 10 minutes. The time to reach anaesthesia of tambaqui juveniles were:  $0.32 \pm 0.11$ ,  $0.38 \pm 0.09$ ,  $0.32 \pm 0.11$ ,  $0.35 \pm 0.09$ ,  $0.39 \pm 0.05$  and  $0.30 \pm 0.08$  minutes when exposed to 35, 50, 65, 85 100 and 135 mg eugenol /L (nominal), respectively. The time to reach anaesthesia for tambaqui sub-adults were:  $1.96 \pm 0.76$ ,  $1.55 \pm 0.32$ ,  $1.48 \pm 0.05$ ,  $1.31 \pm 0.17$ ,  $1.41 \pm 0.18$  and  $1.16 \pm 0.12$  minutes when exposed to 35, 50, 65, 85 100 and 135 mg eugenol /L (nominal), respectively.

Following this short exposure period, there was no mortality in any of the tested doses or in the different exposure times tested in juveniles and sub-adults. For juveniles, recovery time was similar for doses for up to 100 mg/L (approximately 6 – 9 minutes) and significantly greater at a dose of 135 mg/L (approximately 20 minutes). For sub-adults, recovery time was similar for doses for up to 100 mg/L (approximately 3 – 6 minutes) and significantly greater at a dose of 135 mg/L (approximately 8 minutes)

The nominal concentration tested in test 2 (65 mg eugenol/L) led to anaesthesia (no response to mechanical stimulation) of tambaqui juveniles and sub-adults during an exposure period of 10, 20 and 30 minutes. The time to reach anaesthesia was  $1.45 \pm 0.46$  and  $1.48 \pm 0.59$  minutes for juveniles and sub-adults respectively. Following this short period of exposure, 100% of tambaqui juveniles and sub-adults recovered, with a time to recovery equilibrium of  $6.82 \pm 3.74$  and  $3.79 \pm 0.89$  respectively. The time to recover tambaqui juveniles and sub-adults during 20 and 30 minutes of exposure at 65 mg eugenol/L was approximately 6.82 and less than 10 minutes (see figure 1 in report).

### Assessment and conclusion

#### Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported.

No negative neither positive control used.

### **Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

At nominal concentrations of 35 – 135 mg eugenol/L, *Colossoma macropomum* (tambaqui fish) reached anaesthesia after approximately 1 to 2 minutes, and recovered after approximately 6 to 9 minutes. No mortality or negative effects on the fish up to 135 mg eugenol/L after 10 minutes of exposure or at 65 mg eugenol/L after 30 minutes of exposure or in the 96 hours afterwards.

<b>Data point:</b>	CA 9.6.3.4/19 [8.2.1]
<b>Report author</b>	Baldisserotto, B., Parodi, T.V., Don Stevens, E.
<b>Report year</b>	2018
<b>Report title</b>	Lack of postexposure analgesic efficacy of low concentrations of eugenol in zebrafish
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 224 Veterinary Anaesthesia and Analgesia Volume 45, Pages 48-56 (2018)
<b>Guidelines followed in study</b>	Canadian Council on Animal Care guidelines
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

#### Abstract (copied from original literature)

Objective: To test the post-exposure analgesic efficacy of low doses of eugenol in zebrafish.

Study design: A total of 76 large adult zebrafish (*Danio rerio*).

Methods: Fish swimming behavior (median velocity, freeze time, high-speed swimming and distance moved in the vertical direction) was recorded in a 1.6 L video arena before and after exposure to eugenol (0, 1, 2, 5, 10 and 20 mg/L). In a second experiment, fish were anesthetized with 2- phenoxy-ethanol and treated with an injection of 5% acetic acid (noxious stimulus), and then exposed to 0, 1, 2 and 5 mg/L eugenol. The fish swimming behavior was also recorded.

Results: The higher doses (10 and 20 mg/L) reduced the median velocity, high-speed swimming and distance moved in the vertical direction, and increased the freeze time. Zebrafish behavior was not altered by eugenol (1, 2 and 5 mg/L) after noxious stimulation.

Conclusions and clinical relevance: The change in the behavior of zebrafish associated with a noxious stimulus can be monitored and is a good model for studying analgesia in fish. Eugenol (10 and 20 mg/L) induced zebrafish sedation. The response after a noxious stimulus was not affected by post-exposure to lower doses, and thus we cannot recommend its use as an analgesic.

#### Materials and methods

##### Test material

Name:	eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Odontofarma, RS, Brazil.
Active substance content:	99.03 ± 0.15%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

##### Test organism

Species:	adult zebrafish, <i>Danio rerio</i>
Strain/clone:	Not reported
Age at study initiation:	adult zebrafish
Weight/length/height at study initiation:	Not reported
Source:	Local supplier

Feeding during test: No  
 Acclimation: The fish were acclimated to fish housing for 2 months prior to the experimental trials. They were fed flake food three times daily. The conditions of housing were as follows: temperature at  $28.3 \pm 0.4$  °C, 12/12 hour photoperiod.

### *Test conditions*

Hardness: Not reported  
 Test temperature: 25 °C  
 pH: 6.8-7.4  
 Dissolved oxygen: Not reported, but constant aeration  
 Conductivity: Not reported  
 Photoperiod: 12h/12h  
 Light intensity: Not reported

### *Test system*

Study type: Acute toxicity test  
 Duration of study: exposure of 45 minutes, duration of 150 minutes  
 Treatments: 0, 1, 2, 5, 10 or 20 mg eugenol/L (nominal)  
 Analytical determination of test concentrations: No  
 Negative control included: Yes (water with the solvent ethanol)  
 Positive control included: No  
 Parameters measured: Mortality/behaviour  
 Validity criteria: Not reported

Five to seven zebrafish were studied for each concentration (0, 1, 2, 5, 10 or 20 mg eugenol/L). Each fish was transferred to a 100 mL beaker containing eugenol in tank water. After exposure of eugenol (45 minutes) the fish was returned to the video arena and, after a rest time of 5 minutes, the swimming behavior was recorded for 35 seconds (time post1). The fish was then returned to aquarium water without eugenol for 45 minutes, before being transferred to the video arena where, after a rest time of 5 minutes, the swimming behavior was recorded for 35 seconds (time post2). This procedure was repeated for a third recording of the swimming behavior after exposure to eugenol (time post3).

All statistical analyses were performed using the software Statistica Academic Version 10.0 (TIBCO Software Inc., CA, USA) and Minitab Version 17.3 (Minitab Inc., PA, USA).

### **Results**

There were no mortalities or adverse side effects noted during any of the trials in the experiment. The effect of eugenol immersion was only evident at the first observation period 5 minutes after the end of exposure (time post1), and the behavior returned to normal for the later observation periods.

*Post hoc* tests comparing the results of different concentrations with the control showed that the higher concentrations (10 and 20 mg eugenol/L) differed from the control value. However, lower concentrations (1, 2 and 5 mg eugenol/L) did not differ from the control for any of the four variables (Bonferroni  $p = 0.5$  for all tests). The higher doses 10 and 20 mg/L reduced the median velocity, high-speed swimming and distance moved in the vertical direction, and increased the freeze time.

### **Assessment and conclusion**

#### *Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported.

**Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

*Danio rerio*:

No mortalities or adverse side effects occurred after an exposure of 45 minutes up to 20 mg eugenol/L (nominal). The concentration of 20 mg eugenol/L induced sedation on zebrafish and reduced swimming behaviour whereas at concentrations up to 5 mg eugenol/L, no effects on swimming behaviour were observed.

<b>Data point:</b>	CA 9.6.3.4/20 [8.2.1]
<b>Report author</b>	Gonçalves, A.F.N., Santos, E.C.C., Fernandes, J.B.K., Takahashi, L.S.
<b>Report year</b>	2008
<b>Report title</b>	Menthol and eugenol as benzocaine substitutes in anesthetic induction of pacu juveniles
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 235 Acta Sci. Anim. Sci., Volume 30, Pages 339-344 (2008)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

**Abstract (copied from original literature)**

This study aimed to verify the efficacy of natural anesthetic induction of pacu juveniles. Were evaluated four menthol (50, 100, 150 and 200 mg/L), four eugenol (10, 25, 50 and 100 mg/L) and one benzocaine (100 mg/L) concentrations. During the anesthetic procedure, four sedative stages were monitored and evaluated until no reaction of the fish to handling was registered. After performing biometric evaluation on the anesthetized fish, were recorded the recovery time and mortality rate up to 48 hours after the anesthetic experiments. Concentrations of 100, 150 and 200 mg/L of menthol, 50 and 100 mg/L of eugenol showed anesthetic induction time and recovery time similar to that of benzocaine. The obtained results showed that menthol and eugenol are efficient anesthetics for pacu juveniles in substitution of benzocaine, suggesting the concentration of 100 mg/L of menthol and 50 mg/L of eugenol.

**Materials and methods***Test material*

Name:	Eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Not reported
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

*Test organism*

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Species:	Pacu fish ( <i>Piaractus mesopotamicus</i> )
Strain/clone:	Not reported
Age at study initiation:	juveniles
Weight/length at study initiation:	average weight: $110.5 \pm 21.6$ g; average total size: $17.4 \pm 1.4$ cm;
Source:	Aquaculture Centre at São Paulo State University (Caunesp) in Jaboticabal, São Paulo State, , Brazil
Feeding during test:	Not reported
Acclimation:	20 days in polyethylene tanks of 1000 L, static system, fed with fish commercial food twice a day

### *Test conditions*

Hardness:	Not reported
Test temperature:	$25.4 \pm 0.91$ – $26.0 \pm 0.85$ °C (before exposure) $25.5 \pm 0.77$ – $26.0 \pm 0.70$ °C (after exposure)
pH:	8.0 (before exposure) – 7.8 to 8.0 (after exposure)
Dissolved oxygen:	$6.0 \pm 0.41$ – $6.3 \pm 0.36$ mg/L (before exposure) $5.6 \pm 0.27$ – $6.5 \pm 0.56$ mg/L (after exposure)
Conductivity:	Not reported
Photoperiod:	Not reported
Light intensity:	Not reported

### *Test system*

Study type:	Acute toxicity test
Duration of study:	Exposure until fish were anaesthetised (from 23.8 seconds to 214.5 seconds), and then 48 hours for effects
Treatments:	10, 25, 50 and 100 mg L <sup>-1</sup> (nominal)
Analytical determination of test concentrations:	No
Negative control included:	No
Positive control included:	Yes (benzocaine, positive control for anaesthetic effects)
Parameters measured:	Anaesthetics time
Validity criteria:	Not reported

The aim of this study was to determine the best anesthetic concentration of eugenol when compared to a reference compound used in anesthetics, benzocaine.

### **Results**

Exposing pacu juveniles to eugenol, at nominal concentrations of 25 to 100 mg/L, led to induction of anaesthesia in up to 1 hour, not causing mortality within 48 hours of the anaesthetic procedure. The 10 mg/L concentration of eugenol did not enable the fish to reach stage 3, with loss of balance, in less than 1 hour, ruling out the chance of using this concentration in anaesthetic procedures on pacu juveniles. The concentration of 100 mg/L of eugenol, which led the fish to reach stage 4 more quickly than other treatments, resulted in them taking longer than necessary to recover. Up to 48 hours after anaesthesia, no mortality was observed with any treatments.

### **Assessment and conclusion**

#### *Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

There was not an analytical verification of test concentrations and the exposure of fish to eugenol is too short for a real acute toxicity study.

**Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

Nevertheless, results show that there is no mortality when juvenile pacu were exposed to nominally 100 mg eugenol/L.

<b>Data point:</b>	CA 9.6.3.4/21 [8.2.1]
<b>Report author</b>	Perdikaris, C., Nathanailides, C., Gouva, E., Ugwemorubong Ujagwung, G., Bitchava, K., Athanasopoulou, F., Paschou, A., Paschos, I.
<b>Report year</b>	2010
<b>Report title</b>	Size-relative Effectiveness of Clove Oil as an Anaesthetic for Rainbow Trout ( <i>Oncorhynchus mykiss</i> Walbaum, 1792) and Goldfish ( <i>Carassius auratus</i> Linnaeus, 1758)
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 299 Acta Veterinaria Brno, Volume 79, Pages 481-489 (2010)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

**Abstract (copied from original literature)**

The purpose of this work was to investigate the size-relative effectiveness of clove oil as an anaesthetic for rainbow trout and goldfish. In total, 128 rainbow trout (*Oncorhynchus mykiss*) (two groups of 20-23 and 30-33 cm mean fork length) and 160 goldfish (*Carassius auratus*) (four size groups of 1.5-2.5, 5-7, 11-15 and 20-25 cm) were anaesthetized at different clove oil concentrations of 50, 100, 150 mg/L for trouts and 75, 100, 150 mg/L for goldfish. Rainbow trout exhibited total loss of balance and no response to external stimuli with shorter induction time as dosage increased (120.5 seconds, 64.4 seconds and 44.3 seconds, respectively). Goldfish exhibited total loss of balance and no response to external stimuli after induction time that varied with dosage used and body size of fish. The small fish (1.5-7 cm) exhibited shorter induction time which ranged from 84.28 seconds at 75 mg/L clove oil to 41.14 seconds at 150 mg/L clove oil. The larger fish had a longer induction time inversely related to the dosage. Recovery time was longer than induction time in both species. Both species recovered within 6 min after anaesthesia at 150 mg/L clove oil. Clove oil did not produce marked changes ( $P < 0.05$ ) in the physiological indicators of goldfish compared to the control. However, marked changes ( $P < 0.05$ ) were exhibited in the haematocrit of treated rainbow trout that also exhibited hyperkalaemia and hyperglycaemia ( $P > 0.05$ ). For both fish species, clove oil was effective, producing minimum stress and zero mortalities, and can be recommended as an effective anaesthetic.

**Materials and methods***Test material*

Name:	Clove oil
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma Aldrich Co., St. Louis, USA
Active substance content:	88.58% of eugenol
Expiry date of lot/batch:	Not reported

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Storage conditions: Not reported

#### *Test organism 1*

Species: rainbow trout (*Onchorynchus mykiss*)  
Strain/clone: Not reported  
Age at study initiation: Not reported  
Length at study initiation:  $31.06 \pm 2.47$  cm  
Source: Not reported  
Feeding during test: The trout were fed daily with trout pellets at 2% of body weight.  
Acclimation: All fish were starved for 24 h before the experiment.

#### *Test organism 2*

Species: goldfish (*Carassius auratus*)  
Strain/clone: Not reported  
Age at study initiation: Not reported  
Weight/length/height at study initiation:  $6.8 \pm 0.1$  g / 1.5-25 cm  
Source: Not reported  
Feeding during test: carp pellets at a 3% daily ratio  
Acclimation: Acclimation period, conditions (same as test or not), type and amount of food All fish were starved for 24 h before the experiment.

#### *Test conditions 1*

Hardness: Not reported  
Test temperature:  $12 \pm 0.6$  °C  
pH: 7.5 – 7.7  
Dissolved oxygen: above 8.5 ppm  
Conductivity: Not reported  
Photoperiod: 12:12 hours  
Light intensity: Not reported

#### *Test conditions 2*

Hardness: Not reported  
Test temperature:  $18 \pm 0.7$  °C  
pH: 7.5 – 7.7  
Dissolved oxygen: above 7 ppm  
Conductivity: Not reported  
Photoperiod: 12:12 hours

Light intensity: Not reported

*Test system*

Study type: Acute toxicity test (anaesthesia effects)  
Duration of study: 48 hours duration, exposure from 44.30 seconds to 400 seconds for rainbow trout and from 41.15 seconds to 475 seconds for goldfish  
Treatments: rainbow trout: 50, 100, 150 mg clove oil/ L (nominal);  
goldfish: 75, 100, 150 mg clove oil (nominal)  
Analytical determination of test concentrations: No  
Negative control included: Yes  
Positive control included: No  
Parameters measured: Mortality  
Validity criteria: Not reported

Four days before the experiment, ten fish were randomly selected from each tank and sampled for bacteriological and parasitological examinations. Prior to anaesthesia, the goldfish were divided into four fork length classes (1.5-2.5, 5-7, 11-15 and 20-25 cm) and placed in separate glass aquaria (volume 70 l). The trout were divided into two size groups (20-23 and 30-33 cm) and maintained in different holding tanks. Seven to ten fish of each species and size group were exposed to three different concentrations of clove oil (n = 7-10 fish per size group/dosage tested). Rainbow trout were exposed to 50, 100, 150 mg clove oil/L and goldfish were exposed to 75, 100 and 150 mg clove oil/L. Once a fish reached stage 5 of anaesthesia (when fish lost equilibrium and reflex reactivity), it was removed from the experimental tanks, dried and measured. Then the fish was transferred to fresh water in identical tank without rinsing to remove traces of the anaesthetic. Following recovery, the fish were transferred to maintenance tanks and observed for 48 h for potential mortality.

Box plots were plotted for induction and recovery times for the various sizes of fish using SPSS 15.0 software.

**Results**

All nominal concentrations tested (75, 100, 150 mg clove oil) led to anaesthesia (fish lost equilibrium and reflex reactivity). The mean time to reach anaesthesia was 271.90, 249.18, and 92.5714 seconds when exposed to 75, 100 and 150 mg clove oil/L (nominal), respectively (for goldfish). Following this short exposure period, 100% of fish recovered, with a mean time to recovery of 202.23, 304.71 and 251.47 seconds for the groups exposed to 75, 100 and 150 mg clove oil/L (nominal), respectively.

The mean time to reach anaesthesia was 121.27, 167.81 and 145.79 seconds when exposed to 75, 100 and 150 mg clove oil/L (nominal), respectively (for rainbow trout). Following this short exposure period, 100% of fish recovered, with a mean time to recovery of 202.23, 304.71 and 251.47 seconds for the groups exposed to 75, 100 and 150 mg clove oil/L (nominal), respectively.

In this study, clove oil was found to be effective anaesthetic for both rainbow trout and goldfish. Both fish species exhibited normal behavior and remained calm during the induction time with no struggling or rapid swimming, which was a positive sign of their welfare. Additionally, no mortalities or other adverse effects were observed within 48 hours following recovery from anaesthesia.

**Assessment and conclusion**

*Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported.

**Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

At nominal concentrations of 75 – 150 mg clove oil/L *Oncorhynchus mykiss* reached anaesthesia after approximately 2 to 5 minutes, and recovered after approximately 3 to 5 minutes. No mortalities or other adverse effects were observed within 48 hours following recovery from anaesthesia at concentrations of 150 mg clove oil/L.

At nominal concentrations of 75 – 150 mg clove oil/L *Carassius auratus* reached anaesthesia after approximately 2 to 3 minutes, and recovered after approximately 3 to 5 minutes. No mortalities or other adverse effects were observed within 48 hours following recovery from anaesthesia at concentrations of 150 mg clove oil/L.

<b>Data point:</b>	CA 9.6.3.4/22 [8.2.1]
<b>Report author</b>	Keene, J. L., Noakes, D. L.G., Moccia, R. D., Soto, C.G.
<b>Report year</b>	1998
<b>Report title</b>	The efficacy of clove oil as an anaesthetic for rainbow trout, <i>Oncorhynchus mykiss</i> (Walbaum)
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 320 Aquaculture Research Volume 29, Pages 89-101 (1998)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Methodology for static tests from the Biological Test Method for Acute Lethality Tests Using Rainbow Trout (EPS 1/RM/ 91990).
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

**Abstract (copied from original literature)**

The anaesthetic effects of clove-oil-derived eugenol were studied in juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum). Acute lethality and the effects of multiple exposures to eugenol were measured. The estimated 8–96 hours LC<sub>50</sub> for eugenol was found to be approximately 9 mg eugenol/L. Times to induction and recovery from anaesthesia were measured and compared with MS-222 under similar conditions. Eugenol generally induced anaesthesia faster and at lower concentrations than MS-222. The recovery times for fish exposed to eugenol were six to 10 times longer than in those exposed to similar concentrations of MS-222. Clove oil eugenol was determined to be an acceptable anaesthetic with potential for use in aquaculture and aquatic research. Doses of 40–60 mg eugenol/L were found to induce rapid anaesthesia with a relatively short time for recovery in juvenile trout.

**Materials and methods***Test material*

Name:	Eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Hilltech Canada Inc., Vankleek Hill, Ontario, Canada
Active substance content:	Not reported, but naturally eugenol is 70-90%.
Expiry date of lot/batch:	Not reported

Storage conditions: Not reported

#### *Test organism*

Species: rainbow trout, *Oncorhynchus mykiss*  
 Strain/clone: RT94/10/ONT  
 Age at study initiation: sexually immature juvenile (6-month-old)  
 Weight/length at study initiation: 20.46 ± 0.73 g / 12.06 ± 0.14 cm  
 Source: Alma Research Station, Alma, (Ontario, Canada)  
 Feeding during test: No (during anesthesia)  
 Acclimation: They were maintained in aerated well water at a temperature of 9.1 ± 0.2°C with a 16 : 8 h light : dark photoperiod, In well water, fishes were fed 2.1% of their body weight daily with 3g trout pellets.

#### *Test conditions*

Hardness: Not reported  
 Test temperature: 13.9 ± 0.2 °C  
 pH: Not reported  
 Dissolved oxygen: >85% saturation  
 Conductivity: Not reported  
 Photoperiod: 16:8 h light: dark  
 Light intensity: Not reported

#### *Test system*

Study type: Acute toxicity test  
 Duration of study: 72 hours for acute test; max. of 30 min of exposure for anesthesia tests with a study duration of 12 – 14 days  
 Treatments: Mortality 0, 1, 2, 5, 15 and 30 mg eugenol/L; anesthesia 20, 40, 60, 80, 100, 120 and 140 mg eugenol/L (nominal)  
 Analytical determination of test concentrations: No  
 Negative control included: Yes (water containing ethanol)  
 Positive control included: Yes (MS-222 for the anesthetic effects)  
 Parameters measured: Mortality, behaviour  
 Validity criteria: Not reported

Six test aquaria were filled to 20 L with continuously aerated well water. Clove oil stock solution was added to produce final concentrations of 0, 1, 2, 5, 15 and 30 ppm eugenol among the six aquaria. Ten fish were randomly selected from a pool of 750 and placed in each tank. Total mortalities, behaviours, temperature and oxygen were measured and noted hourly for the first 12 hours of the experiment, every 3 hours for the next 12 hours, and every 6 hours for the remaining 72 hours. A fish was considered dead when no opercular beats were witnessed for a 15-min period of continuous observation. The fish were then euthenized with an overdose of MS-222 (> 200 ppm), and wet weights to the nearest 0.01 g and fork lengths to the nearest mm were measured. The 96-h LC<sub>50</sub> experiment was repeated three times.

The time to onset of anaesthesia was measured for both clove oil and MS-222 under the same experimental conditions. The following concentrations were tested: 20, 40, 60, 80, 100, 120 and 140 ppm eugenol/L. Ten fish, randomly selected from a pool of 500, were individually placed in the test aquarium and the time to loss of equilibrium and time to stage 5 anaesthesia were measured to the nearest second with a stopwatch. The test fish were then placed individually in 10 L of aerated well water in recovery aquaria. The time to recovery of equilibrium and recovery of the fear response was measured to the nearest second for each test fish. Fish from this experiment were transferred to 0.7 m<sup>3</sup> holding tanks, grouped by treatment, and were observed daily for any abnormal behaviours and mortalities for 12–14 days after recovery.

The effects of varying times of exposure and varying concentrations of clove oil on recovery times in fish were measured in 20 L of aerated well water. Ten fish were randomly selected from a pool of 350 and placed within the test aquarium for 3, 6, 10, 20 or 30 min. Fish were then removed and placed individually in recovery tanks. The times to recovery of equilibrium and recovery of fear response were measured to the nearest second and any

mortality was recorded. The eugenol concentrations tested in this experiment were 40, 60, 80 and 100 ppm. Recovered fish were transferred to 0.7m<sup>3</sup> holding tanks, grouped by treatment, and observed for 12–14 days for any signs of mortality or abnormal behaviours.

#### Results

All fish exposed to 1, 2 and 5 ppm eugenol solutions survived the 96 hours of the test, and no mortalities or abnormal behaviours were observed in these fish for a period of 12–14 days after its completion. One hundred per cent mortalities were observed for both the 15 and 30 ppm eugenol treatments for all three replications at the end of the 96-h LC<sub>50</sub> test. The LC<sub>50</sub> value was reported to be 9.0 ppm eugenol (based on nominal concentrations).

Doses of 40–140 ppm eugenol tended to elicit a similar response time to the loss of equilibrium (between 30 and 50 seconds) in the fish tested.

There was a positive exponential relationship between exposure time and the time to recovery of both equilibrium and fear response for all concentrations of eugenol tested. No mortalities were observed among groups of 10 fish exposed to 40 ppm eugenol for 20 min or less. No mortalities were observed for fish exposed to 60 ppm eugenol for 10 min or less. No mortalities were observed for fish exposed to 80 or 100 ppm eugenol for 6 min or less.

#### Assessment and conclusion

##### Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported.

Purity of test item not reported, only natural occurrence.

#### **Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

*Oncorhynchus mykiss*: LD<sub>50</sub> value was reported to be 9.0 mg eugenol/L (based on nominal concentrations).

<b>Data point:</b>	CA 9.6.3.4/23 [8.2.1]
<b>Report author</b>	Wang, W., Dong, H., Sun, Y., Cao, M., Duan, Y., Li, H., Liu, Q., Gu, Q., Zhang, J.
<b>Report year</b>	2019
<b>Report title</b>	The efficacy of eugenol and tricaine methanesulphonate as anaesthetics for juvenile Chinese sea bass ( <i>Lateolabrax maculatus</i> ) during simulated transport
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 321 Journal Appl Ichthyol., Volume 35, Pages 551-557 (2019)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

#### Abstract (copied from original literature)

The purpose of this study was to evaluate the anaesthesia effects of eugenol and MS-222 sedatives applied on juvenile *Lateolabrax maculatus* during simulated transport. In experiment 1, the juveniles were divided into two groups, with seven concentrations tested on each group (eugenol [4, 6, 8, 10, 12, 14 and 16 mg/L] and MS-222 [20, 30, 40, 50, 60, 70 and 80 mg/L]). Induction and recovery times were recorded. The time for anaesthesia was shortened, and the time for complete recovery was prolonged as the anaesthetic concentration increased. The optimal transport concentration for each anaesthetic tested was 6 mg/L of eugenol and 30 mg/L for MS-222. In experiment 2, the 5-hr simulated transport test showed that the survival rate of *L. maculatus* juveniles with anaesthesia was 100%, and without anaesthesia, survival was 60%. After 24 hr of recovery following transport,

the fish showed 100% survival for the group with added anaesthetic and 40% for the group without added anaesthetic. Compared to the non-anaesthetized groups, the anaesthetized transport groups showed significant increases in the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) ( $p < 0.05$ ). The levels of AST, ALT and alkaline phosphatase (AKP) were significantly higher in the MS-222 transport group than in the eugenol transport group ( $p < 0.05$ ). The levels of AKP were significantly higher in the non-anaesthetized transport group than in the anaesthetized group ( $p < 0.05$ ). According to the present experiment results, eugenol was an efficient anaesthetic in *L. maculatus*, and we recommend eugenol instead of MS-222 as an anaesthetic for the short-time transport of *L. maculatus*.

### Materials and methods

#### Test material 1

Name:	Eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Shanghai Medical Instruments Co., Ltd. Shanghai, China
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

#### Test organism

Species:	Chinese sea bass, <i>Lateolabrax maculatus</i>
Strain/clone:	Not reported
Age at study initiation:	juveniles
Weight at study initiation:	100 ± 10 g
Source:	fish hatchery in Zhuhai, Guangdong Province, China.
Feeding during test:	No
Acclimation:	recirculation aquaculture system at the density 5 g/L for 2 weeks (4.5-m diameter, 0.7-m depth with biofilter linked). The water exchange rate was 500 L/hr. The juveniles were fed at 3% ± 1% of the fish biomass per day during the acclimation period with commercial feed for perch (Foshan Shunde District Haihuang Industry Co., Ltd.).

#### Test conditions

Hardness:	Not reported
Test temperature:	28 ± 0.5°C
pH:	Not reported
Dissolved oxygen:	Not reported
Conductivity:	Not reported
Photoperiod:	Not reported
Light intensity:	Not reported

#### Test system

Study type:	Anaesthetic test
Duration of study:	5 hours and 24 hour recovery
Treatments:	4, 6, 8, 10, 12, 14 and 16 mg eugenol/L (nominal)
Analytical determination of test concentrations:	No
Negative control included:	Yes
Positive control included:	No
Parameters measured:	Mortality following anaesthetic
Validity criteria:	Not reported

The aim of this study was to evaluate the anaesthesia effects of eugenol applied on juvenile *Lateolabrax maculatus* during simulated transport. Following anaesthesia, the survival rate after 24 hours were measured.

### Results

Nominal concentrations of eugenol at 4 and 6 mg eugenol/L lead to light effects of anaesthesia after approximately 100 to 220 seconds exposure, but recovery occurred within approximately 30 seconds and there was no mortality to fish over the following 24-hour period. Nominal concentrations of eugenol at up to 12 mg eugenol/L lead to effects of deep anaesthesia after approximately 900 seconds exposure, but recovery occurred within approximately 50 seconds and there was 10% mortality over the following 24-hour period. Similar effects of anaesthesia were observed after short exposure to nominally 14 mg eugenol/L and 16 mg eugenol/L, and over the following 24 hours mortality was 30% and 50%, respectively. All concentrations are based on nominal concentrations.

### Assessment and conclusion

#### Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable as the analytical verification of test concentrations was not reported.

### Assessment and conclusion by applicant:

The study is not acceptable (not reliable).

Chinese sea bass, *Lateolabrax maculatus*: At nominal concentrations up to 16 mg eugenol/L, effects of anaesthesia were observed after approximately 300 seconds exposure and over the following 24 hours mortality was 50%.

<b>Data point:</b>	CA 9.6.3.4/24 [CA 8.2.1]
<b>Report author</b>	Yousefi, M., Hoseini, S. M., Vatnikov, Y. A., Nikishov, A. A., Kulikov, E. V.
<b>Report year</b>	2018
<b>Report title</b>	Thymol as a new anesthetic in common carp ( <i>Cyprinus carpio</i> ): Efficacy and physiological effects in comparison with eugenol
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 335 Aquaculture Volume 495, Pages 376-383 (2018)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

### Abstract (copied from original literature)

The aim of the present study was to investigate thymol anesthetic efficiency and biochemical effects in common carp in comparison to eugenol. In the first experiment, time of induction of and recovery from anesthesia were recorded in the fish anesthetized with either eugenol or thymol at the concentrations of 6.25, 12.5, 25, 50, 75, 100, 125, 150 and 200 mg / L (ten fish per concentration). In the second experiment, stress responses, oxidative stress and biochemical effects of eugenol and thymol were investigated in the fish after short-term (5 min; 43 mg / L eugenol or 52 mg / L thymol) anesthesia and long-term (3 h; 10 or 20 mg / L of either of the anesthetics) anesthetic exposure. Eugenol anesthetized the fish within 600–90 s (with recovery time of 190–380 s) at the concentrations of 25–150 mg / L. Thymol induced anesthesia within 850–60 s (with recovery time of 210–1200 s) at the concentrations of 25–200 mg / L. Eugenol resulted in a significant increase in plasma aspartate transaminase (AST), lactate dehydrogenase (LDH), malondialdehyde (MDA) and phosphate levels compared to thymol after the short-term anesthesia. After the long-term exposure, eugenol led to significant elevations in

cortisol, glucose, lactate, AST, LDH, total antioxidant capacity (TAC), MDA, superoxide dismutase (SOD) and phosphate and decrease in catalase (CAT) compared to thymol. In conclusion, thymol is an efficient anesthetic in carp allowing for rapid sampling as well as surgery. In addition, thymol has fewer side effects compared to eugenol in the case of induction of stress, oxidative stress and tissue damage, thus is recommended for carp anesthesia.

## Materials and methods

### *Test material 1*

Name:	eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Aldrich, Milwaukee, USA
Active substance content:	99%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

### *Test material 2*

Name:	thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma, St. Louis, USA
Active substance content:	98.5%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

### *Test organism*

Species:	common carp, <i>Cyprinus carpio</i>
Strain/clone:	Not reported
Age at study initiation:	Not reported
Weight/length/height at study initiation:	110 ± 5.65 g
Source:	Not reported
Feeding during test:	No
Acclimation:	10 days during which they were fed with commercial feed.

### *Test conditions*

Hardness:	Not reported
Test temperature:	25.3 ± 1.25 °C
pH:	7.58 ± 0.45
Dissolved oxygen:	6.32 ± 0.87 mg O <sub>2</sub> /L
Conductivity:	Not reported
Photoperiod:	Not reported
Light intensity:	Not reported

### *Test system*

Study type:	Anaesthetic effect
Duration of study:	Approximately 10-30 minutes

Treatments: 6.25, 12.5, 25, 50, 75, 100, 125, 150 and 200 mg thymol/L; 6.25, 12.5, 25, 50, 75, 100, 125, 150 and 200 mg eugenol/L

Analytical determination of test concentrations: No

Negative control included: No

Positive control included: No

Parameters measured: Mortality/behaviour

Validity criteria: Not reported

Fish were exposed to nominally 6.25, 12.5, 25, 50, 75, 100, 125, 150 and 200 mg/L anesthetics eugenol and thymol (10 fish of each tank per concentration) to record induction and recovery time. Anesthetic and recovery chambers were 10-L tanks equipped with aeration. The fish were individually caught from the holding tanks and placed into the anesthetic chamber. The time to reach stages 3 and 4 anesthesia were recorded and then the fish was caught and placed into the recovery tank to record recovery time.

### Results

Both eugenol and thymol were not able to induce any anesthesia stages in the fish at the nominal concentration of 6.25 mg/L. Thymol at the nominal concentration of 12.5 mg/L induced stage 3 anesthesia; however, eugenol failed to induce anesthesia at this concentration. Eugenol induced stage 4 anesthesia within 600–90 s (with recovery time of 190–380 s) at the nominal concentrations of 25–150 mg/L; however, there was no significant difference in induction between the nominal concentrations 125 and 150 mg/L. Thymol induced stage 4 anesthesia within 850–60 s (with recovery time of 210–1200 s) at the nominal concentrations of 25–200 mg/L. There were relationships between the anesthetics' concentrations and induction time, and between the anesthetics' concentrations and recovery time. In general, induction time of the anesthetics was similar (except at the concentration 50 mg / L); however, the thymol-anesthetized fish recovered within longer period compared to eugenol. Eugenol failed to anesthetize the fish in <90 s, but thymol induced anesthesia in the fish within 60 s at the nominal concentration of 200 mg/L.

### Assessment and conclusion

#### Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported.

### **Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

At nominal concentrations of 25 – 200 mg thymol/L, *Cyprinus carpio* (common carp) reached anaesthesia after approximately 1 to 15 minutes, and recovered after approximately 3 to 20 minutes.

At nominal concentrations of 25 – 150 mg eugenol/L, *Cyprinus carpio* (common carp) reached anaesthesia after approximately 1.5 to 10 minutes, and recovered after approximately 3 to 6 minutes.

No mortality was reported.

<b>Data point:</b>	CA 9.6.3.4/25 [8.2.1]
<b>Report author</b>	Cao, X., Wang, Y., Yu, N., Le, Q., Hu, J., Yang, Y., Kuang, S., Zhang, M., Sun, Y., Gu, W., Yan, X.
<b>Report year</b>	2019
<b>Report title</b>	Transcriptome analysis reveals the influence of anaesthetic stress on the immune system of crucian carp ( <i>Carassius auratus</i> ) under the process of treatment and low concentration transport by MS-222 and Eugenol
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 366 Aquaculture Research Volume 50, Pages 3138-3153 (2019)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

#### Abstract (copied from original literature)

Anaesthetics are widely used in aquaculture as a method of stress relief. However, few studies have reported the influence of anaesthetics on the immune system of fish. Therefore, in the present study, we selected two different anaesthetics, MS-222 (100 mg/L) and eugenol (20 mg/L), to anaesthetize crucian carp and analyse the transcriptome. It was found that over 137 million high-quality reads were generated and de novo assembled into a final set of 97,556 unigenes. A total of eight differentially expressed genes (DEGs) related to the immune response were co-expressed in the two anaesthetic groups. GO and the KEGG revealed that these genes functioned primarily to enrich antigen processing and presentation pathways, including *MHCI*, *MHCIIa*, *p-MHCIIa*, *P-MHCIIa*, *CD74*, *MHCII antigen* and *MHCII antigena*. Moreover, *LRRFIP2* was found to be associated with the immune response. Consistent with the transcriptome findings, qPCR verified the changes in the relative level of expression of these genes. Furthermore, long-term exposure to low concentrations of MS-222 (30 mg/L) and eugenol (8 mg/L) showed an impact on these immune genes. In conclusion, anaesthetics used in high or low concentrations for treatment and transport could affect the immune system in fish species. While eugenol was associated with an earlier activation of immune gene expression, MS-222 exhibited more significant effects on the immune response. These findings improve the understanding of the mechanisms of anaesthetics on immune damage and will be of great value for future studies involving anaesthetic selection and treatment for fish.

#### Materials and methods

##### Test material 1

Name:	eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Not reported
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

##### Test organism

Species:	crucian carp, <i>Carassius auratus</i>
Strain/clone:	Not reported
Age at study initiation:	One-year-old

Weight/length/height at study initiation: as  $65.8 \pm 5.7$  g /  $15.2 \pm 2.0$  cm  
 Source: cultured in the laboratory at Ningbo University (Ningbo, China)  
 Feeding during test: No  
 Acclimation: Fish were cultured in 1,000 L fibreglass-reinforced plastic tanks and maintained in continuously aerated water at  $25 \pm 1^\circ\text{C}$  for 1 week. During the acclimation period, crucian carp were fed commercial feed (Tianbang, Ningbo) twice daily, which was equivalent to 1.5% of their weight.

### *Test conditions*

Hardness: Not reported  
 Test temperature: Not reported  
 pH:  $7.8 \pm 0.2$   
 Dissolved oxygen:  $8.2$  mg O<sub>2</sub> /L  
 Conductivity: Not reported  
 Photoperiod: Not reported  
 Light intensity: Not reported

### *Test system*

Study type: Acute toxicity test (anaesthesia effects)  
 Duration of study: 25 hours (exposure duration unclear)  
 Treatments: 6, 7, 8 and 9 mg eugenol/L (nominal)  
 Analytical determination of test concentrations: No  
 Negative control included: Yes  
 Positive control included: No  
 Parameters measured: Mortality (gene expression parameters are not reported here as not considered relevant)  
 Validity criteria: Not reported  
 25 fishes were exposed to different concentrations of anaesthetic eugenol, 6, 7, 8 and 9 mg/L and one control group for transportation simulation. Time of exposure is unclear from the paper.

### **Results**

When the behavioural performance of crucian carp was observed after 25 hours, a nominal concentration of 6 mg eugenol/L had no influence on the fish. A nominal concentration of 9 mg eugenol/L eugenol resulted in a partial loss of equilibrium in the fish. The optimal lowest concentration of eugenol to induce anesthesia for transportation of fish was reported as 8 mg eugenol/L (nominal).

### **Assessment and conclusion**

#### *Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.  
 Proposed category: 3 not reliable  
 Analytical verification of test concentrations was not reported.  
 Purity of eugenol not reported. Time of exposure in treated test media is unclear.

### **Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

*Carassius auratus* (crucian carp): When the behavioural performance of crucian carp was observed after 25 hours, a nominal concentration of 6 mg eugenol/L had no influence on the fish. A nominal concentration of 9 mg eugenol/L eugenol resulted in a partial loss of equilibrium in the fish. The optimal lowest concentration of eugenol to induce anesthesia for transportation of fish was reported as 8 mg eugenol/L (nominal).

<b>Data point:</b>	CA 9.6.3.4/26 [8.2.1]
<b>Report author</b>	Ögretmen, F., Gölbası, S., Inanan, B. E., Kizak, V., Kayim, M.
<b>Report year</b>	2014
<b>Report title</b>	Use of Clove Oil and Eugenol to Anesthetize Fingerling Shabut <i>Barbus grypus</i>
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 377 North American journal of aquaculture Volume 76, Pages 9-13 (2014)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

#### Abstract (copied from original literature)

In this study, the efficacy of four doses of clove oil and eugenol were compared to sedate fingerling Shabut *Barbus grypus* to various stages of sedation and recovery. The results from the present study indicated that effective concentrations were at 50 µL/L for eugenol (induction time: 58 ± 8 s; recovery time: 199 ± 15 s [mean ± SD]) and clove oil (induction time: 76 ± 6 s; recovery time: 161 ± 34 s). The induction times decreased significantly with the increasing concentrations of clove oil and eugenol, while recovery times increased with increasing concentrations of anesthetic ( $P < 0.05$ ). Exposure of fish to 75 or 100 µL/L clove oil or to 50, 75, or 100 µL/L eugenol resulted in mean induction times ≤ 1 min. Fish did not become fully sedated within 3 min when treated with 25 µL/L clove oil. Other concentrations of both anesthetics, fish reached to full induction at ≤ 3 min and recovered from anesthesia ≤ 5 min. Our results showed the clove oil and eugenol are effective anesthetics for fingerling Shabut when used at concentrations of 50–100 µL/L.

#### Materials and methods

##### Test material 1

Name:	clove oil
Formulation type:	Not relevant
Source and lot/batch no.:	Biopont, Budapest, Hungary
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

##### Test material 2

Name:	eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Merck, KGaA, Darmstadt, Germany
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

##### Test organism

Species:	Shabut, <i>Barbus grypus</i>
Strain/clone:	Not reported

Age at study initiation: Fingerling  
 Weight/length/height at study initiation:  $30.36 \pm 8.47$  g /  $15.30 \pm 1.28$  cm  
 Source: obtained from earthen ponds at General Directorate of State Hydraulic Works, Fish Production Station, Ataturk Dam Lake, Urfa, Turkey  
 Feeding during test: No  
 Acclimation: Fish were transferred to the hatchery and acclimated for 2 weeks before the start of the experiment. During the acclimatization period fish were fed *ad libitum* with 40% protein commercial pellets twice a day. Fish were not fed the day before the start of the study.

### *Test conditions*

Hardness: Not reported  
 Test temperature:  $23.0 \pm 0.10$  °C  
 pH:  $8.23 \pm 0.20$   
 Dissolved oxygen:  $8.20 \pm 0.11$   
 Salinity: 1 ‰  
 Conductivity: Not reported  
 Photoperiod: Not reported  
 Light intensity: Not reported

### *Test system*

Study type: Acute toxicity test (anaesthesia effects)  
 Duration of study: Minutes  
 Treatments: 25, 50, 75 and 100 µL clove oil/L; 25, 50, 75 and 100 µL eugenol/L (nominal)  
 Analytical determination of test concentrations: No  
 Negative control included: No  
 Positive control included: No  
 Parameters measured: Mortality/behaviour  
 Validity criteria: Not reported

Seven fingerlings of Shabut were exposed to clove oil and eugenol concentrations to determine induction and recovery times of anesthetics. (Total number = 84 fish). Each replicate consisted of seven fish exposed separately. Experiments were prepared in triplicate. Four nominal concentrations of these two anesthetics were adjusted to 25, 50, 75, and 100 µL/L. The recovery stage was recorded after transferring the fish to aerated water in the recovery aquarium (30 L). Induction and recovery times were measured using a digital stopwatch. Kruskal–Wallis test was used to assess the differences in induction and recovery times. Statistics were performed using SPSS version 15.0.

### **Results**

Nominal concentrations of eugenol or clove oil at 25, 50, 75 and 100 µL/L lead to effects of anaesthesia after approximately 30 to 800 seconds exposure, but recovery occurred within approximately 150 to 200 seconds and there was no observed fish mortality.

### **Assessment and conclusion**

#### *Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported. Purity of the active substances not reported.

**Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

*Barbus grypus* (Shabut fish, fingerlings): No mortality was observed during the application of 100 µL clove oil/L up to 3 minutes of exposure, total recovery of the normal swimming was observed after 5 minutes.

*Barbus grypus* (Shabut fish, fingerlings): No mortality was observed during the application of 100 µL eugenol/L up to 3 minutes of exposure, total recovery of the normal swimming was observed after 5 minutes.

<b>Data point:</b>	CA 9.6.3.4/27 [8.2.1]
<b>Report author</b>	Takatsuka, V., Costa D.G.C., Oliveira, N.Y., Sanches, E.G., Azevedo, V. G.
<b>Report year</b>	2019
<b>Report title</b>	Use of eugenol for anesthesia of lesser guitarfish <i>Zapteryx brevirostris</i> (Rhinobatidae)
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 379 Brazilian Journal of Biology Volume 79, Pages 516-520 (2019)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

**Abstract (copied from original literature)**

Anesthesia can be utilized as a non-lethal procedure to allow easy handling of teleosts and elasmobranchs in captivity or in the wild. For this, anesthetic protocols need to be established according to the species. The aim of this study was to determine the ideal concentration of eugenol for anesthesia of *Zapteryx brevirostris*. Four concentrations were tested: 21.25, 42.50, 85.00 and 170.00 mg/L (ratio of 1:5 with absolute ethanol). The perfect concentration of eugenol for this species was 85.0 mg/L, which enabled up to 300 seconds of work on the fish, without any response to handling.

**Materials and methods***Test material 1*

Name:	eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	K-Dent
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

*Test organism*

Species:	lesser guitarfish (ray), <i>Zapteryx brevirostris</i> (Rhinobatidae)
Strain/clone:	Not reported
Age at study initiation:	adult
Weight/length/height at study initiation:	mean weight was 541.16 ± 119.18 g and their

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	mean total length was $44.47 \pm 4.48$ cm.
Source:	The fish were obtained through a trawl fishery in Ubatuba (Brazil)
Feeding during test:	No, the first meal was offered 24 hours after recovery from anesthesia, and it was observed that the consumption was not different from the amount prior to the experiment (50 g).
Acclimation:	The rays were acclimatized and maintained in three circular fiberglass tanks with a capacity of 3000 L, at a density of seven rays per tank (approximately 1 kg/m <sup>3</sup> ) and at a temperature of $25 \pm 2$ °C and salinity of $30 \pm 1$ . Feed was offered once a day in the morning, consisting of Atlantic seabob shrimp ( <i>Xiphopenaeus kroyeri</i> ) without head and Frigate tuna ( <i>Auxis thazard</i> ) in cubes, at the amount of 1% of the biomass of the tank. Food leftovers and feces were siphoned out, 5 minutes after feeding started.

### *Test conditions*

Hardness:	Not reported
Test temperature:	$25 \pm 2$ °C
pH:	Not reported
Dissolved oxygen:	Not reported
Salinity:	$30 \pm 1$
Conductivity:	Not reported
Photoperiod:	Not reported
Light intensity:	Not reported

### *Test system*

Study type:	Anaesthetic effect
Duration of study:	maximum exposure of 10 minutes, followed by 72 hours recovery in clean water
Treatments:	21.25, 42.50, 85.00 and 170.00 mg eugenol/L (nominal)
Analytical determination of test concentrations:	No
Negative control included:	No
Positive control included:	No
Parameters measured:	Mortality/behaviour
Validity criteria:	Not reported

Lesser guitarfish (rays) were exposed to different concentrations of eugenol; 21.25, 42.50, 8.00 and 170.00 mg/L. The rays were sorted and immersed individually in each concentration of anesthetic solution (5-6 rays per concentration). The chronometer was triggered to establish the time taken to induce anesthesia, which was counted from the time of transferring the ray to the tank containing eugenol, until anesthesia stage III was reached. After reaching stage III, each ray was transferred to a 30 L plastic box containing seawater, and the anesthesia recovery period began. Biometry was performed while the ray was in this box and, afterwards, it was transferred to a box containing 100 L of water for final recovery monitoring. The anesthesia recovery time was monitored until the ray exhibited normal swimming movements and the frequency of the spiracle beats reached the same level as in the first measurement before induction of anesthesia. The data were subjected to the ANOVA and Tukey test.

### **Results**

Nominal concentrations of 42.50, 85.00 and 170.00 mg eugenol/L lead to effects of anaesthesia (stage III – total loss of reaction to external stimuli) after approximately 75 to 400 seconds exposure, but recovery occurred within approximately 250 to 400 seconds and there was no observed fish mortality in the following 72 hours. At the lowest tested nominal concentration of 21.25 mg eugenol/L, the fish did not reach anaesthesia stage III after the maximum exposure time of 600 seconds (10 minutes).

### **Assessment and conclusion**

*Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported. Active substance content was not reported.

**Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

*Zapteryx brevirostris* (lesser guitarfish): Nominal concentrations of 42.50, 85.00 and 170.00 mg eugenol/L lead to effects of anaesthesia (stage III – total loss of reaction to external stimuli) after approximately 75 to 400 seconds exposure, but recovery occurred within approximately 250 to 400 seconds and there was no observed fish mortality in the following 72 hours. At the lowest tested nominal concentration of 21.25 mg eugenol/L, the fish did not reach anaesthesia stage III after the maximum exposure time of 600 seconds (10 minutes).

<b>Data point:</b>	CA 9.6.3.4/28 [8.2.1]
<b>Report author</b>	Gomes, D. P., Chaves, B. W., Becker, A. G., Baldisserotto, B.
<b>Report year</b>	2011
<b>Report title</b>	Water parameters affect anaesthesia induced by eugenol in silver catfish, <i>Rhamdia quelen</i>
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 396 Aquaculture Research Volume 42, Pages 878-886 (2011)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

**Abstract (copied from original literature)**

The present study investigated the effects of water pH (5.0, 7.0 and 9.0), hardness (0, 20 and 120mg CaCO<sub>3</sub>/L) and temperature (15, 23 and 30 °C) on the induction of sedation and anaesthesia, and subsequent recovery, of silver catfish exposed to eugenol. Moreover, the blood gas tensions (PvO<sub>2</sub> and PvCO<sub>2</sub>) and blood pH in silver catfish acclimated to these temperatures were investigated after exposure to eugenol. Water pH, hardness, temperature and fish size affect the efficacy of eugenol in silver catfish, particularly at the lower concentrations tested (20 and 30 mg/L). Sedation of this species can be induced at concentrations as low as 20 mg/L, but for anaesthesia a concentration of at least 40 mg/L of eugenol must be used to compensate for the influence of fish size and water quality. Blood gas tension and pH were affected by eugenol anaesthesia, but only in fish acclimated to 30 ±1 °C.

**Materials and methods***Test material 1*

Name:	eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Not reported
Active substance content:	99.03%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

*Test organism*

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Species:	silver catfish, <i>Rhamdia quelen</i>
Strain/clone:	Not reported
Age at study initiation:	juvenile
Weight/length/height at study initiation:	3.5 ± 0.7 g / 7.7 ± 0.8 cm
Source:	Bela Vista Fish Culture (Santa Maria, RS, Brazil)
Feeding during test:	No
Acclimation:	Fish were maintained for 2 weeks in continuously aerated 250 L tanks. Fish were fed once per day with commercial fish feed until apparent satiety, up to 24 h before an experiment.

### *Test conditions*

Hardness:	0, 20 and 120 mg/L calcium carbonate.
Test temperature:	15, 23 and 30 °C
pH:	5.0, 7.0 and 9.0
Dissolved oxygen:	5.8 - 7.2 mg O <sub>2</sub> / L
Conductivity:	Not reported
Photoperiod:	12 hours: 12 hours (light/dark)
Light intensity:	Not reported

### *Test system*

Study type:	Anaesthetic test
Duration of study:	30 minutes
Treatments:	20, 30 and 40 mg eugenol/L (nominal)
Analytical determination of test concentrations:	No
Negative control included:	No
Positive control included:	No
Parameters measured:	Mortality/behaviour
Validity criteria:	Not reported

Fish were exposed to 20, 30 and 40 mg eugenol/L to evaluate the time to sedation. The maximum observation time was 30 minutes. After induction, juveniles were transferred to anaesthetic-free aquaria to measure anaesthesia and recovery times. The effects of pH, temperature and water hardness on sedation, anesthesia and recovery were compared by one way analysis of variance and Tukey's test; or, when homogeneity of variances was not obtained, by Kruskal-Wallis ANOVA and the Mann-Whitney test.

### **Results**

Nominal concentrations of 20, 30 and 40 mg eugenol/L lead to effects of anaesthesia after approximately 300 to 800 seconds exposure, with the time also influenced by water quality parameters. However, recovery occurred within approximately 80 to 300 seconds at all concentrations tested and there was no observed fish mortality.

### **Assessment and conclusion**

#### *Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported.

**Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

At nominal concentrations of 20 – 40 mg eugenol/L, silver catfish, *Rhamdia quelen*, reached anaesthesia after approximately 1 to 3 minutes, and recovered after approximately 2 to 5 minutes.

There was no mortality in the experiment up to 40 mg eugenol/L (nominal), when fish were exposed during 1 to 3 minutes of sedation and during recovery.

<b>Data point:</b>	CA 9.6.3.4/29 [CA 8.2.2.1]
<b>Report author</b>	Hoskonen, P., Heikkinen, J., Eskelinen, P., Pirhonen, J.
<b>Report year</b>	2015
<b>Report title</b>	Efficacy of clove oil and ethanol against <i>Saprolegnia</i> sp. and usability as antifungal agents during incubation of rainbow trout <i>Oncorhynchus mykiss</i> (Walbaum) eggs
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 146 Aquaculture Research volume 46, Pages 581—589 (2015)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

**Abstract (copied from original literature)**

Inhibitory concentrations of clove oil and ethanol against growth of *Saprolegnia* sp. Hyphae were screened by a modification of the hemp (*Cannabis sativa* L.) seed MicroPlate (HeMP) method and their usability as antifungal agents during incubation of rainbow trout *Oncorhynchus mykiss* eggs was tested. *In vitro* experiment showed that in continuous static exposure, clove oil at 100 mg/L significantly inhibited the growth of *Saprolegnia*, whereas in bath exposures, clove oil at 500 mg/L had no significant effect at any exposure time tested (15, 60 and 240 minutes), but clove oil at 10 000 mg/L significantly inhibited growth at all exposure times. Clove oil and ethanol treatments had no visible effects on the onset or spread of the fungus during incubation of rainbow trout eggs. Clove oil at 1000 mg/L resulted in 95-100% mortality before the eyed stage was reached. Sublethal concentrations of clove oil and ethanol had no effects on the development rate of the embryo or growth and yolk utilization efficiency after hatching. This study suggests that clove oil and ethanol may not be options in controlling aquatic fungi infestations during incubation of rainbow trout eggs.

**Materials and methods***Test material 1*

Name:	Clove oil
Formulation type:	Not relevant
Source and lot/batch no.:	Not reported
Active substance content:	eugenol 75%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

*Test organism*

Species:	Fertilized and unsterilized rainbow trout eggs
Strain/clone:	eggs from each parent pair
Age at study initiation:	Not reported
Weight/length/height at study initiation:	Not reported
Source:	Savon Taimen fish farm in Rautalampi, Finland
Feeding during test:	No
Acclimation:	Not reported

*Test conditions*

Hardness:	Not reported
Test temperature:	12.5 °C
pH:	7.04
Dissolved oxygen:	Not reported
Conductivity:	Not reported
Photoperiod:	variable but the eggs were shielded from bright lights and disturbance
Light intensity:	Not reported

*Test system*

Study type:	Non-standard fish early life stage study, in which eggs also exposed to fungal zoospores
Duration of study:	30 days approximately (exposure to clove oil mixture was limited to 15 minutes once or three times per week)
Treatments:	500 mg clove oil/L + 7100 mg ethanol/L; 1000 mg clove oil/L + 7100 mg ethanol/L
Analytical determination of test concentrations:	No
Negative control included:	Yes (water and ethanol)
Positive control included:	No
Parameters measured:	Mortality Egg mortality; hatching success; fry mortality, weight, length.
Validity criteria:	Not reported

100 of eggs rainbow trout were exposed to clove oil concentrations of 500 and 1000 mg/L and ethanol 7110 mg ethanol/L one or three times per week in baths of 15 minutes, from 30<sup>th</sup> of April to 18<sup>th</sup> of May (hatching). The control was well water. After the eyed stage the dead eggs were manually removed from the incubation vessels and counted once a week, all un-hatched eggs were counted and all hatched fry were visually classified as healthy, abnormal or dead. In the *in vivo* experiment, the numbers of hatched healthy and abnormal fry, wet body weight, length and dry weight of body and yolk sac were analysed with one-way ANOVA and *post hoc* comparisons were performed with Fisher's LSD test.

**Results**

The treatments (exposure to clove oil at nominally 500 or 1000 mg/L for 15 minutes once or three times a week) did not influence the timing of the eyed stage or hatching. There were no significant differences in the survival to hatch or in the amount of visibly abnormal fry.

Mortalities of eggs exposed to mixture of 1000 mg clove oil/L and 7110 mg ethanol/L, one or three times per week were 95 and 100%.

**Assessment and conclusion***Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported, and actual exposure is difficult to determine as only short 15-minute exposure periods once or twice a week in a study of approximately 30 days duration. All treatment groups also included exposure to a fungus, which grows on and inhibits fish eggs.

**Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

Mortalities of rainbow trout eggs exposed to short (15-minute) treatments with mixture of 1000 mg clove oil/L and 7110 mg ethanol/L (and a fungus), one or three times per week for approximately 30 days were 95 and 100%.

<b>Data point:</b>	CA 9.6.3.4/30 [8.2.4.2]
<b>Report author</b>	Jiang, S., Zhou, F., Yang, W., Wu, Z., Yin, L., Yang, Q., Yebing, Y. and Jiang, S.
<b>Report year</b>	2020
<b>Report title</b>	Anaesthetic effect of eugenol at different concentrations and temperatures on black tiger shrimp ( <i>Penaeus monodon</i> )
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 29 Aquaculture Research volume 53, Pages 3268-3273 (2020)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

**Abstract (copied from original literature)**

The anaesthetic effects of eugenol on *Penaeus monodon* were investigated at the different eugenol concentrations (60, 110, 160 and 210 mg/L), water temperature (21, 26 and 31°C), air exposure time (3, 6, 9 and 12 min) and body weight ( $2.62 \pm 0.27$ ,  $6.34 \pm 0.36$  and  $11.43 \pm 0.33$  g). The anaesthesia and recovery time were recorded. The results showed that the anaesthesia time of the shrimp decreased with the increase in the eugenol concentration and water temperature, and the recovery time increased with the increase of the eugenol concentration and the decrease of water temperature. Under the same eugenol concentrations, the recovery time increased with the increase of air exposure time and body weight. Under the eugenol concentration range of 60–210 mg/L, the recovered rate was 100%. The results indicated that eugenol is a safe and efficient anaesthetic for *P. monodon*.

**Materials and methods***Test material 1*

Name:	Eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Shanghai Medical Instrument Co., Ltd
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

*Test organism*

Species:	<i>Penaeus monodon</i> , shrimp
Strain/clone:	Not reported
Age at study initiation:	average body weight $3.21 \pm 0.18$ g
Weight/length/height at study initiation:	Not reported

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Source:	Cultured at Shenzhen Experimental Station of South China Sea Fisheries Research Institute of Chinese Academy of Fishery Sciences
Feeding during test:	Shrimps were temporarily fed in cement ponds before the experiment and stopped feeding 1 day before the experiment. Fed for 5 days during the recovery phase of the test.
Acclimation:	Not reported

#### *Test conditions*

Hardness:	Not reported
Test temperature:	28 °C in all tests, except specific test aiming to assess effects of different temperatures (21, 26 and 31 °C)
pH:	8.2
Dissolved oxygen:	Not reported
Salinity:	29 ppt
Conductivity:	Not reported
Photoperiod:	Not reported
Light intensity:	Not reported

#### *Test system*

Study type:	Acute toxicity test (anaesthesia effects)
Duration of study:	5 days (exposure period was approximately only 2 – 30 minutes; see further details below)
Treatments:	60, 110, 160 and 210 mg eugenol/L (nominal)
Analytical determination of test concentrations:	No
Positive control included:	No
Parameters measured:	Anaesthesia (no response to mechanical stimulation); Mortality
Validity criteria:	Not reported

Eugenol was mixed with 95% ethanol at a volume ratio of 1:9 and then dissolved in sand-filtered natural seawater. The concentrations of eugenol were set at 60, 110, 160 and 210 mg/L. The anaesthesia and recovery time of each shrimp were recorded. There were three parallel groups with 10 shrimps in each parallel group. The anaesthesia time was defined as there was no response to mechanical stimulation to shrimp. After anaesthesia, the shrimp were immediately moved to fresh sea water for recovery, and the recovery time was the balance swimming time until all the steps of the shrimp were restored. The proportion of shrimp resuscitated after the anaesthesia test compared to the total number of anaesthetized shrimp is called recovery rate. At the end of the experiment, the shrimp were placed in a clean plastic barrel, and the survival rate was observed after 5 days of feeding. Data from each treatment were subjected to one-way analysis of variance (ANOVA).

In addition to the first experiment at 28 °C, the above test was also repeated at water temperatures of 21, 26 and 31 °C.

Furthermore, a third experiment was carried out in which after anaesthesia, prior to placing the shrimps in clean seawater for recovery, the shrimps were placed on a wet gauze to expose them to air for 3, 6, 9 or 12 minutes. A fourth experiment was the same as the first, but tested only a single concentration of 100 mg eugenol/L and used shrimps of differing body weights ( $2.62 \pm 0.27$ ,  $6.34 \pm 0.36$  and  $11.43 \pm 0.33$  g).

### **Results**

All nominal concentrations tested (60, 110, 160 and 210 mg eugenol/L) led to anaesthesia (no response to mechanical stimulation) of the shrimps. The mean time to reach anaesthesia was 25.49, 13.51, 4.84 and 2.86 minutes when exposed to 60, 110, 160 and 210 mg eugenol/L (nominal), respectively (in the main test at 28 °C, mean shrimp size 3.21 g and no air exposure before recovery phase). Following this short exposure period, 100% of shrimps recovered, with a mean time to recovery of 25.47, 17.01, 11.31 and 6.43 minutes for the groups exposed to 60, 110, 160 and 210 mg eugenol/L (nominal), respectively. Univariate analysis of variance showed that there were significant differences in anaesthesia time and recovery time between different concentrations of eugenol ( $p < .05$ ). No mortality occurred for any treatment group in the following 5 days in untreated seawater.

Whilst the additional experiments found significant effects on anaesthesia and recovery times when shrimps were exposed at different water temperatures, with different air exposures after eugenol exposure, or for shrimp of differing body weights, in all cases the mean anaesthesia time was no less than approximately 2 minutes, and

the mean recovery time was no more than approximately 30 minutes (Figures 2 to 4 of the paper). 100% of shrimps recovered and survived the eugenol exposures.

#### Assessment and conclusion

##### Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported and test item purity is not known.

#### **Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

At nominal concentrations of 60 – 210 mg eugenol/L, *Penaeus monodon* (shrimp) reached anaesthesia after approximately 2 to 30 minutes, and recovered after approximately 5 to 30 minutes. No mortality or other negative effects on the shrimp occurred in any of the eugenol treatments, up to 210 mg eugenol/L, during anaesthetic exposure (approximately 2 to 30 minutes) or in the 5 days afterwards.

<b>Data point:</b>	CA 9.6.3.4/31 [8.2.4.2]
<b>Report author</b>	Li, Y., She, Q., Han, Z., Sun, N., Liu, X., Li, X.
<b>Report year</b>	2018
<b>Report title</b>	Anaesthetic effects of eugenol on grass shrimp ( <i>Palaemonetes sinensis</i> ) of different sizes at different concentrations and temperatures
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 30 Scientific Reports volume 8:11007, Pages 2045-2322
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

#### Abstract (copied from original literature)

Essential oil derivatives are widely used for anaesthetising aquatic animals. However, the effectiveness of anaesthesia often varies according to the anaesthetic agent, species, temperature, dosage, and interactions among these factors. This study evaluated the effects of eugenol on three sizes of the shrimp *Palaemonetes sinensis* at different concentrations and temperatures. Eugenol dose, water temperature, and shrimp size were found to significantly influence anaesthesia in *P. sinensis*. Induction time decreased linearly with increasing water temperature and eugenol concentration, while it increased with body weight. However, recovery times lengthened with increasing concentration and temperature, and shortened with lower body size. At 100 and 200 µL/L eugenol concentrations, the survival rates of medium and large shrimps were maintained at over 80% at all temperatures studied over 72 h recovery. However, the survival rates of small shrimps were below 60% at 24 °C and 28 °C over 5 days of recovery. These results suggest that eugenol is an effective and rapid anaesthetic for *P. sinensis*, but it might have disadvantages such as slow recovery and possible mortality in small shrimps and at higher temperatures and dosages.

#### Materials and methods

##### Test material

Name: eugenol  
 Formulation type: Not relevant  
 Source and lot/batch no.: Jiangxi Xuesong Natural Medicinal Oil Co., Ltd. (China)

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Active substance content:	99%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

### *Test organism*

Species:	<i>Palaemonetes sinensis</i> , Chinese grass shrimp
Strain/clone:	Not reported
Age at study initiation:	Not reported
Weight/length/height at study initiation:	small (1.5–2.5 cm), medium (2.5–3.5 cm), and large (3.5–4.5 cm)
Source:	rice field in Panjin City, Liaoning Province, China
Feeding during test:	No, all shrimps were starved for 24 h prior to anaesthetic experimentation
Acclimation:	30 days prior to experimentation. Freshwater oligochaetes were offered as food at a rate of 2% of body weight every 24 h. After the 30-day period elapsed, every 5 tanks of the 30 tanks were acclimated and maintained for 2 weeks at 8 °C, 12 °C, 16 °C, 20 °C, 24 °C, and 28 °C, with 1 °C rise per day until the target temperature was reached.

### *Test conditions*

Hardness:	Not reported
Test temperature:	8 °C, 12 °C, 16 °C, 20 °C, 24 °C, and 28 °C.
pH:	Not reported
Dissolved oxygen:	Not reported
Salinity:	Not reported
Conductivity:	Not reported
Photoperiod:	12:12 h light: dark cycle
Light intensity:	Not reported

### *Test system*

Study type:	Acute toxicity test (anaesthesia effects)
Duration of study:	5 days (exposure period was approximately only 20 – 120 minutes; see further details below)
Treatments:	100, 200, 300, 400, and 500 µL eugenol/L
Analytical determination of test concentrations:	No
Negative control included:	No
Positive control included:	No
Parameters measured:	Anaesthesia: induction stage 1 (partial loss of equilibrium), induction stage 2 (complete loss of equilibrium), recovery stage 1 (partial regained control of equilibrium), recovery stage 2 (complete regained control of equilibrium); Mortality; Body weight change

Ten shrimps of each group were held in five separate plastic tanks (5 L capacity) containing different concentrations of eugenol (100, 200, 300, 400, and 500 µL /L). Water used in the experiment was supplied from the acclimatisation tanks, and temperature was maintained by using six constant-temperature incubators (GXZ-500; Ningbojiangnan Co. Ltd., Ningbo City, Zhejiang Province, China). The two stages of induction and the two recovery stages from anaesthesia were conducted under identical experimental conditions. The four stages recorded were: induction stage 1 (partial loss of equilibrium), induction stage 2 (complete loss of equilibrium), recovery stage 1 (partial regained control of equilibrium), recovery stage 2 (complete regained control of equilibrium).

The time required to reach each stage was recorded by one observer per stage. After shrimps showed a complete loss of equilibrium and non-reactivity to stimuli, they were removed from the anaesthetic tank to a recovery tank (20 × 40 × 30 cm) using a hand net. The survival rate for each recovery tank was measured every 24 h over the subsequent 5 days. Change in wet weight was calculated based on the body weight of each individual measured before and after induction. One-way and three-way ANOVAs were used to detect differences in induction and recovery times. Statistical analyses were carried out using SPSS 17.0 software.

## Results

All nominal concentrations tested (100, 200, 300, 400, and 500 µL eugenol/L) led to anaesthesia (no response to mechanical stimulation) of the shrimps. The mean time to reach anaesthesia was 40 minutes when exposed to 100 µL eugenol/L (nominal). The time required for anaesthetic induction stage 2 (complete loss of equilibrium) for small shrimps is approximately 40 minutes at eugenol concentration of 100 µL eugenol/L. Above this concentration the induction time to reach stage 2 (complete loss of equilibrium) for small shrimps is approximately 20 minutes or lower. For medium shrimps after an exposure period of 20-60 minutes reached Stage I (partial loss of equilibrium) at concentration 100 µL eugenol/L. The time required for anaesthetic induction stage 2 (complete loss of equilibrium) at concentration above 100 µL eugenol/L is approximately 20 minutes. For large shrimps after an exposure period of approximately 90 minutes reached Stage I at concentration ≥ 100 µL eugenol/L. Above this concentration the induction time to reach stage I for large shrimps is 30 minutes at lower temperature and 20 minutes approximately at higher temperatures.

The induction and recovery times for anaesthesia were greater at low temperatures than at high temperatures. The time to induction stage 1 for all groups was less than 40 min, but caused higher mortality, especially at concentrations above 300 µL eugenol /L.

Shrimps at low temperature took a long time to reach anaesthesia stage 2 under 100 µL eugenol /L, and the interaction of temperature and size had significant effects on both induction and recovery times.

Similar to induction time, recovery time was less variable at 500 µL eugenol /L than at other concentrations, across all size classes and temperatures. For recovery stage 1, the recovery time of large shrimps increased linearly as concentration and temperature increased. At temperatures of 8 °C and 12 °C, recovery time to stage 2 required more than 2 h for all sizes of shrimp at all concentrations.

The survival rates of medium and large shrimps were above 80% over 5 days at all temperatures tested. However, the survival rates of smaller shrimps were below 60% at temperatures above 20 °C, over 5 days of recovery. At concentration of 300 eugenol µL/L, the survival rates of shrimps of all sizes at 16 °C and 20 °C were maintained above 70% over 5 days. At eugenol concentrations over 400 µL eugenol/L, the highest survival rates were achieved at 20 °C, 20 °C, and 24 °C for small, medium, and large shrimps, respectively.

Concentrations ranging from 100 to 500 µL/L were all found to be effective in immobilizing shrimps of different size classes and temperature conditions. Although induction times for *P. sinensis* were significantly shorter at higher concentrations, the safe dose of eugenol was found to be 200 µL eugenol/L, which yielded adequate anaesthesia, and ensured optimized survival over a 5-day recovery period. This indicates that this dose provides effective results with an appropriate margin of safety

## Assessment and conclusion

### Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported, no negative control included to check that any effects on mortality were not related to handling stress.

### **Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

At nominal concentrations of 100 – 300 µL eugenol/L, *P. sinensis* (shrimp) reached anaesthesia after approximately 20 to 30 minutes, and recovered after approximately 5 to 120 minutes. No mortality or other negative effects on the shrimp occurred in any of the eugenol treatments, up to 200 µL eugenol/L, during anaesthetic exposure (approximately 20 to 90 minutes) or in the 5 days afterwards.

<b>Data point:</b>	CA 9.6.3.4/32 [8.2.4.2]
<b>Report author</b>	Darbyshire, A.K., Oliver, K.H., Dupont, W.D., Plummer, W.D., Jones, C.P., Boyd, K.L.
<b>Report year</b>	2019
<b>Report title</b>	Anesthesia and Euthanasia of Brine Shrimp ( <i>Artemia franciscana</i> )
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 35 Journal of the American Association for Laboratory Animal Science volume 58, Pages 58-64.
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

#### Abstract (copied from original literature)

Invertebrates are often overlooked as laboratory animals, yet they are commonly used in toxicology, developmental, cellular and molecular biology, and radiation studies with euthanasia as an endpoint. Little is known regarding appropriate euthanasia methods for invertebrate species, particularly for *Artemia*. Here, we evaluated the AVMA-recommended 2-step method of euthanasia in brine shrimp (*Artemia franciscana*). *Artemia* were exposed first to anesthetic solutions of 60% alcohol, 2.5 mg/L eugenol, or 4 g/L tricaine methanesulfonate (TMS) and then were transferred to euthanasia solutions of 70% alcohol, 95% alcohol, or 10% neutral buffered formalin. We examined time to anesthesia, behavioral response to anesthesia, anesthesia recovery, and time to euthanasia. Our results show that 2.5 mg/L eugenol and 4 g/L TMS inconsistently achieved anesthesia. Although 60% alcohol produced anesthesia, the time to anesthesia varied among replicate groups, and exposure resulted in an increase in abnormal behavior. We therefore do not recommend any of the tested anesthetic solutions for use in *Artemia*. Although all 3 euthanasia solutions were effective, more research is needed to provide recommendations regarding euthanasia for this species.

#### Materials and methods

##### Test material 1

Name:	Eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	ACROS Organics, Morris, NJ USA/AC119110050
Active substance content:	99%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

##### Test organism

Species:	<i>Artemia franciscana</i> , brine shrimp
Strain/clone:	Not reported
Age at study initiation:	Adult
Weight/length/height at study initiation:	Not reported
Source:	The Aquatic Critter, Nashville, TN
Feeding during test:	No
Acclimation:	Acclimation period not reported. The <i>Artemia</i> were fed spirulina (Whole Foods, Nashville, TN). The conditions to maintain the culture were pH = 8.0, T = 25 °C, salinity: 1030

g/dL (40 ppt) and 12:12-h light:dark cycle

### *Test conditions*

Hardness:	Not reported (actual test conditions not reported)
Test temperature:	25 °C
pH:	Not reported (actual test conditions not reported)
Salinity:	Not reported (actual test conditions not reported)
Conductivity:	Not reported (actual test conditions not reported)
Photoperiod:	Not reported (actual test conditions not reported)
Light intensity:	Not reported (actual test conditions not reported)

### *Test system*

Study type:	Acute toxicity test (anaesthesia effects)
Duration of study:	2 hours (exposure period was 5 - 30 minutes; see further details below)
Treatments:	1.3 and 2.5 mg eugenol/L (nominal)
Analytical determination of test concentrations:	No
Negative control included:	Yes (tank water)
Positive control included:	No
Parameters measured:	Anesthesia (which was defined as a lack of forward motion and lack of response to a wooden probe); Mortality
Validity criteria:	Not reported

In an initial test, *Artemia* were individually exposed in wells of 24-well plates to nominal concentrations of 1.3 or 2.5 mg eugenol/L. The paper describes subsequent methods used to assess effects of 2.5 mg eugenol/L on i) the time to anesthesia; and ii) the ability to recover following a 5-minute exposure. However, further results for eugenol were excluded from the paper due to the inability to repeat anesthesia at this concentration (2.5 mg eugenol/L).

### **Results**

Time to anesthesia of *Artemia franciscana* decreased from 30 minutes at 1.3 mg/L eugenol (nominal) to approximately 5 minutes at 2.5 mg/L (nominal) with no obvious difference in behavior. 2.5 mg/L eugenol was therefore used as the nominal concentration for subsequent tests, but the paper reports that subsequent trials using eugenol over a 7-month period failed to reproduce reliable anesthesia and further results on time to reach anaesthesia and recovery ability were therefore not reported.

### **Assessment and conclusion**

#### *Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported. The paper itself highlights that the results for eugenol could not be reliably reproduced and full results were not reported.

#### **Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

*Artemia franciscana* (brine shrimp): No reproducible effects of eugenol as anesthesia.

<b>Data point:</b>	CA 9.6.3.4/33 [8.2.4.2]
<b>Report author</b>	Cansian, R. L., Vanin, A. B., Orlando, T., Piazza, S. P., Puton, B. M. S., Cardoso, R. I., Gonçalves, I. L., Honaiser, T.C., Paroul, N., Oliveira, D.
<b>Report year</b>	2017
<b>Report title</b>	Toxicity of clove essential oil and its ester eugenyl acetate against <i>Artemia salina</i>
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 351 Brazilian Journal of Biology Volume 77, Pages 155-161 (2017)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

#### Abstract (copied from original literature)

The production of compounds via enzymatic esterification has great scientific and technological interest due to the several inconveniences related to acid catalysis, mainly by these systems do not fit to the concept of “green chemistry”. Besides, natural products as clove oil present compounds with excellent biological potential. Bioactives compounds are often toxic at high doses. The evaluation of lethality in a less complex animal organism can be used to a monitoring simple and rapid, helping the identification of compounds with potential insecticide activity against larvae of insect vector of diseases. In this sense, the toxicity against *Artemia salina* of clove essential oil and its derivative eugenyl acetate obtained by enzymatic esterification using Novozym 435 as biocatalyst was evaluated. The conversion of eugenyl acetate synthesis was 95.6%. The results about the evaluation of toxicity against the micro-crustacean *Artemia salina* demonstrated that both oil ( $LC_{50}= 0.5993 \mu\text{g/mL}$ ) and ester ( $LC_{50}= 0.1178 \mu\text{g/mL}$ ) presented high toxic potential, being the eugenyl acetate almost 5 times more toxic than clove essential oil. The results reported here shows the potential of employing clove oil and eugenyl acetate in insecticide formulations.

#### Materials and methods

##### Test material 1

Name:	Clove oil
Formulation type:	Not relevant
Source and lot/batch no.:	Viafarma (São Paulo-Brazil)
Active substance content:	eugenol 85.5%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

##### Test organism

Species:	brine shrimp, <i>Artemia salina</i>
Strain/clone:	Not reported
Age at study initiation:	nauplii
Weight/length/height at study initiation:	Not reported
Source:	Not reported
Feeding during test:	Not reported
Acclimation:	The cysts of <i>Artemia salina</i> were placed in a plastic container with artificial saline solution (23 g of marine salt/1 liter of

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distilled/deionized water/0.7 g of sodium bicarbonate) with artificial illumination, under aeration, with control of temperature (20-30 °C) during 24 hours of incubation for the hatching

#### *Test conditions*

Hardness:	Not reported
Test temperature:	20 – 30 °C
pH:	Not reported
Dissolved oxygen:	Not reported
Salinity:	artificial saline solution
Conductivity:	Not reported
Photoperiod:	Not reported
Light intensity:	Not reported

#### *Test system*

Study type:	Acute toxicity test
Duration of study:	24 hours
Treatments:	0.10, 0.20, 0.30, 0.40, 0.50, 0.60, 0.70, 0.80, 0.90 and 10.0 µg clove oil/mL (nominal)
Analytical determination of test concentrations:	No
Negative control included:	Yes
Positive control included:	No
Parameters measured:	Mortality
Validity criteria:	Not reported

The organisms-test were exposed to different concentrations of both products to be tested (clove essential oil ) for 24 hours, using test tubes, each one containing at least 10 nauplii of *Artemia salina*, at 10 different concentration of products, in triplicate runs. In a first assay, the range of concentrations to be tested was determined (0.10, 0.20, 0.30, 0.40, 0.50, 0.60, 0.70, 0.80, 0.90 and 10.0 µg clove oil/mL). After 24 hours of exposure, the counting of alive and dead nauplii was carried out.

The LC<sub>50</sub> values were determined in triplicate employing non-linear regression model available in GraphPad Prism 6.0 software.

**Results**

*Artemia salina* (brine shrimp) 24-hour LC<sub>50</sub> value was reported to be 0.5993 µg clove oil/mL (based on nominal concentrations).

**Assessment and conclusion***Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported.

**Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

*Artemia salina* (brine shrimp) 24-hour LC<sub>50</sub> = 0.5993 µg clove oil/mL based on nominal concentrations

<b>Data point:</b>	CA 9.6.3.4/34 [8.2.8]
<b>Report author</b>	Guenette, S.A., Helie, P., Beaudry, F., Vachon, P.
<b>Report year</b>	2007
<b>Report title</b>	Eugenol for anesthesia of African clawed frogs ( <i>Xenopus laevis</i> )
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 166 Veterinary Anesthesia and Analgesia volume 34, Pages 164-170 (2007)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

**Abstract (copied from original literature)**

Objective: To determine the level of anesthesia attained in *Xenopus laevis* frogs with eugenol at different doses and by different routes of administration.

Study design: Prospective experimental trial.

Animals: Sixty *X. laevis* non breeding female frogs weighing between 90 and 140 g.

Methods: Three different routes of administration were tested – subcutaneous injections into the dorsal lymph sacs, topical administration using a gauze patch, and immersion in a bath containing eugenol. Following the determination of the best route of administration, the acetic acid test, the withdrawal reflex, righting reflex, heart rate, and respiratory frequency were used to evaluate central nervous system depression following eugenol bath administration. In an additional group, the response to a surgical incision of the abdominal wall was evaluated. The pharmacokinetics of eugenol were determined following bath immersion administration, and pharmacokinetic parameters were calculated following blood concentration determination by tandem liquid chromatography/mass spectrometry analyses.

Results: It was not possible to induce anesthesia with subcutaneous and patch administration, independent of the eugenol dose administered. The immersion bath was the only efficacious route for anesthesia inducing surgical anesthesia for at least 30 minutes with postoperative analgesia. Histopathology of selected tissues (heart, lung, liver, kidneys, and eyes) showed no evidence of lesions 24 hours following bath immersion. The elimination half-life (T<sub>1/2</sub>) was 4 hours.

Conclusion: When administered as a single-bath immersion (dose 350 mg/L) for 15 minutes, eugenol may serve as an effective anesthetic in *X. laevis* frogs for short surgical procedures.

**Materials and methods***Test material*

Name: Eugenol

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Formulation type:	Not relevant
Source and lot/batch no.:	Sigma Inc. (St Louis, MO, USA)
Active substance content:	99 %
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

#### *Test organism*

Species:	<i>Xenopus laevis</i> , African clawed frogs
Strain/clone:	Not relevant
Age at study initiation:	Non-breeding females
Weight at study initiation:	90-140 g
Source:	Xenopus I, Dexter, MI, USA
Feeding during test:	No
Acclimation:	Frogs were fed every other day with commercial blood worms (Bloodworms Tropical Fish Food, Hayward, CA, USA)

#### *Test conditions*

Hardness:	Not reported
Test temperature:	21 ± 2 °C
pH:	Not reported
Dissolved oxygen:	Not reported
Conductivity:	Not reported
Photoperiod:	Not reported
Light intensity:	Darkness for exposure in the immersion bath

#### *Test system*

Study type:	Acute toxicity test (anaesthesia effects)
Duration of study:	15 minutes of exposure for the immersion bath
Treatments:	350 mg eugenol/L (nominal)
Analytical determination of test concentrations:	No
Negative control included:	No
Positive control included:	No
Parameters measured:	Behaviour (reflex, muscle contraction, heart rate and respiration frequency).
Validity criteria:	Not reported

One group of nine frogs (three frogs/time point) was used to evaluate the surgical level of anesthesia following immersion in eugenol at 350 mg eugenol/L. To test for surgical anesthesia, skin and abdominal muscle incisions were performed at 15, 30, and 60 minutes following bath immersion. Muscle contraction, heart rate and respiration frequency were monitored. Statistical analysis was performed with SAS.

#### **Results**

Eugenol produces anesthesia in *Xenopus laevis* frogs when administered for 15 minutes by immersion at a concentration of 350 mg eugenol/L. Following this short exposure period, the behavioral tests revealed that the duration of analgesia inducing surgical anesthesia ranged from 15 to 30 minutes, and the mean recovery time for normal breathing frequency was 66 ± 40 minutes.

#### **Assessment and conclusion**

##### *Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported. No control reported.

**Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

At nominal concentrations of 350 mg eugenol/L, *Xenopus laevis* (African clawed frog) reached anesthesia after approximately 15 minutes, and mean recovery time for normal breathing frequency approximately 66 minutes. No mortality or adverse effects on the frog occurred at 350 mg eugenol/L during anaesthetic exposure of 15 minutes.

<b>Data point:</b>	CA 9.6.3.4/35 [8.2.8]
<b>Report author</b>	Goulet, F., Vachon, P., Hélie, P.
<b>Report year</b>	2011
<b>Report title</b>	Evaluation of the Toxicity of Eugenol at Anesthetic Doses in African Clawed Frogs ( <i>Xenopus laevis</i> )
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 178 Toxicologic Pathology volume 39, Pages 471-477 (2011)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

**Abstract (copied from original literature)**

Eugenol has been shown to induce anesthesia in African clawed frogs (*Xenopus laevis*). The toxicity of eugenol, administered at anesthetic doses, was evaluated in *Xenopus* frogs with an average body weight of  $28.2 \pm 13.7$  g. Frogs were immersed in 250 mL of an aqueous solution containing 350  $\mu$ L/L of eugenol for ten minutes and received a single administration (group 1, twelve animals) or three consecutive daily administrations (group 2, twelve animals). In each group, six frogs were scheduled to be euthanized the following day (subgroup A) and the other six were scheduled to be euthanized after a one-week recovery period (subgroup B). Morphologic changes consistent with renal tubular apoptosis affecting distal tubules in the medulla were observed in all subgroup A animals, ranging from mild to moderate in group 1, and from mild to severe in group 2. In subgroup B, renal tubular regeneration was present in all but one animal examined. These findings suggest that eugenol toxicity in amphibians is first manifested by renal tubular apoptosis. Other eugenol-related lesions were massive hepatic necrosis in group 2 (n = 6), hyaline membranes in the lung (n = 5), and adipose tissue hemorrhages in group/subgroup 2B (n = 4).

**Materials and methods***Test materia*

Name:	eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma-Aldrich St. Louis, MO, USA
Active substance content:	99%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

*Test organism*

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Species:	African Clawed Frogs, <i>Xenopus laevis</i>
Strain/clone:	Not relevant
Age at study initiation:	Medium-sized female African clawed frogs
Weight at study initiation:	28.2 ± 13.7 g
Source:	Xenopus 1; Dexter, MI, USA
Feeding during test:	No
Acclimation:	Animals were fed every other day with commercial <i>Xenopus</i> food (Xenopus Express, Brooksville, FL, USA).

### *Test conditions*

Hardness:	no hardness (normal values 70 to 150 mg/mL)
Test temperature:	21 ± 2 °C
pH:	6.8 – 7.3
Dissolved oxygen:	Not reported
Conductivity:	Not reported
Photoperiod:	Darkness during exposure
Light intensity:	Not reported
<i>Test system</i>	
Study type:	Acute toxicity test
Duration of study:	10 minutes of exposure in bath immersion, observations after 7 days
Treatments:	350 µL eugenol/L
Analytical determination of test concentrations:	No
Negative control included:	Yes
Positive control included:	No
Parameters measured:	Mortality Behaviour (clinical observation)
Validity criteria:	Not reported

Two groups of twelve frogs each were anesthetized with eugenol by bath immersion. Six frogs kept in water from the same source were used as controls. In group 1, animals received a single administration of eugenol, whereas in group 2, animals received three consecutive daily administrations of eugenol. Each group was further subdivided as follows: in subgroup A, animals were scheduled to be euthanized twenty-four hours after the last eugenol bath, whereas in subgroup B, animals were scheduled to be euthanized after a one-week recovery period. Six control animals were used; two were euthanized every week, starting on day 1 of the experimental period. After a ten-minute immersion period, the frog was removed from the solution, thoroughly rinsed with purified water, and placed in a water pan for the recovery period.

### **Results**

Group 1 animals (single administration) were clinically normal and exhibited normal behavior throughout the study. The only observed side effect related to eugenol anesthesia was vomiting in two of the twelve animals during the anesthetic recovery phase of 24 hours. One frog in group 1B (single administration, one-week recovery) was found dead in its aquarium on the fourth day following eugenol immersion.

In group 2 (three consecutive daily administrations), frogs showed depression, were reluctant to move, and swam less vigorously than control animals from day 3 up to the end of the experimental period. In group 2A (three administrations, no recovery), one animal was found dead on the scheduled date of euthanasia (day 2). In group 2B (three administrations, one-week recovery), two animals were found dead on the scheduled date of euthanasia (day 8). All control frogs remained clinically normal throughout the experimental period.

### **Assessment and conclusion**

#### *Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported.

**Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

At nominal concentrations of 350 µL eugenol/L, *Xenopus laevis* (African Clawed Frog) reached anesthesia after approximately 10 minutes. No mortality on the frog occurred at 350 µL eugenol/L during anaesthetic exposure of 10 minutes with small effects after 24 hours of recovery.

<b>Data point:</b>	CA 9.6.3.4/36 [8.3.2]
<b>Report author</b>	Toledo, P.F.S.; Jumbo, L.O.V.; Rezende, S.M.; Haddi, K.; Silva, B.A.; Mello, T.S.; Della Lucia, T.M.C.; Aguiar, R.W.S.; Smaghe, G.; Oliveira, E.E.
<b>Report year</b>	2020
<b>Report title</b>	Disentangling the ecotoxicological selectivity of clove essential oil against aphids and non-target ladybeetles
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 119 Science of the Total Environment, Volume 718, 137328 Pages 1-12 (2020)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	Supporting information only

**Abstract (copied from original literature)**

The plant-based biopesticides have been proposed as insect pest control tools that seem to be safer for the environment and human health when compared to synthetic conventional molecules. However, such assumptions are generally made without considering the absence of detrimental effects on sublethally-exposed non-target organisms or showing the physiological basis of the selective action of such botanical products. Thus, by using in silico-based and in vivo toxicological approaches, the present investigation aimed to disentangle the ecotoxicological selectivity of clove, *Syzygium aromaticum*, essential oil against the aphid *Rhopalosiphum maidis* and the non-target ladybeetle, *Coleomegilla maculata*. We also investigated whether the sublethal exposure to clove essential oil would affect the locomotory and predatory abilities of *C. maculata*. We found that the clove essential oil concentration estimated to kill 95% (LC<sub>95</sub>: 0.17 µL/cm<sup>2</sup>) of the aphids was lethal to <18% of *C. maculata*. Indeed, our in silico results reinforced such differential susceptibility, as it predicted that eugenol and β-caryophyllene (i.e., the clove essential oil major components) bound to three potential molecular targets (i.e., transient receptor potential (TRP) channels, octopamine, and gamma-aminobutyric acid (GABA) receptors) of the aphids but only to the octopamine receptors of the ladybeetles. Additionally, the ladybeetles that were exposure to the clove essential oil exhibited unaffected abilities to locomote and to prey upon *R. maidis* aphids when compared to unexposed ladybeetles. Thus, by displaying lower toxicity against the ladybeetles, the clove essential oil represents a safer alternative tool to be integrated into programs aiming to manage aphid infestations.

**Materials and methods***Test material 1*

Name: Clove oil

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Formulation type:	Not relevant
Source and lot/batch no.:	Extracted by steam distillation from clove buds following a method described in a separate literature reference (Viteri Jumbo <i>et al.</i> 2014). Batch no. not reported.
Active substance content:	87.4% eugenol; 11.5% $\beta$ -caryophyllene
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

#### *Test organism*

Species:	Ladybeetle ( <i>Coleomegilla maculata</i> )
Strain/clone:	Not reported
Age at study initiation:	adults
Weight/length/height at study initiation:	Not reported
Source:	adults collected from a maize field at the Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais, Brazil, research experimental unit and reared under laboratory conditions.
Feeding during test:	No
Acclimation:	ladybeetles were fed with a combination of <i>Ephestia kuehniella</i> Zeller eggs and a mixture of yeast with <i>Apis mellifera</i> L. honey (1:1). $25 \pm 2$ °C, relative humidity: $65 \pm 5$ %, and photoperiod 12 hours light/12 hours darkness

Note, the study also assessed effects on the aphid species, *Rhopalosiphum maidis*, but this aspect of the study is not discussed further in this summary as effects on *R. maidis* (a target pest) are not relevant for the ecotoxicological assessment of non-target organisms.

#### *Test conditions*

Test temperature:	$25 \pm 2$ °C
Photoperiod:	12 hours light/12 hours darkness
Light intensity:	Not reported
<i>Test system</i>	
Study type:	1) Acute toxicity test (on impregnated filter papers) 2) Locomotor bioassay (on treated Petri dish) 3) Predatory bioassay (on treated Petri dish)
Duration of study:	1) Acute toxicity: 12 hours 2) Locomotor (walking activity) – not clear but understood that adults exposed and assessed for a 10 minute period 3) Predatory bioassay – exposed for 12 hours, then held for 12 hours without treatment or prey, then assessed upto 24 hours after offered prey (aphids) for next four consecutive days
Treatments:	control, 0.17 $\mu$ L clove oil/cm <sup>2</sup> (nominal)
Analytical determination of test concentrations:	No
Negative control included:	Yes
Positive control included:	No
Parameters measured:	1) Mortality 2) Walking activity 3) Predatory activity (consumption of prey)
Validity criteria:	Not reported

## Results

12-hour exposure to dry residues of clove oil (containing 87.4% eugenol; 11.5%  $\beta$ -caryophyllene) applied to filter paper at a rate of 0.17  $\mu\text{L}$  clove oil/ $\text{cm}^2$  caused less than 18% mortality to adult ladybeetles (*Coleomegilla maculata*). No significant effects on locomotory behaviour (walking distance, time or average speed) or predatory activity (daily number of aphid prey consumed) of *C. maculata* were observed at an application rate of 0.17  $\mu\text{L}$  clove oil/ $\text{cm}^2$  (nominal) compared to untreated controls.

## Assessment and conclusion

### Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 2 reliable with restrictions.

No positive control, full details of test item extraction not included in this paper and test item was clove oil (but contains high % of eugenol (87.4%) so may be useful as supporting information).

## Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).

12-hour exposure to dry residues of clove oil (containing 87.4% eugenol; 11.5%  $\beta$ -caryophyllene) applied to filter paper at a rate of 0.17  $\mu\text{L}$  clove oil/ $\text{cm}^2$  caused less than 18% mortality to adult ladybeetles (*Coleomegilla maculata*). No significant effects on locomotory behaviour (walking distance, time or average speed) or predatory activity (daily number of aphid prey consumed) of *C. maculata* were observed at an application rate of 0.17  $\mu\text{L}$  clove oil/ $\text{cm}^2$  (nominal) compared to untreated controls.

<b>Data point:</b>	CA 9.6.3.4/37 [8.6]
<b>Report author</b>	Xuan, T.D., Toyama, T., Fukuta, M., Khanh, T.D., Tawata, S.
<b>Report year</b>	2009
<b>Report title</b>	Chemical Interaction in the Invasiveness of Cogongrass ( <i>Imperata cylindrica</i> (L.) Beauv.)
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 77 Journal of Agricultural and Food Chemistry Volume 57, Pages 9448-9453 (2009 Oct)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

## Abstract (copied from original literature)

From gas chromatography-mass spectrometry (GC-MS), numerous plant growth inhibitors were found in the rhizome and root exudates of cogongrass, one of the most problematic weeds in the world. iso-Eugenol, iso-ferulic acid, linoleic acid, ferulic acid, and vanillin were the major chemicals in the rhizome (88.1-392.2  $\mu\text{g/g}$  of fresh root), while 4-acetyl-2-methoxyphenol was the principle substance (872.6  $\mu\text{g/plant}$ ) in the root exudates. In fields, the use of cutting and plowing reduced weed biomass and weed density of cogongrass >70%. However, the alternative invasion of beggar tick might be a problem, because its density and biomass increased 33.3 and 62.5%, respectively. Chemicals from cogongrass showed selective effects against tested invasive species. Of them, 2,4-di-tert-butylphenol was the most potent (78.3-100% of inhibition), followed by iso-eugenol and 4-acetyl-2-methoxyphenol. These compounds may play important roles in the invasiveness of cogongrass and might be promising parent constituents of synthesis to develop novel herbicides for control of invasive plants.

## Materials and methods

### Test material

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Name: iso-Eugenol  
Formulation type: Not relevant  
Source and lot/batch no.: Purchased from Wako Pure Chemical Industries, Japan.  
Active substance content: Not reported  
Expiry date of lot/batch: Not reported  
Storage conditions: Not reported

*Test organism 1*

Species: Beggar tick (*Bidens pilosa* L.)  
Strain/clone: Not reported  
Age at study initiation: Seed (after germination)  
Weight/length/height at study initiation: Not reported  
Source: The seeds were harvested in the fields of the Agricultural Farm, University of the Ryukyus, Japan, in 2007.  
Feeding during test: Not relevant  
Acclimation: Not relevant

*Test organism 2*

Species: Barnyardgrass (*E. crus-galli*)  
Strain/clone: Not reported  
Age at study initiation: Seed (after germination)  
Weight/length/height at study initiation: Not reported  
Source: Collected in the field of Miyazaki Prefecture, Japan, in 2006.  
Feeding during test: Not relevant  
Acclimation: Not relevant

*Test organism 3*

Species: Leucaena (*L. leucocaphala*)  
Strain/clone: Not reported  
Age at study initiation: Seed (after germination)  
Weight/length/height at study initiation: Not reported  
Source: The seeds were harvested in the fields of the Agricultural Farm, University of the Ryukyus, Japan, in 2007.  
Feeding during test: Not relevant  
Acclimation: Not relevant

*Test organism 4*

Species: Cogongrass (*I. cylindrica*)  
Strain/clone: Not reported  
Age at study initiation: Seed (after germination)  
Weight/length/height at study initiation: Not reported  
Source: The seeds were harvested in the fields of the Agricultural Farm, University of the Ryukyus, Japan, in 2007.

Feeding during test: Not relevant  
Acclimation: Not relevant

### *Test conditions*

Test temperature: 25°C  
Photoperiod: Dark during the test  
Light intensity: Not reported

### *Test system*

Study type: Effects on plant growth when seeds exposed to impregnated filter paper in a Petri dish  
Duration of study: 5 days  
Treatments: 0.1 mg iso-Eugenol/mL (nominal), applied at a volume of 10 mL to a Petri dish lined with filter paper  
Analytical determination of test concentrations: No  
Negative control included: Yes (distilled water)  
Positive control included: No  
Parameters measured: Growth (elongation of the shoot and root)  
Validity criteria: Not reported

Before testing, the seeds were air-dried and hermetically stored at -25 °C for one month. These seeds were sterilized with 1% sodium hypochlorite for 30 minutes and well rinsed several times with distilled water before use. The harvested spikelets of *I. cylindrica* were kept at -25 °C for 1 month, then spread on the surface of a 0.5% potato dextrose agar (PDA) box, and kept in the dark at 25 °C, humidity at 70%, at 2-3 days for germination stimulation. The germination percentages of these invasive species were randomly checked and showed over 90%.

An aliquot of 10 mL of the test solution (0.1 mg iso-eugenol/mL) was put in a Petri dish (9 cm in diameter), lined with filter paper, and sowed with 10 seeds. The plates were transferred to an incubator (25 °C) placed in the dark. Treatments with distilled water only were used as the controls. After 5 days, shoot and root lengths were measured. The inhibitory activity was expressed as the average of the suppressive magnitude on elongation of the shoot and root of the tested species. This experiment was repeated twice.

## **Results**

When seeds were placed on filter papers impregnated with test solutions in a Petri dish (10 mL of nominally 0.1 mg iso-eugenol/mL), after 5 days the mean inhibition on the growth of root and shoot length was 76.3, 70.4, 44.2 and 99.3 for beggar tick (*B. pilosa*), cogongrass (*I. cylindrica*), leucaena (*L. leucocaphala*) and barnyardgrass (*E. crus-galli*), respectively.

## **Assessment and conclusion**

### *Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Test item purity not reported and analytical verification of test concentrations was not reported. Not clear if effects are reported in terms of % inhibition to control or otherwise. Study is non-standard so of limited reliability for a quantitative risk assessment and no positive control to confirm sensitivity of the test.

**Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

When seeds were placed on filter papers impregnated with test solutions in a Petri dish (10 mL of nominally 0.1 mg iso-eugenol/mL), after 5 days the mean inhibition on the growth of root and shoot length was 76.3, 70.4, 44.2 and 99.3 for beggar tick (*B. pilosa*), cogongrass (*I. cylindrica*), leucaena (*L. leucocaphala*) and barnyardgrass (*E. crus-galli*), respectively.

<b>Data point:</b>	CA 9.6.3.4/38 [8.6]
<b>Report author</b>	Darabi, H.R., Mohandessi, S., Balavar, Y., Moghaddam M.M., Aghapoor, K., Mohsenzadeh, F., Nourinia, A.A.
<b>Report year</b>	2011
<b>Report title</b>	Clove bud oil: an efficient, economical and widely available oil for the inhibition of wheat seed germination
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 81 Environmental Chemistry Letters : Official Journal of the European Association of Chemistry and the Environment Volume 9, Issue 4, Pages 519-524 (2011)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

**Abstract (copied from original literature)**

Pre-harvest sprouting refers to the precocious germination of the grain in the spike prior to harvest as a result of moist weather conditions at harvest time. From the agricultural viewpoint, it is necessary to impose an exogenous dormancy to wheat seeds in order to improve the resistance of seed to pre-harvest sprouting. In this regard, we found that clove bud essential oil is a strong inhibitor for wheat seed germination. The extract obtained from clove bud by supercritical fluid extraction using CO<sub>2</sub> as solvent minimized the number of extracts to two compounds, eugenol and eugenyl acetate. Eugenol, as the main constituent of the oil, was responsible for its strong inhibitory activity in wheat seeds. The aqueous solution of clove bud oil was submitted to germination assay at various concentrations from 50 to 400 mg/L. Complete inhibition of seed germination was recorded when the concentration was 400 mg/L. Roots and sprouts have similar sensitivity to inhibitory effect. In an empirical study, the synergistic cooperation of eugenol and eugenyl acetate from clove bud oil in the inhibition of seed germination was found to be a 1:1 ratio. The clove bud essential oil is widely available and will broaden the horizon of applications for natural and safe inhibitors in the fields.

**Materials and methods***Test material*

Name:	Eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	From commercial source, but no further details given
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

*Test organism*

Species:	One variety of non-dormant sprouting-susceptible wheat cultivar, <i>Shiroodi</i> .
Strain/clone:	Not reported
Age at study initiation:	Seed stage
Weight/length/height at study initiation:	Not reported
Source:	From 2006 harvest in Golestan County in Iran.
Feeding during test:	Not relevant
Acclimation:	Not reported

### *Test conditions*

Test temperature:	20°C
Photoperiod:	Continuous lighting
Light intensity:	15-W fluorescent lamp
<i>Test system</i>	
Study type:	Germination inhibitory effect of eugenol on wheat seeds (grown in Petri dishes and exposed to the test item solution in the bottom of the dish (no growth medium))
Duration of study:	4 days
Treatments:	7 mL of test solutions containing 50, 100, 200, 300 and 400 mg eugenol/L (nominal)
Analytical determination of test concentrations:	No
Negative control included:	Yes (distilled water)
Positive control included:	No
Parameters measured:	Inhibition of germination. The germinated seeds (2–3-mm root and hypocotyls lengths) were counted daily for 4 days, and germination assays were stopped when no new seed germinated for 5 days consecutively.
Validity criteria:	Not reported

### **Results**

When seeds were exposed to 7 mL test solution in Petri dishes, eugenol inhibited the germination of wheat seeds completely at 400 mg eugenol/L (nominal). Germination was inhibited in a concentration-response dependent manner, with approximately 90%, 80%, 70% and 10% germination observed at 50, 100, 200 and 300 mg eugenol/L (nominal), respectively (approximate values based on Fig. 5 of paper).

### **Assessment and conclusion**

#### *Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported. There is no value for the purity of the active. % germination in the negative control was not reported for comparison.

### **Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

When seeds were exposed to 7 mL test solution in Petri dishes, eugenol inhibited the germination of wheat seeds completely at 400 mg eugenol/L (nominal). Germination was inhibited in a concentration-response dependent manner, with approximately 90%, 80%, 70% and 10% germination observed at 50, 100, 200 and 300 mg eugenol/L (nominal), respectively (approximate values based on Fig. 5 of paper).

<b>Data point:</b>	CA 9.6.3.4/39 [8.6]
<b>Report author</b>	Ahuja, N., Batish, D.R., Singh, H. P., Kohli, R. K.
<b>Report year</b>	2015
<b>Report title</b>	Herbicidal activity of eugenol towards some grassy and broad-leaved weeds
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 204 Journal of pest science volume 88, Pages 209-218 (2015)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	Supporting information only

#### Abstract (copied from original literature)

The present study investigated the phytotoxic potential of eugenol, a major component from the essential oil of clove [*Syzygium aromaticum* (L.) Merrill and Perry], towards four grassy [*Echinochloa crus-galli* (L.) Beauv., *Phalaris minor* Retz., *Sorghum halepense* (L.) Pers. and *Leptochloa chinensis* (L.) Nees] and four broad-leaved [*Ageratum conyzoides* L., *Commelina benghalensis* L., *Cassia occidentalis* L. and *Bidens pilosa* L.] weeds. The effect of eugenol (50–1000 µM) on the growth and development of seedlings after 7 days of treatment was studied in terms of percent germination, root and shoot length, total chlorophyll content and cellular respiration. Eugenol at 1000 µM caused 55–70 and 42–90% decrease in percent germination in grassy and broad-leaved weeds, respectively. Likewise, root length declined by 55–90 and 57–71%, whereas shoot length was decreased by 50–83 and 36–73% in grassy and broad-leaved weeds, respectively, in response to 1000 µM eugenol. The observed reduction in the plant growth was accompanied by a decline in the total chlorophyll content (37–53%) and cellular respiration (36–57%) in the test plants. However, the inhibitory effect was stronger towards grassy weeds than towards the broad-leaved ones. Thus, future research can be focused on developing eugenol, a natural plant product, as an environmentally safe herbicide in replacement to the harmful chemical herbicides.

#### Materials and methods

##### Test material

Name:	eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Alfa Aesar, Lancashire, UK,
Active substance content:	98%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

##### Test organism

Species:	Seeds of <i>P. minor</i> , <i>E. crus-galli</i> , <i>A. conyzoides</i> , <i>L. chinensis</i> , <i>C. benghalensis</i> , <i>S. halepense</i> , <i>C. occidentalis</i> and <i>B. pilosa</i>
Strain/clone:	Not reported
Age at study initiation:	Not relevant
Weight/length/height at study initiation:	Not relevant
Source:	Seeds were collected locally from plants growing wildly in agricultural fields around Chandigarh, India
Feeding during test:	No

Acclimation: Before use, *P. minor*, *E. crus-galli*, *S. halepense*, *L. chinensis*, *C. benghalensis* and *B. pilosa* seeds were preimbibed in water for 24 h and *A. conyzoides* for 48 h, and *C. occidentalis* seeds were scarified with sulphuric acid and imbibed overnight in water.

### Test conditions

Test temperature: 25 ± 2 °C (except 15 ± 2°C in case of *P. minor*)  
 Photoperiod: 16h/8h light/dark  
 Light intensity: 240 µmol photons/m<sup>2</sup> s  
*Test system*  
 Study type: Phytotoxicity test  
 Duration of study: 7 days  
 Treatments: 50, 100, 250, 500 and 1000 µM eugenol (nominal)  
 Analytical determination of test concentrations: No  
 Negative control included: Yes, distilled water with ~ 0.1% of Tween-20  
 Positive control included: No  
 Parameters measured: Seedling emergence (number of seeds germinated) and growth (root and shoot length)  
 Validity criteria: Not reported

The solutions of eugenol were prepared using Tween-20 (final concentration ~ 0.1%) as a surfactant. Eugenol in the concentrations of 50, 100, 250, 500 and 1000 µM was evaluated for phytotoxicity against the selected weed species in a dose– response manner under laboratory conditions. Distilled water with ~ 0.1% of Tween-20 was used as a control.

The pre-imbibed weed seeds (15 of *C. occidentalis*, 20 each of *P. minor*, *E. crus-galli* and *S. halepense*, 25 each of *L. chinensis*, *C. benghalensis* and *A. conyzoides*) were placed in Petri dishes (15 cm in diameter) lined with a thin layer of cotton wad and Whatman #1 filter paper. Each Petri dish was moistened with 12 mL of respective eugenol solution or distilled water. The Petri dishes were then sealed with Cellotape<sup>®</sup> to avoid loss of the eugenol due to volatilization.

Statistical analyses were performed using SPSS software version 16.0.

### Results

Eugenol (≥50 µM) caused a significant reduction in the emergence of both grassy and broadleaved weed species, except in *S. halepense*, *E. crus-galli* and *B. pilosa* (reduction significant at ≥100 µM) and *C. occidentalis* (reduction significant at ≥250 µM). At 1000 µM eugenol, nearly 75, 69, 62 and 55% reduction in emergence was observed in *P. minor*, *S. halepense*, *L. chinensis* and *E. crus-galli*, respectively. In case of broad-leaved weeds, a reduction of nearly 89, 61, 60 and 42% was observed in *C. benghalensis*, *B. pilosa*, *A. conyzoides* and *C. occidentalis*, respectively, at 1000 µM eugenol.

Eugenol significantly inhibited the root growth in the grassy weeds, *E. crus-galli*, *L. chinensis* and *P. minor*, by nearly 13–89, 12–72 and 18–81%, respectively, over that of control at a concentration range from 50 to 1000 µM. Likewise, the root length of broad-leaved species also declined by 11–63, 7–57 and 11–69% in *A. conyzoides*, *C. occidentalis* and *B. pilosa* (significant at ≥100 µM eugenol), and by 25–71% in *C. benghalensis* (significant at ≥50 µM eugenol), respectively, in response to 50 to 1000 µM of eugenol.

The maximum decline in the shoot length was observed in case of *P. minor* and *C. benghalensis* ranging from ~16–83 and 20–73% (significant at ≥50 µM eugenol) at 50–1000 µM, respectively. *C. occidentalis*, *A. conyzoides* (broad-leaved) and *S. halepense* (grassy) started at a lower level, but they declined as well (significant at ≥100 µM eugenol), registering a decline of ~11–36, 19–54 and 9–50%, respectively, in response to 50–1000 µM eugenol.

In general, grassy weeds were more sensitive towards eugenol. *E. crus-galli* and *P. minor* were the most sensitive to eugenol when a comparison between grassy weeds was done. In broad-leaved weeds, *C. benghalensis* was the most sensitive followed by *B. pilosa*, *A. conyzoides* and *C. occidentalis*. However, a stronger inhibition in the root growth was observed as compared to the shoot growth in all the test weeds.

### Assessment and conclusion

#### Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 2 reliable with restrictions

No standard study design assessing effects on plants only in Petri dishes. No positive control, no analytical verification.

**Assessment and conclusion by applicant:**

The study is considered as supporting information only (reliable with restrictions).

Eugenol at 1000 µM caused 55–70 and 42–90% decrease in percent germination in grassy and broad-leaved weeds, respectively. Likewise, root length declined by 55–90 and 57–71%, whereas shoot length was decreased by 50–83 and 36–73% in grassy and broad-leaved weeds, respectively, in response to 1000 µM eugenol. Grassy plants are more sensitive than broad-leaved ones.

<b>Data point:</b>	CA 9.6.3.4/40 [CA 8.6]
<b>Report author</b>	Waliwitiya, R., Isman, M. B., Vernon, R.S., Riseman, A.
<b>Report year</b>	2020
<b>Report title</b>	Insecticidal Activity of Selected Monoterpenoids and Rosemary Oil to <i>Agriotes obscurus</i> (Coleoptera: Elateridae)
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 218 Journal of Economic Entomology volume 98, Pages 1560-1665 (2020)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	Supporting information only

**Abstract (copied from original literature)**

Acute toxicities of three naturally occurring monoterpenoid essential oil constituents and the essential oil of rosemary were tested against late instars of *Agriotes obscurus* (L.) (Coleoptera: Elateridae). Both contact and volatile toxicities of thymol, citronellal, eugenol, and rosemary oil were determined. Also, phytotoxicity of these compounds was evaluated on corn germination and seedling development. Thymol had the greatest contact toxicity ( $LD_{50} = 196.0 \mu\text{g/larva}$ ), whereas citronellal and eugenol were less toxic ( $LD_{50} = 404.9$  and  $516.5 \mu\text{g/larva}$ , respectively). Rosemary oil did not show any significant contact toxicity, even at  $1600 \mu\text{g/larva}$ . In terms of volatile toxicity, citronellal was the most toxic to wireworm larvae ( $LC_{50} = 6.3 \mu\text{g/cm}^3$ ) followed by rosemary oil ( $LC_{50} = 15.9 \mu\text{g/cm}^3$ ), thymol ( $LC_{50} = 17.1 \mu\text{g/cm}^3$ ), and eugenol ( $LC_{50} = 20.9 \mu\text{g/cm}^3$ ). Thymol, eugenol, and citronellal significantly inhibited corn seed germination and development, whereas rosemary oil had only minimal phytotoxic effects.

**Materials and methods***Test material 1*

Name:	thymol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma, St. Louis, MO
Active substance content:	> 98%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

*Test material 2*

Name:	eugenol
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Formulation type:	Not relevant
Source and lot/batch no.:	Arylessence. Inc., Marietta, GA
Active substance content:	> 95%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

### *Test organism*

Species:	Corn seed
Strain/clone:	Pioneer Brand Hybrid #39T68
Age at study initiation:	Not relevant
Weight/length/height at study initiation:	Not relevant
Source:	Pioneer Hi-Bred Ltd
Feeding during test:	Not relevant
Acclimation:	Corn seed were presoaked in water for 24 hours.

Note, study also tested effects on the beetle larvae, *Agriotes obscurus*, but this not discussed further in this summary as it's a target pest species so not relevant to the ecotoxicological assessment of non-target organisms.

### *Test conditions*

Test temperature:	22 °C
Photoperiod:	Not reported
Light intensity:	Not reported
<i>Test system</i>	
Study type:	Phytotoxicity test
Duration of study:	6 days
Treatments:	200, 400, 800, 1,600, and 3,200 µg eugenol and thymol/seed (nominal)
Analytical determination of test concentrations:	No
Negative control included:	Yes (methanol)
Positive control included:	No
Parameters measured:	Germination percentage, cotyledon length, and radicle length
Validity criteria:	Not reported

Corn seeds were coated with 20 µL of the test solutions using a micropipettor. 10 corn seeds were exposed to concentrations of 200, 400, 800, 1600, and 3200 µg eugenol and thymol/seed with three complete replications. Plastic pots (14 cm in diameter) with cleaned sand were used to test the phytotoxicity of the compounds, eugenol and thymol to corn. After treating seeds, they were planted 2.5 cm in depth in the sand. Pure methanol was used as the control treatment. ANOVA procedures were performed by SPSS software.

### **Results**

Differences in corn seed germination percentage, cotyledon length, and radicle length were observed among the treatments ( $P > 0.005$ ) and concentrations ( $P > 0.0001$ ). Thymol and eugenol inhibited germination and reduced cotyledon and radical growth as concentration increased. Thymol and eugenol, applied to corn seed at 400 µg/seed, caused no phytotoxicity.

The seed germination was reduced by 3%, 3%, 30%, 23% and 80% when exposed at 200, 400, 800, 1600 and 3200 µg thymol/seed, respectively. The cotyledon length was reduced by about 11% and 56% when exposed respectively to 1600 and 3200 µg thymol/seed. The radicle length was reduced by about 18% and 63% when exposed respectively to 1600 and 3200 µg thymol/seed.

The seed germination was reduced by 0%, 3%, 10%, 10% and 43% when exposed at 200, 400, 800, 1600 and 3200 µg eugenol/seed, respectively. The cotyledon length was reduced by about 13% and 24% when exposed

respectively to 1600 and 3200 µg eugenol/seed. The radicle length was reduced by about 11% and 25% when exposed respectively to 1600 and 3200 µg eugenol/seed.

Thymol and eugenol, applied to corn seed at 400 µg/seed, caused no phytotoxicity.

Thymol, applied to corn seed at 3200 µg/seed, causes a reduction in germination of 80% and in growth (more than 50% inhibition on cotyledon and radicle lengths).

Eugenol applied to corn seed at 3200 µg/seed, causes a reduction of 43% in germination and in growth (about 25% inhibition on cotyledon and radicle lengths).

#### Assessment and conclusion

##### Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 2 reliable with restrictions

No analytical verification of test concentrations.

#### Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).

Corn seed. No effects on seed germination at concentrations of 400 µg thymol/seed.

Corn seed. No effects on seed germination at concentrations of 400 µg eugenol/seed.

<b>Data point:</b>	CA 9.6.3.4/41 [8.6]
<b>Report author</b>	Kalinova , J., Triska, J., Vrchotova, N.
<b>Report year</b>	2011
<b>Report title</b>	Occurrence of eugenol, coniferyl alcohol and 3,4,5-trimethoxyphenol in common buckwheat( <i>Fagopyrum esculentum</i> Moench) and their biological activity
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 254 Acta Physiol Plant, Volume 33, Pages 1679–1685 (2011)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	Supporting information only

#### Abstract (copied from original literature)

Common buckwheat (*Fagopyrum esculentum* Moench) is well known as a weed suppressing crop due to its strong competitive and allelopathic characteristics. The possible allelopathic compounds in buckwheat include compounds from different groups, such as flavonoids, fatty acids, phenolic acids, etc. Less attention has been paid to other phenolic compounds, specifically eugenol, *o*-eugenol, coniferyl alcohol and 3,4,5-trimethoxyphenol as possible allelochemicals. The effects of eugenol, *o*-eugenol, coniferyl alcohol, and 3,4,5-trimethoxyphenol on germination and plant growth were tested on seven plant species. The results of our study showed that eugenol, coniferyl alcohol, or 3,4,5-trimethoxyphenol are minority components of the buckwheat plant. Eugenol reached the highest concentration (1.16 µg/g DW in buckwheat leaves) from these compounds and they probably do not have a significant function in the allelopathy of common buckwheat. However, due to the inhibitory effects on germination and plant growth, eugenol could be utilized in the plant protection of sustainable agriculture.

#### Materials and methods

##### Test material

Name: Eugenol  
 Formulation type: Not relevant  
 Source and lot/batch no.: Sigma-Aldrich

Active substance content: Not reported  
 Expiry date of lot/batch: Not reported  
 Storage conditions: Not reported

### Test organism

Species: yarrow (*Achilea millefolium* L.), ribwort plantain (*Plantago lanceolata* L.), Dutch clover (*Trifolium repens* L.), perennial ryegrass (*Lolium perenne* L.), barnyardgrass (*E. crus-galli* (L) P.B.), and redroot pigweed (*Amaranthus retroflexus* L. AMARE). White mustard (*Sinapis alba* L.) was used for the bioassays as a control test plant because this species is sensitive to phytotoxins.

Strain/clone: Not reported  
 Age at study initiation: Seeds  
 Weight/length/height at study initiation: Not reported  
 Source: Not reported  
 Feeding during test: Not relevant  
 Acclimation: Not reported

### Test conditions

Test temperature:  $22 \pm 2^\circ\text{C}$   
 Photoperiod: darkness  
 Light intensity: not relevant

*Test system*

Study type: Phytotoxicity test (germination test)  
 Duration of study: 3 days  
 Treatments: 1, 5, 10, 50, 100, 500, 1000, 5000, and 10000  $\mu\text{M}$  eugenol in water (nominal)

Analytical determination of test concentrations: No  
 Negative control included: Yes (methanol as solvent)  
 Positive control included: No  
 Parameters measured: growth (length of the roots and hypocotyl)  
 Validity criteria: Not reported

Different concentrations of the tested compounds were applied on filter paper. Thirty seeds of each species were sown on filter paper in Petri dishes (9 cm diameter). Every dish was enclosed with parafilm in order to reduce evaporation. There were at least four replicated plates for each tested concentration of the compounds. The tests were repeated in two different dates. The germination was evaluated at concentrations, 1, 10, 100, 1000, and 10000  $\mu\text{M}$  of eugenol in water.

The data were evaluated using the analysis of variance (ANOVA, software Statistica 6.0). The effective concentration required for 50% inhibition ( $\text{EC}_{50}$ ) was established based on the fitted regression equations.

### Results

The effects of the eugenol on selected plant species were similar. Eugenol reduced the growth of radicle more than the growth of hypocotyl. Dutch clover (*T. repens* L.) and redroot pigweed (*A. retroflexus* L.) were the most resistant, and perennial ryegrass (*L. perenne* L.) was the most sensitive.

Seed germination (% of control) of selected species following exposure:

Concentration $\mu\text{M}$ eugenol	Control ( <i>S.alba</i> )	<i>T. repens</i>	<i>E. crus-galli</i>	<i>L. perenne</i>	<i>A. milefolium</i>	<i>P. lanceolata</i>	<i>A. retroflexus</i>
1	114	99	113	125	106	98	100

10	106	83	100	100	101	88	90
100	91	87	89	42	71	71	82
1000	49	56	58	34	37	53	57
10000	0	0	0	0	0	0	0

Weed growth at different eugenol concentrations:

Weed	Part	EC <sub>50</sub> (µM eugenol)
<i>A. milefolium</i>	root	75
	hypocotyl	24
<i>E. crus-galli</i>	root	28
	hypocotyl	685
<i>L. perenne</i>	root	11
	hypocotyl	54
<i>P. lanceolata</i>	root	86
	hypocotyl	430
<i>A. retroflexus</i>	root	220
	hypocotyl	1067
<i>T. repens</i>	root	359
	hypocotyl	448

### Assessment and conclusion

#### Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 2 reliable with restrictions

No standard study design assessing effects on plants only in Petri dishes. No positive control, no analytical verification and test item purity not reported.

### Assessment and conclusion by applicant:

The study is considered as supporting information only (reliable with restrictions).

*A. milefolium* EC<sub>50</sub> (root) = 75 µM eugenol based on nominal concentrations

*A. milefolium* EC<sub>50</sub> (hypocotyl) = 24 µM eugenol based on nominal concentrations

*E. crus-galli* EC<sub>50</sub> (root) = 28 µM eugenol based on nominal concentrations

*E. crus-galli* EC<sub>50</sub> (hypocotyl) = 685 µM eugenol based on nominal concentrations

*L. perenne* EC<sub>50</sub> (root) = 11 µM eugenol based on nominal concentrations

*L. perenne* EC<sub>50</sub> (hypocotyl) = 54 µM eugenol based on nominal concentrations

*P. lanceolata* EC<sub>50</sub> (root) = 86 µM eugenol based on nominal concentrations

*P. lanceolata* EC<sub>50</sub> (hypocotyl) = 430 µM eugenol based on nominal concentrations

*A. retroflexus* EC<sub>50</sub> (root) = 220µM eugenol based on nominal concentrations

*A. retroflexus* EC<sub>50</sub> (hypocotyl) = 1067 µM eugenol based on nominal concentrations

*T. repens* EC<sub>50</sub> (root) = 359 µM eugenol based on nominal concentrations

*T. repens* EC<sub>50</sub> (hypocotyl) = 448 µM eugenol based on nominal concentrations

*Ecotoxicology: Methyl eugenol (4 literature papers)*

<b>Data point:</b>	CA 9.6.3.4/42 [CA 8.6]
<b>Report author</b>	Amri, I.; Mancini, E.; De Martino, L.; Marandino, A.; Lamia, H.; Mohsen, H.; Bassem, J., Scognamiglio, M., Reverchon, E. and De Feo, V.
<b>Report year</b>	2012
<b>Report title</b>	Chemical Composition and Biological Activities of the Essential Oils from Three <i>Melaleuca</i> Species Grown in Tunisia
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 73 International Journal of Molecular Sciences Volume 13, Pages 16580-16591 (2012)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	Supporting information only

#### Abstract (copied from original literature)

The chemical composition of the essential oils of *Melaleuca armillaris* Sm., *Melaleuca styphelioides* Sm. and *Melaleuca acuminata* F. Muell., collected in Tunisia, was studied by means of GC and GC-MS analysis. In all, 46 compounds were identified, 38 for *M. armillaris*, 20 for *M. acuminata* and eight for *M. styphelioides*, respectively. The presence of a sesquiterpenic fraction (52.2%) characterized the oil from *M. armillaris*; *M. styphelioides* oil was rich in methyl eugenol, a phenolic compound (91.1%), while *M. acuminata* oil is mainly constituted by oxygenated monoterpenoids (95.6%). The essential oils were evaluated for their *in vitro* potentially phytotoxic activity against germination and initial radicle growth of *Raphanus sativus* L., *Lepidium sativum* L., *Sinapis arvensis* L., *Triticum durum* L. and *Phalaris canariensis* L. seeds. The radicle elongation of five seeds was inhibited at the highest doses tested, while germination of all seeds was not affected. Moreover, the essential oils showed low antimicrobial activity against eight selected microorganisms.

#### Materials and methods

##### Test material

Name:	Essential oil of <i>Melaleuca styphelioides</i>
Formulation type:	Not relevant
Source and lot/batch no.:	<i>Melaleuca styphelioides</i> essential oil contains 91.1% of methyl eugenol after hydrodistillation of leaves collected from the Botanical Garden of the National Institute of Researches on Rural Engineering, Water and Forests (Ariana, Tunisia) in April 2011.
Active substance content:	91.1 % methyl eugenol (plus approx. 2% other compounds identified)
Expiry date of lot/batch:	Not reported
Storage conditions:	+4°C in the dark

Only the essential oil of *Melaleuca styphelioides* (91.1% methyl eugenol) is considered in this summary (the other essential oils included in the paper are not considered relevant to this dossier).

*Test organism group 1:* Phytotoxicity assay on seed germination

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Species:	Wild mustard ( <i>Sinapis arvensis</i> ), durum wheat ( <i>Triticum durum</i> ), canary grass ( <i>Phalaris canariensis</i> ), radish ( <i>Raphanus sativus</i> ), garden cress ( <i>Lepidium sativum</i> )
Strain/clone:	Not reported
Age at study initiation:	Seed stage
Weight/length/height at study initiation:	Not relevant
Source:	The seeds of radish and garden cress were purchased from Blumen srl (Piacenza, Italy), while mustard, wheat and canary grass were collected from wild plants.
Acclimation:	Not reported

### *Test conditions group 1*

Test temperature:	20 ± 1°C
Photoperiod:	Natural photoperiod
Light intensity:	Not reported
<i>Test system group 1</i>	
Study type:	Phytotoxic activity against germination and radicle elongation of terrestrial plant seeds (grown in Petri dishes and exposed to the test item on impregnated filter paper)
Duration of study:	120-hours exposure (5 days)
Treatments:	The <i>Melaleuca styphelioides</i> essential oil, in a water–acetone mixture (99.5:0.5), was assayed at the doses of 2.5, 1.25, 0.625, 0.25, 0.125 and 0.062 µg <i>Melaleuca styphelioides</i> essential oil/mL (equivalent to concentration of 2.28, 1.14, 0.57, 0.23, 0.11 and 0.06 µg methyl eugenol/mL based on content of 91.1% methyl eugenol).
Analytical determination of test concentrations:	No but concentration of methyl eugenol in <i>Melaleuca styphelioides</i> essential oil has been measured. Methyl eugenol was identified by gas chromatography in the natural extract and relative concentrations were obtained by peak area normalization.
Negative control included:	Yes. Controls performed with water-acetone mixture alone showed no appreciable differences in comparison with controls in water alone.
Positive control included:	Not reported
Parameters measured:	Effects on seed germination and radicle elongation
Validity criteria:	Not reported

The seeds were surface sterilized in 95% ethanol for 15 seconds and sown in Petri dishes (diameter = 90 mm) containing five layers of Whatman filter paper impregnated with distilled water (7 mL, control) or a tested solution of the essential oil (7 mL) at the different assayed doses. Seed germination was observed directly in Petri dishes, each 24 hours. A seed was considered germinated when the protrusion of the root became evident. After 120 hours (on the fifth day), the effects on radicle elongation were measured in centimetres. Each determination was repeated three times, using Petri dishes containing 10 seeds each. Data are expressed as the mean ± SD for both germination and radicle elongation. Data were ordered in homogeneous sets, and the Student's *t* test of independence was applied.

## **Results**

### *Phytotoxic Activity*

When seeds were placed on filter papers impregnated with test solutions in a Petri dish, there were no significant effects on seed germination for all five plant species tested compared to the controls when exposed to any of the tested concentrations of *Melaleuca styphelioides* essential oil (containing 91.1% of methyl eugenol), up to the highest concentration tested of 2.5 µg *M. styphelioides* oil/mL (equivalent to 2.28 µg methyl eugenol/mL). Similarly, there were no significant effects on radicle elongation for seeds of wild mustard, durum wheat or canary grass.

Significant inhibition of initial radicle elongation to *Raphanus sativus* (radish) seeds over a 120-hour period occurred at 0.125 and 1.25 µg *M. styphelioides* oil/mL (equivalent to concentrations of 0.11 and 1.14 µg methyl eugenol/mL), but there was no clear dose-response, and no significant effects observed at the highest concentration tested.

Significant inhibition of initial radicle elongation to *Lepidium sativum* (garden cress) seeds was observed at all concentrations tested, with radicles measuring  $2.8 \pm 1.8$  cm in controls compared to  $1.9 \pm 1.0$  cm in those seeds exposed to the highest concentration of *Melaleuca styphelioides* essential oil, equivalent to  $2.28 \mu\text{g}$  methyl eugenol/mL.

#### Assessment and conclusion

##### Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 2 reliable with restrictions

Analytical verification of test concentrations was not reported. Study is non-standard so of limited reliability for a quantitative risk assessment and no positive control to confirm sensitivity of the test.

#### **Assessment and conclusion by applicant:**

The study is considered as supporting information only (reliable with restrictions).

When seeds were placed on filter papers impregnated with test solutions in a Petri dish, there were no significant effects on seed germination for all five plant species tested compared to the controls when exposed to any of the tested concentrations of *Melaleuca styphelioides* essential oil (containing 91.1% of methyl eugenol), up to the highest concentration tested of  $2.5 \mu\text{g}$  *M. styphelioides* oil/mL (equivalent to  $2.28 \mu\text{g}$  methyl eugenol/mL). Similarly, there were no significant effects on radicle elongation for seeds of wild mustard, durum wheat or canary grass.

Significant inhibition of initial radicle elongation to *Raphanus sativus* (radish) seeds over a 120-hour period occurred at  $0.125$  and  $1.25 \mu\text{g}$  *M. styphelioides* oil/mL (equivalent to concentrations of  $0.11$  and  $1.14 \mu\text{g}$  methyl eugenol/mL), but there was no clear dose-response, and no significant effects observed at the highest concentration tested.

Significant inhibition of initial radicle elongation to *Lepidium sativum* (garden cress) seeds was observed at all concentrations tested, with radicles measuring  $2.8 \pm 1.8$  cm in controls compared to  $1.9 \pm 1.0$  cm in those seeds exposed to the highest concentration of *Melaleuca styphelioides* essential oil, equivalent to  $2.28 \mu\text{g}$  methyl eugenol/mL.

<b>Data point:</b>	CA 9.6.3.4/43 [CA 8.6]
<b>Report author</b>	Gogoi, R., Loying, R., Sarma, N., Begum, T., Pandey, S.K. and Lal, M.
<b>Report year</b>	2020
<b>Report title</b>	Comparative Analysis of <i>In-Vitro</i> Biological Activities of Methyl Eugenol Rich <i>Cymbopogon khasianus</i> Hack., Leaf Essential Oil with Pure Methyl Eugenol Compound
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 90 Current Pharmaceutical Biotechnology, volume 21, No. 10, Pages927-938 (2020)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

#### Abstract (copied from original literature)

**Background:** The essential oil of methyl eugenol rich *Cymbopogon khasianus* Hack. was evaluated and its bioactivities were compared with pure methyl eugenol. So far, methyl eugenol rich essential oil of lemongrass was not studied for any biological activities; hence, the present study was conducted.

**Objective:** This study examined the chemical composition of essential oil of methyl eugenol rich *Cymbopogon khasianus* Hack., and evaluated its antioxidant, anti-inflammatory, antimicrobial, and herbicidal properties and genotoxicity, which were compared with pure compound, methyl eugenol.

**Material and Methods:** Methyl eugenol rich variety of *Cymbopogon khasianus* Hack., with registration no. INGR18037 (c.v. Jor Lab L-9) was collected from experimental farm CSIR-NEIST, Jorhat, Assam (26.7378°N, 94.1570°E). The essential oil was obtained by hydro-distillation using a Clevenger apparatus. The chemical composition of the essential oil was evaluated using GC/MS analysis and its antioxidant (DPPH assay, reducing power assay), anti-inflammatory (Egg albumin denaturation assay), and antimicrobial (Disc diffusion assay, MIC) properties, seed germination effect and genotoxicity (*Allium cepa* assay) were studied and compared with pure Methyl Eugenol compound (ME).

**Results:** Major components detected in the Essential Oil (EO) through Gas chromatography/mass spectroscopy analysis were methyl eugenol (73.17%) and  $\beta$ -myrcene (8.58%). A total of 35 components were detected with a total identified area percentage of 98.34%. DPPH assay revealed considerable antioxidant activity of methyl eugenol rich lemongrass essential oil ( $IC_{50} = 2.263 \mu\text{g/mL}$ ), which is lower than standard ascorbic acid ( $IC_{50} 2.58 \mu\text{g/mL}$ ), and higher than standard Methyl Eugenol (ME) ( $IC_{50} 2.253 \mu\text{g/mL}$ ). Methyl eugenol rich lemongrass EO showed  $IC_{50} 38.00 \mu\text{g/mL}$ , ME  $36.44 \mu\text{g/mL}$ , and sodium diclofenac  $22.76 \mu\text{g/mL}$ , in *in-vitro* anti-inflammatory test. Moderate antimicrobial activity towards the 8 tested microbes was shown by methyl eugenol rich lemongrass essential oil whose effectiveness against the microbes was less as compared to pure ME standard. Seed germination assay further revealed the herbicidal properties of methyl eugenol rich essential oil. Moreover, *Allium cepa* assay revealed moderate genotoxicity of the essential oil.

**Conclusion:** This paper compared the antioxidant, anti-inflammatory, antimicrobial, genotoxicity and herbicidal activities of methyl eugenol rich lemongrass with pure methyl eugenol. This methyl eugenol rich lemongrass variety can be used as an alternative of methyl eugenol pure compound. Hence, the essential oil of this variety has the potential of developing cost-effective, easily available antioxidative/ antimicrobial drugs but its use should be under the safety range of methyl eugenol and needs further clinical trials.

#### Materials and methods

##### Test material 1

Name:	Lemongrass ( <i>Cymbopogon khasianus</i> (cv. Jorlab L-9)) oil
Formulation type:	Not relevant
Source and lot/batch no.:	Extracted from fresh lemongrass leaves collected from the experimental farm of CSIR-NEIST, Jorhat
Active substance content:	methyl-eugenol 73.17%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

##### Test material 2

Name:	methyl-eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma-Aldrich Company (Steinheim, Germany),
Active substance content:	$\geq 98\%$
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

##### Test organism

Species:	<i>Vigna radiata</i> L., Mung bean
Strain/clone:	Not reported
Age at study initiation:	Not relevant (seeds)
Weight/length/height at study initiation:	Not relevant
Source:	Not reported
Feeding during test:	Not relevant
Acclimation:	Not relevant

Note, the other tests included in the paper (such as antimicrobial screening tests), are excluded from this summary as these are not relevant to the ecotoxicological risk assessment of non-target organisms.

### *Test conditions*

Test temperature: 25 ±1°C.  
 Photoperiod: Not reported  
 Light intensity: Not reported

### *Test system*

Study type: Seed germination test (applied on filter papers in Petri dishes)  
 Duration of study: 24 hours  
 Treatments: 1 mL lemongrass oil and 1 mL pure methyl-eugenol poured onto filter papers (equivalent concentrations or application rates are not reported, but the paper reports that ‘different concentrations’ were evaluated)  
 Analytical determination of test concentrations: No  
 Negative control included: Yes (water)  
 Positive control included: No  
 Parameters measured: germination percentage and radical emergence; seedling length  
 Validity criteria: Not reported

The effects of lemongrass essential oil (73% methyl eugenol) and pure (≥98%) methyl eugenol compound were evaluated after 24 hours of the treatment. Seeds of *Vigna radiata* L. were air-dried with moisture content below 12%, and for the assay, the seeds were imbibed in tap water for a period of 24 hours. Three layers of filter paper were stacked in all Petri plates and moistened by 3 mL distilled water. Then 1 mL of each test item was poured on the filter paper and allowed to diffuse by covering the plates. Each of the treatment combinations had three replications. Twenty seeds were kept in each plate maintaining equidistance. Plates were incubated at 25 ±1°C. The Petri plates were examined for germination regularly. The germination percentage and radical emergence were recorded.

### **Results**

The lemongrass essential oil (73% methyl eugenol) and the pure (≥98%) methyl eugenol significantly (P<0.05) affected seedling lengths and germination rates. The paper reports that ‘the data revealed decrease in germination rate and radical emergence with increasing concentration of methyl eugenol rich lemongrass EO and pure compound ME’, but the concentrations tested, and % effects observed relative to the control, are not reported. ‘On the other hand, water treated *Vigna radiata* showed uniform germination and growth rate’. ANOVA analysis was computed and the results showed similarities between the function of both the essential oil and methyl eugenol pure compound.

### **Assessment and conclusion**

#### *Reliability assessment*

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Test concentrations were not reported, and limited details of the results are available.

Positive control was not reported.

### **Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

Paper reported significant effects of lemongrass essential oil (73% methyl eugenol) and pure (≥98%) methyl eugenol on seedling lengths and germination rates of *Vigna radiata* L., Mung bean. However, concentrations of test items tested, and % effects observed relative to the control, are not reported.

<b>Data point:</b>	CA 9.6.3.4/44 [CA 8.2.1]
<b>Report author</b>	Soares Vilhena, C., Santos do Nascimento, L.A., de Aguiar Andrade, E.H., do Rosário da Silva, J.K., Hamoy, M., Ferreira Torres, M., Luz Barbas, L.A.
<b>Report year</b>	2019
<b>Report title</b>	Essential oil of <i>Piper divaricatum</i> induces a general anaesthesia-like state and loss of skeletal muscle tonus in juvenile tambaqui, <i>Colossoma macropomum</i>
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 164 Aquaculture volume 510, Pages 169–175 (2019)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (see details in summary below) – critically, no analytical verification of test concentrations, and acute toxicity tests only 90 to 462 seconds
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

#### Abstract (copied from original literature)

This study investigated the anaesthetic potential of the essential oil from the leaves of *Piper divaricatum* (EOPD) through evaluation of the behaviour and electromyographic (EMG) recordings from the fish *Colossoma macropomum* which was used as an animal model. Initially, fish ( $3.9 \pm 0.3$  g;  $6.4 \pm 0.49$  cm, total length) were subjected to short-term anaesthetic baths in five different concentrations of the EOPD: 25, 30, 35, 40 and 45  $\mu\text{L/L}$  to record the latencies for deep anaesthesia and recovery. Ten fish per concentration were used ( $n=10$ ) and each animal was considered a replicate and used only once. Thereafter, for the evaluation of EMG, fish ( $5.6 \pm 1.8$  g;  $8.7 \pm 0.52$  cm, total length) were assigned to the following groups: a) sham control (basal recordings) and b) fish exposed to the EOPD at 40  $\mu\text{L/L}$  and subsequent recovery in anaesthetic-free water. Nine fish per analysis ( $n=9$ ) were used. The EOPD presented a high concentration of methyleugenol (71.36%) and prompted immediate behavioural changes in fish. Initially, hyperactivity was observed, followed by loss of the righting reflex and full body immobilization. All concentrations tested were capable to promote an anaesthetic-like state in tambaqui, with 40  $\mu\text{L/L}$  being the minimal concentration necessary to induce a rapid immobilization, i.e. < 3 min. The EMG showed a marked and reversible myorelaxation effect, confirming this oil as an indisputable muscle relaxant agent. The EOPD was capable of promoting a general anaesthesia-like state, with complete body immobilization of *Colossoma macropomum* at all concentrations tested. Gradual increases in frequency and amplitude of EMG tracings confirm the reversibility and uneventful resumption of normal swimming behaviour observed during recovery. Our results underscore the anaesthetic potential and myorelaxant effects of *Piper divaricatum* essential oil.

## Materials and methods

### Test material

Name:	<i>Piper divaricatum</i>
Formulation type:	Not relevant
Source and lot/batch no.:	Aromatic and Oleaginous Plants of the Amazon” of the Federal University of Pará, Belém, Brazil
Active substance content:	methyl-eugenol 71.36%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

### Test organism

Species:	<i>Colossoma macropomum</i> , Tambaqui juveniles
Strain/clone:	Not reported
Age at study initiation:	juveniles
Weight/length at study initiation:	3.9 ± 0.3 g / 6.4 ± 0.49 cm
Source:	commercial fish farm in Northern Brazil,
Feeding during test:	Yes, fish were fed twice daily with commercial feed
Acclimation:	During acclimation (30 days), the water quality parameters were monitored daily. The initial stocking density was 1 g fish.L <sup>-1</sup>

### Test conditions

Hardness:	60 ± 1.08 mg/L calcium carbonate.
Test temperature:	27.5 ± 0.7 °C (only acclimation conditions reported)
pH:	7.1 ± 0.2 (only acclimation conditions reported)
Dissolved oxygen:	5.8 ± 0.2 mg O <sub>2</sub> / L (only acclimation conditions reported)
Conductivity:	Not reported
Photoperiod:	12 h light: 12h dark
Light intensity:	Not reported

### Test system

Study type:	Acute toxicity test
Duration of study:	exposure: 90 to 462 seconds to reach A3 stage, 48 hours
Treatments:	25, 30, 35, 40 and 45 µL EOPD/L (nominal)
Analytical determination of test concentrations:	No
Negative control included:	Yes (water)
Positive control included:	No
Parameters measured:	Mortality
Validity criteria:	Not reported

Juvenile fish were exposed to concentrations of ethanolic solution of essential oil *P. divaricatum* at 25, 30, 35, 40 and 45 µL EOPD/L. A control group was used and animals were transferred to aquaria with anaesthetic-free water and observed for 30 min.

The maximum observation time during induction was 30 min, after which fish were no longer followed for signs of anaesthesia. In the case A3 stage was achieved, fish were transferred to tanks with anaesthetic-free water, and the time elapsed for recovery was registered. After recovery, fish were observed for 48 hours to check for mortalities

## Results

All nominal concentrations tested (20, 30, 35, 40 and 45 µL EOPD/L) led to anaesthesia of fish. The time to reach deep anesthesia was 462 ± 9, 260 ± 7, 210 ± 1, 156 ± 2, 90 ± 6 seconds when exposed to 20, 30, 35, 40 and

45mg  $\mu$ L EOPD/L. Following this short exposure period, 100% of fish recovered, with a mean time to recovery of  $221 \pm 3$ ,  $149 \pm 2$ ,  $215 \pm 2$ ,  $188 \pm 2$  and  $128 \pm 1$  seconds. No mortality occurred during or after exposure to EOPD. During the behaviour evaluation, no signs of anesthesia were observed in fish exposed to anaesthetic-free water, including the vehicle control. Fish were considered fully recovered after resumption of the righting reflex and normal swimming after 5 minutes. One-way ANOVA and Turkey's test were used to compare times to anesthesia induction and recovery.

#### Assessment and conclusion

##### Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported.

#### **Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

Test item was an essential oil of *Piper divaricatum* (referred to as EOPD), containing 71.36% methyl-eugenol.

At nominal concentrations of 20 – 45  $\mu$ L EOPD/L, *Colossoma macropomum* (tambaqui freshwater fish), reached anaesthesia after approximately 2 to 5 minutes, and recovered after approximately 2 to 4 minutes. No mortality after 5 minutes of exposure at concentrations up to 45  $\mu$ L EOPD/L.

<b>Data point:</b>	CA 9.6.3.4/45 [CA 8.3.2]
<b>Report author</b>	Xu, H.X., Zheng, X.-S., Yang, Y.-J., Tian, J.-C., Lu, Y.-H., Tan, K.-H., Heong, K.-L., Lu, Z.-X.
<b>Report year</b>	2015
<b>Report title</b>	Methyl eugenol bioactivities as a new potential botanical insecticide against major insect pests and their natural enemies on rice ( <i>Oriza sativa</i> )
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 240 Crop Protection, Volume 72, Pages 144-149 (2015)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	Supporting information only

#### Abstract (copied from original literature)

The bioactivities of methyl eugenol (ME) against rice insect pests, the brown planthopper (BPH), *Nilaparvata lugens*, the leaf folder (RLF), *Cnaphalocrocis medinalis*, the striped stem borer (SSB), *Chilo suppressalis*, and their major natural enemies, a predator *Cyrtorhinus lividipennis* and parasitoids *Anagrus nilaparvatae* and *Trichogramma japonicum* were determined under laboratory conditions. The results showed ME had repellent, systemic and contact insecticidal activities against BPH. The nymphal mortality increased with raised ME concentrations. The  $LC_{50}$  value for BPH using an impregnated filter paper bioassay was 1025 mg/L which was much lower than that of 3778 mg/L obtained from an immersion bioassay. ME also had a contact toxicity against RLF larvae with a  $LC_{50}$  of 250 mg/L. However, bioactivity of ME against SSB larvae was much lower than against RLF. Only 21.8% of 2<sup>nd</sup> instar SSB larvae died at 24 h after being treated with 8000 mg/L ME. In addition, ME also had contact toxicity to predator *C. lividipennis* and parasitic natural enemies *A. nilaparvatae*, *T. japonicum*, with  $LC_{50}$  values of 527, 105 and 123 mg/L, respectively. Even so, the toxicity of ME to natural enemies was much lower than that of chemical insecticides.

## Materials and methods

### Test material

Name:	Methyl eugenol (ME)
Formulation type:	Not relevant.
Source and lot/batch no.:	Sigma-Aldrich Corporation
Active substance content:	98%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

### Test organism

Species:	<i>Cyrtorhinus lividipennis</i> Reuter (Hemiptera: Miridae) <i>Anagrus nilaparvatae</i> Pang & Wang (Hymenoptera: Mymaridae) <i>Trichogramma japonicum</i>
Strain/clone:	Not reported
Age at study initiation:	female adults; 48 hours old for <i>C. lividipennis</i> and 4 hours old for <i>A. nilaparvatae</i> and <i>T. japonicum</i> .
Weight/length/height at study initiation:	Not reported
Source:	<i>Cyrtorhinus lividipennis</i> were collected from a paddy field in Hangzhou <i>Anagrus nilaparvatae</i> was collected from rice fields in Hangzhou. After identification and separation, it was reared by brown planthopper eggs for more than one generation for experiments. <i>Trichogramma japonicum</i> Ashmead (Hymenoptera: Trichogrammatidae) was trapped from a paddy field in Xiaoshan and maintained in the laboratory by using eggs of the rice moth, <i>Corcyra cephalonica</i> Stainton (Lepidoptera: Pyralidae).
Feeding during test:	For <i>C. lividipennis</i> : Kimura B nutrient solution. For <i>A. nilaparvatae</i> and <i>T. japonicum</i> : 10% honey solution
Acclimation:	The rearing condition for all the above insect species was maintained at $26 \pm 1$ °C, 70-80% relative humidity and a photoperiod of 14:10 h (L:D).

### Test conditions

Test temperature:	$26 \pm 1$ °C (all tests)
Photoperiod:	14/10 hours (light/darkness) (all tests)
Light intensity:	Not reported
Relative humidity:	70-80%
<i>Test system</i>	
Study type:	Contact toxicity
Duration of study:	24 hours for <i>C. lividipennis</i> ; 12 hours for <i>A. nilaparvatae</i> and <i>T. japonicum</i>
Treatments:	For contact bioassays, methyl eugenol was dissolved in acetone. - Against <i>C. lividipennis</i> – 0, 250, 500, 1000, 2000 mg methyl eugenol/L

- Against the egg parasitoids (*A. nilaparvatae* and *T. japonicum*) - 0, 50, 100, 200, 400 mg methyl eugenol/L

Analytical determination of test concentrations: No

Negative control included: Yes

Positive control included: No

Parameters measured: Mortality

Validity criteria: Not reported

For all tests, an 8 x 13 cm<sup>2</sup> filter paper was immersed in a required concentration of methyl eugenol for 5 seconds. For the control, the filter paper was dipped into acetone. The papers were then naturally air dried at room temperature, then finally shaped into a cylinder and placed in a glass tube.

Ten female adults of *C. lividipennis* emerged within 48 hours were introduced into each filter paper cylinder in a test tube with a rice tiller containing sufficient brown planthopper eggs and a 5 mL Kimura B nutrient solution. Six replications were prepared for each treatment. Ten female adults of *A. nilaparvatae* or twenty female adults of *T. japonicum* emerged within 4 hours were introduced into each treated filter paper cylinder and transferred into a test tube fed with 10% honey solution. The numbers of surviving parasitoids were recorded after 12 hours, and six replicates for each treatment were performed.

All statistical tests were performed by using ProStat software (Poly Software International).

The lethal concentration LC<sub>50</sub> values were calculated by using SAS-probit program (SAS Institute Inc., 1997).

### Results

In the contact toxicity test against *C. lividipennis*, *A. nilaparvatae*, and *T. japonicum*, the mortality of *C. lividipennis* was >50% at >1000 mg methyl eugenol/L. In addition, the results indicated that LC<sub>50</sub> value of methyl eugenol against female adults of *C. lividipennis* was 527 mg methyl eugenol/L. The mortality of the parasitoids, *A. nilaparvatae* and *T. japonicum*, was >50% at the concentrations >100 and 200 mg methyl eugenol/L, respectively. The LC<sub>50</sub> value of methyl eugenol to female adults of *A. nilaparvatae* was 105 mg methyl eugenol/L. Furthermore, the LC<sub>50</sub> value of methyl eugenol to female adults of *T. japonicum* was 123 mg methyl eugenol/L.

### Assessment and conclusion

#### Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 2 reliable with restrictions

Non-standard study design testing non-standard non-target arthropod species for EU assessments. No positive control and no validity criteria reported.

#### **Assessment and conclusion by applicant:**

The study is considered as supporting information only (reliable with restrictions).

*Cyrtorhinus lividipennis*: 24h-LC<sub>50</sub> (contact toxicity) = 527 mg methyl eugenol/L (95% confidence limits: 449-601 mg methyl eugenol/L) based on nominal concentrations

*Anagrus nilaparvatae*: 12h-LC<sub>50</sub> (contact toxicity) = 105 mg methyl eugenol/L (95% confidence limits: 88-121 mg methyl eugenol/L) based on nominal concentrations

*Trichogramma japonicum*: 12h-LC<sub>50</sub> (contact toxicity) = 123 mg methyl eugenol/L (95% confidence limits: 111-136 mg methyl eugenol/L) based on nominal concentrations

<b>Data point:</b>	CA 9.6.3.4/46 [CA 8.2.1]
<b>Report author</b>	Zahran, E., Risha,E., Rizk,A.
<b>Report year</b>	2021
<b>Report title</b>	Comparison propofol and eugenol anesthetics efficacy and effects on general health in Nile Tilapia
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 9 complementary Aquaculture, Volume 534, 736251 (2021)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

#### Abstract (copied from original literature)

Propofol is increasingly used as a fish anesthetic and pain management tool. However, studies concerning its effect on fish health biomarkers are lacking. Therefore, we aimed to investigate the propofol anesthetic efficacy and its effect on the health biomarkers in Nile tilapia compared to eugenol, an efficient and safe fish anesthetic commonly used in daily aquaculture practices. Nile tilapia were exposed to 2.5 mg/L propofol (PR) and 30 mg/L eugenol (EU). Induction, recovery times, and opercular movements (OM) were recorded. Blood and tissue samples were collected at time 0 (control) after anesthesia was achieved, and after a full recovery for each anesthetic agent, to assess the health biomarkers of the Nile tilapia. Results demonstrated extended induction and anesthetic duration along with rapid recovery in the propofol group compared to eugenol. OM showed a similar trend in both groups, with a significant frequency decrease during anesthesia that was restored during the recovery phase. The PR-group showed a significant transient increase of hematological indices in contrast to the EU group. However, TLC (total leukocytic count) and heterophils showed no statistical changes in the PR group compared to the EU group. A significant transient increase of glucose, cortisol, reduced glutathione (GSH), and catalase (CAT) levels compared to their baseline levels was observed during anesthesia in PR and EU groups with full reversal during recovery. No significant changes were noticed in liver function enzymes, Malondialdehyde (MDA), Superoxide dismutase (SOD), and mRNA tumor necrosis factor-  $\alpha$  (*tnf- $\alpha$* ) expression level in fish exposed to both anesthetics at any stage. The head kidney mRNA interleukin-8 (*il-8*) level was upregulated significantly during recovery compared to the baseline level in the PR group. However, during anesthesia, it showed no significant changes in the PR group compared to its control and recovery level, in contrast to the EU group. The spleen mRNA *il-8* exhibited no significant changes in the PR group during anesthesia and recovery compared to the control, contrary to the EU group during recovery. However, splenic *il-8* was upregulated during recovery compared to anesthesia in the PR group, in contrast to the EU group. Overall, propofol showed advantages in different scenarios compared to eugenol and vice versa. Eugenol appears better suited for use during minor procedures, while propofol may be used when smoother induction and deeper anesthesia are of interest, with a lack of alterations on cytokines expressions during anesthesia.

#### Materials and methods

##### Test material 1

Name:	eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Sigma-Aldrich Corporation (St. Louis, USA)
Active substance content:	99%
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

*Test organism*

Species:	Nile tilapia, <i>O. niloticus</i>
Strain/clone:	Not reported
Age at study initiation:	Not reported
Weight at study initiation:	35 ± 2.3 g (maintenance data only)
Source:	private farm (Kafr El-Elsheikh, Egypt)
Feeding during test:	No. Fish were starved for 24 h before starting the experiment
Acclimation:	Water quality parameters were maintained throughout the acclimation and experimental period. Fish were fed with a commercial pellet diet (Uccma feed, Egypt) at a rate of 3% body weight.

*Test conditions*

Hardness:	Not reported
Test temperature:	24 ± 2 °C
pH:	7.1–7.3
Dissolved oxygen:	6.5–7.8 mg /L
Conductivity:	Not reported
Photoperiod:	12 h light/ dark
Light intensity:	Not reported

*Test system*

Study type:	Acute toxicity test (anaesthesia effects)
Duration of study:	90 minutes approximately (exposure period of 5 to 20 minutes; see further details below)
Treatments:	eugenol 30 mg/L (nominal)
Analytical determination of test concentrations:	No
Negative control included:	No
Positive control included:	No
Parameters measured:	Anaesthesia (or complete lack of voluntary movement); Mortality/behaviour
Validity criteria:	Not reported

Total of 12 fish/group were netted quietly and placed in a transfer bucket (10 L) containing water from the housing tank, and the basal frequency opercular movement (OM) was recorded visually for one minute (min) at 5, 10, 15, and 20 min time points. Fish was removed from the bucket into the induction tank containing 10 L of anesthetic solution (propofol or eugenol). The induction time (time to the total loss of equilibrium) was evaluated with a mild touch of the lateral side of the fish with a pipette, and with a response to a tail pinch by gently pressing the caudal fin with forceps; and time to reach stage IV of anesthesia (complete lack of voluntary movement) were recorded. The fish was immediately transferred into the anesthetic-free and aerated recovery tank after reaching stage IV, and the time to restore normal swimming activity was recorded. Both induction and recovery were carried out in aquarium tanks (40× 20 × 20 cm) filled with continuously aerated dechlorinated tap water. OM was recorded per each fish during the anesthesia and recovery similarly as performed before fish exposure. Behavioral measures were followed to evaluate stages of anesthesia and recovery. The anesthetic bath and water in the recovery aquaria were renewed after each fish to ensure convenient exposure dosages. All observations of anesthesia and recovery were investigated by the same observer (AR) throughout the study. A-EU and R-EU were referred to the anesthetic and recovery stages in the eugenol group, respectively.

**Results**

Nominal concentrations of eugenol at 30.0 mg eugenol/L (exposure for 5 to 20 minutes) caused anesthesia in Nile tilapia fish. The times to reach induction and anaesthesia (complete lack of voluntary movement) were 2.5 ± 1.3 and 19.87 ± 3.4 min; respectively. Following this short exposure period, the recovery time for eugenol was 45.6 ± 12.02 min.

Compared to the baseline the OM (operculum movement) exhibited similar pattern during anaesthesia at 10–20 min but was lower than the baseline ( $P \leq 0.0001, 0.0001, 0.0056$ ) at 5–15 min after recovery then restored near to baseline values at 20 min. There were no statistical changes during anaesthesia.

**Assessment and conclusion***Reliability assessment*

For full details and justification, please refer to KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported.

**Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

At nominal concentrations of 30 mg eugenol/L (exposure for 5 to 20 minutes), *Oreochromis niloticus* (Nile tilapia) reached anaesthesia after approximately 20 minutes, and recovered after approximately 45 minutes. No mortality or other negative effects on *Oreochromis niloticus* (Nile tilapia) at 30 mg eugenol/L during the anesthetic exposure (approximately 3 minutes).

<b>Data point:</b>	CA 9.6.3.4/47 [CA 8.2.1]
<b>Report author</b>	Sobrinho Ventura, A., Caetano Corrêa Filho, R. A., Teodoro, G. C., Menes, L., Leite Barbosa, P. T., Rodrigues Stringheta, G., Jerônimo, G. T., Aparecido Povh, J.
<b>Report year</b>	2020
<b>Report title</b>	Essential oil of <i>Ocimum basilicum</i> and eugenol as sedatives for Nile Tilapia
<b>Report No</b>	-
<b>Document No</b>	Internal reference: Study 11 complementary  Journal of Agricultural Studies, Volume 8, No 2, ISSN 2166-0379 (2020)
<b>Guidelines followed in study</b>	-
<b>Deviations from current test guideline</b>	Yes (non-standard study type; see details in summary below)
<b>Previous evaluation</b>	No, not previously submitted
<b>GLP/Officially recognised testing facilities</b>	No, not conducted under GLP
<b>Acceptability/Reliability:</b>	No

**Abstract (copied from original literature)**

This study evaluated the modulation of the ventilatory frequency of Nile tilapia sedated with essential oil of *Ocimum basilicum* and eugenol. The fish were exposed to the following treatments: control (water only); ethanol 200 µL/L (concentration used to dilute the anesthetic); eugenol 10 µL/L and 20 µL/L; essential oil of *O. basilicum* at the concentration of 10 µL/L and 20 µL/L. After 90 minutes of exposure to the treatments, water quality, mortality and respiratory rate were determined. The concentration of 20 µL/L of the essential oil of *O. basilicum* and eugenol showed a sedative effect and reduced the excretion of metabolic ammonia in Nile tilapia. There was no mortality in fish exposed to the treatments. The respiratory rate did not differ between the different treatments. It is concluded that the concentration of 20 µL/L of the essential oil of *O. basilicum* and eugenol shows the best result in the inducing sedative effect for Nile tilapia, and that the essential oil of *O. basilicum* and eugenol in the concentrations of 10 µL/L and 20 µL/L in an exposure period up to 90 minutes do not alter the ventilatory frequency of Nile tilapia.

**Materials and methods***Test material 1*

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Name:	eugenol
Formulation type:	Not relevant
Source and lot/batch no.:	Maquira® (batch not reported)
Active substance content:	Not reported
Expiry date of lot/batch:	Not reported
Storage conditions:	Not reported

### *Test organism*

Species:	Nile Tilapia, <i>O. niloticus</i>
Strain/clone:	Not reported
Age at study initiation:	Not reported
Weight/length at study initiation:	344.58 ± 28.49 g / 23.84 ± 1.39 cm
Source:	Fish were purchased from a commercial fish farm located in Dourados, Mato Grosso do Sul –Brazil.
Feeding during test:	No
Acclimation:	The fish were acclimated for seven days in 5000 L tanks with constant oxygenation. They were fed until apparent satiation with a commercial diet (Do peixe Douramix®), with 7 to 9 mm pellets (28.0% crude protein, 5.0% ether extract, 3.5% crude fiber, 12.0% of moisture, 10.0% mineral matter, 3.0% calcium). Feeding was suspended 24 hours before the beginning of the experiment.

### *Test conditions*

Hardness:	Not reported
Test temperature:	26.59 ± 0.04 – 26.65 ± 0.09
pH:	8.17 ± 0.06 – 8.23 ± 0.10
Dissolved oxygen:	6.06 ± 0.46 – 6.14 ± 0.98
Conductivity:	49.43 ± 2.85 – 50.99 ± 5.30 µS/cm
Photoperiod:	Not reported
Light intensity:	Not reported

### *Test system*

Study type:	Acute toxicity (anesthesia test)
Duration of study:	48 hours (exposure duration of 30 – 90 minutes)
Treatments:	0, 10 and 20 µL eugenol/L (nominal)
Analytical determination of test concentrations:	No
Negative control included:	Yes (water and solvent controls)
Positive control included:	No
Parameters measured:	Mortality/behaviour (ventilatory frequency)
Validity criteria:	Not reported

The experimental design was completely randomized with two treatments of eugenol (n = 8) 10 and 20 µL eugenol/L, a water control and solvent (ethanol) control, with four aquaria, each aquarium contains two fish. Fish were acclimated for 10 minutes in 8 L aquariums (width 40 cm x height 60 cm x length 65 cm) with constant oxygenation. The ventilatory frequency was quantified in 0, 30, 60 and 90 minutes of exposure to the respective treatments, considering the time elapsed for the occurrence of 20 consecutive opercular beats and then transformed in frequency per minute. Each fish was used only once. After the experimental period the fish were allocated in net cages of 2.00 m x 2.00 m x 1.20 m (Max Telas®) and the mortality was assessed up to 48 hours.

The results were evaluated using the Shapiro-Wilk test to assess normality and the Levene test to assess homoscedasticity. After observing that the data did not meet the assumptions of normality and homogeneity of variances, the Kruskal-Wallis non-parametric analysis was used, followed by the Dunn test. The data were analyzed using the software SAS® (Statistical Analysis System) at a significance level of  $p < 0.05$  (SAS, 2009).

#### Results

Nominal concentrations of eugenol at 10 – 20  $\mu\text{L}$  eugenol/L (exposure for 30 – 90 minutes) caused sedative effect. The fish presented only mild sedation, maintained the ability to react to external stimuli, with reduced movements, but with normal balance.

Nominal concentrations of eugenol at 20  $\mu\text{L}/\text{L}$  are capable of reducing the excretion of metabolic ammonia and inducing sedative effect in Nile tilapia. Eugenol at the concentrations of 10  $\mu\text{L}/\text{L}$  and 20  $\mu\text{L}/\text{L}$  during an exposure period of up to 90 minutes, the ventilatory frequency of Nile tilapia *O. niloticus* did not change.

After this short period of exposure, no mortality to fish was observed.

#### Assessment and conclusion

##### Reliability assessment

For full details and justification, please refer to Document KCA 9.4.2/02.

Proposed category: 3 not reliable

Analytical verification of test concentrations was not reported.

#### **Assessment and conclusion by applicant:**

The study is not acceptable (not reliable).

At nominal concentrations of 20  $\mu\text{L}$  eugenol/L (exposure period of 30 – 90 minutes), *Oreochromis niloticus* (Nile tilapia) reached mild sedative effects. Following this short period of time no adverse effects were observed after 90 minutes neither in ventilator frequency nor mortality.