# KBBPPS

# Knowledge Based Bio-based Products' Pre-Standardization

**Work package 6**

**Biodegradability**

## Deliverable N° 6.3:

# Eco-toxicological impact study

## Public

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## List of abbreviations, acronyms and used terms

| | |
|---|---|
| CLP: | Classification, Labelling and Packaging of substances and mixtures |
| ECXX: | Effect concentration XX |
| ELXX: | Effect load XX |
| HOSO: | High oleic sunflower oil |
| ICXX: | Inhibition concentration XX |
| ILXX: | Inhibition load XX |
| LCXX: | Lethal concentration XX |
| LLXX: | Lethal load XX |
| WAF: | Water accommodated fraction |
| WSF: | Water soluble fraction |

# 1 Publishable summary

The objective of work package 6 “Biodegradability” of the KBBPPS project is to develop and validate a biodegradation test methodology and a method in order to evaluate environmental safety in freshwater and soil for bio-based lubricants and solvents.

The results of the questionnaires and the contacts with the CEN committees revealed that no further needs exist with regard to environmental safety of bio-based solvents. For bio-based lubricants needs exist with regard to biodegradability, but no specific issues with regard to environmental safety were mentioned.

In a first phase the criteria with regard to environmental safety of the Blue Angel ecolabel and the European ecolabel for lubricants were investigated. It can be concluded that these labelling systems for environmentally friendly lubricants evaluate their toxicity already satisfactorily by means of mainly aquatic test organisms on different trophic levels (bacteria, algae, daphnia and/or fish). Moreover also the European legislation is taken into account in order to ensure that substances labelled as harmful for the environment (according the CLP (Classification, Labelling and Packaging) regulation) are not present or only present at very limited concentration in the labelled lubricants.

This approach differs from the approach used for biodegradable plastics (e.g. compostable plastics or plastics biodegradable in soil). For such materials, environmental safety by means of ecotoxicity tests is evaluated on possible residuals or metabolites obtained at the end of the biodegradation phase. This can be explained by the fact that the different constituents of plastic materials are normally not available in the product as such. However, during the biodegradation certain constituents can be released in the environment and toxicity can increase. For lubricants (fluid or paste), this is less possible since the different constituents are probably already available in the product as such and consequently evaluation of the product before biodegradation can probably be considered as the most stringent approach.

In spite of the fact that lubricants are in some cases (intentionally or accidently) released in a soil environment, toxicity by means of terrestrial organisms (soil bacteria, earthworms or plants) is not prescribed due to the fact that toxicity of substances present in lubricants to terrestrial organisms is generally lower than the toxicity to aquatic organisms. This is generally expected for organic substances due to adsorption to organic material in soil as mentioned in the background document with regard to the European Ecolabel for lubricants.

In our opinion the procedure used in the labelling systems is very detailed and takes into account the current legislation in order to avoid that toxic compounds enter in the environment.

Therefore only a few preliminary tests were executed in order to (1) evaluate if toxic effects of lubricants towards terrestrial organisms are indeed less significant when compared to aquatic organisms and to (2) compare toxicity before and after the biodegradation phase. From the first preliminary results it can indeed be concluded that terrestrial organisms (earthworms and plants) are less sensitive to lubricants when compared to aquatic organisms.

The plant ecotoxicity tests performed by the Agricultural University of Athens on natural soil where reference bio-lubricants (e.g. sunflower oil) at various concentrations were biodegraded indicate a special sensitivity of the cress plants to the presence of non-biodegraded bio-lubricants. The mechanism of the inhibition of the plants growth will be analysed following the completion of the on-going ecotoxicity tests.

The earthworm based ecotoxicity tests executed by the Agricultural University of Athens did not show any negative effect of the biodegradation of reference bio-lubricants in natural soil. Provided that bio-based lubricants other than positive reference are non-toxic (tested for ecotoxicity as materials) no negative effect is expected towards earthworms from their biodegradation in natural soil.

In the next phase of the project the samples used for the interlaboratory biodegradation test will be added in a 1000 mg/l concentration to freshwater and immediate toxicity and toxicity after the biodegradation phase will be evaluated and compared. Also further research toward ecotoxicity in soil will be executed in order to further investigate difficulties observed in these first preliminary tests.

Website: www.KBBPPS.eu

# 2 Introduction

This deliverable was compiled as part of task 6.3 “Evaluation of environmental safety, including requirements, tests and criteria, of bio-lubricants and bio-solvents” of the KBBPPS project.

It serves the following purposes:

- Review of characteristics of bio-based lubricants and bio-based solvents relevant to evaluation of environmental safety;
- Summary of the results of questionnaires executed in order to map the needs with regard to biodegradation, ecotoxicity and labelling of bio-based lubricants and bio-based solvents;
- Summary of CEN meetings related to bio-based lubricants and bio-based solvents;
- Overview of first preliminary ecotoxicity tests executed in order to investigate the environmental safety in freshwater and soil;
- Development of draft test methodology in order to evaluate environmental safety of bio-based lubricants in freshwater and soil under aerobic conditions;
- Definition of the test set-up for the interlaboratory testing for the next phase of the project.

The first 3 above mentioned topics are discussed in detail for both biodegradation and environmental safety in Chapter 3 and Chapter 4 of deliverable 6.2 “Draft biodegradability standard”.

This deliverable, as part of the work carried out within the framework of WP6 “Biodegradability” of the EU KBBPPS project, aims to evaluate environmental safety of bio-based lubricants in freshwater and soil.

In spite of the fact that the DOW mentioned that also environmental safety of bio-based solvents would be evaluated, this was cancelled as recommended by CEN TC411/WG2 “Bio-based solvents”.

Chapter 3 and Chapter 4 contain an overview of the first preliminary ecotoxicity tests executed in order to develop a method in order to evaluate environmental safety of bio-based lubricants in freshwater and soil, respectively. Chapter 5 and Chapter 6 give the proposed evaluation method for environmental safety of (bio-based) lubricants in freshwater and soil, respectively, which will be used for the interlaboratory testing in the next phase of the project.

This deliverable focusses only on environmental safety in freshwater and soil as these environments are frequently affected by the release of lubricants. Also a marine environment can be affected by the release of lubricants. This was not evaluated in this study.



# 3 Evaluation of environmental safety in aquatic environment

## 3.1 Evaluation of environmental safety in current labelling systems

An overview of the existing requirements with regard to environmental safety of the international specification ISO 15380, the German Blue Angel ecolabel and the European ecolabel is given in Table 1.

These labelling systems and specifications for environmentally friendly lubricants evaluate toxicity by means of mainly aquatic test organisms on different trophic levels (bacteria, algae, daphnia and/or fish). Moreover the labelling systems also include principles of European legislation in order to ensure that substances labelled as harmful for the environment (according the CLP (Classification, Labelling and Packaging) regulation) are not or only very limited present in the environmentally friendly lubricants.

Another approach is used for the evaluation of the eco-toxicological impact of biodegradable plastics. A few examples:

- European standards for compostable plastics or packaging (EN 13432 “Packaging—requirements for packaging recoverable through composting and biodegradation—test scheme and evaluation criteria for the final acceptance of packaging” or EN 14995 “Plastics - Evaluation of compostability - Test scheme and specifications”) require that ecotoxicological effects are evaluated after a composting phase of 12 weeks. The obtained composts, which contain possibly residuals or metabolites of the degraded plastics, need to be used for a subsequent plant toxicity test. Besides ecotoxicity tests after the biodegradation phase, also requirements with regard to heavy metals and fluorine content of the plastic are prescribed.
- French standard for materials biodegradable in soil (NF U 52-001 “Biodegradable materials for use in agriculture and horticulture - Mulching products - Requirements and test methods”) prescribes that environmental safety should be evaluated after a stabilisation phase of 90 days. The stabilisation phase is necessary in order to evaluate toxicity of metabolites formed during the biodegradation phase. Environmental safety is evaluated by means of different trophic levels (plants, earthworms & water algae). Besides the ecotoxicity tests after the biodegradation phase, also requirements with regard to heavy metals, fluorine and organic parameters of the plastic are prescribed.
- The European standard for plastics disposable in waste water treatment plants (EN 14987 (2006) “Plastics – Evaluation of disposability in waste water treatment plants – Test scheme for final acceptance and specifications”) only requires that biodegradation and solubility/dispersibility is evaluated. No requirements with regard to environmental safety are prescribed.

➔ **CONCLUSION:** The ecotoxicological impact of biodegradable lubricants is evaluated on the test item as such, while ecotoxicological impact of biodegradable plastics is evaluated after the biodegradation phase.

This can be explained by the fact that the different constituents of polymers are normally not available in the product as such. However, during the biodegradation certain components can be released in the environment and toxicity can increase. For lubricants (fluid or paste), this is probably less the case as the different constituents are probably already available in the product as such and consequently evaluation of the product before biodegradation can be considered as the most stringent approach.

Moreover residuals of compostable products will only be released in the environment after the composting phase when the compost (possibly containing residuals of the test materials) is used as fertilizer in agriculture or in gardens. During the biodegradation phase, compostable plastics (or residuals of compostable plastics) remain in industrial composting installations. Lubricants, in contrast, are immediately released (unavoidable or accidentally) in the environment and consequently, the lubricant may also not be toxic before the biodegradation phase. The evalution of environmental safety only after biodegradation would clearly be insufficient for lubricants and therefore evaluation of the ecotoxicity of the product as such (as required in the labellings systems and the specifications) is clearly needed.

Besides the evaluation of the lubricant as such, also evaluation of toxicity after the biodegradation phase could be investigated additionally. Although it already needs to be mentioned that in case very toxic intermediates would be formed during the biodegradation of a sample this would be detected in the biodegradation test as the activity of the bacteria (= the inoculum of an aquatic biodegradation test) would decrease or stop and biodegradation of the sample would decrease significantly. The evaluation of the ecotoxicity after biodegradation could be interesting for products that are characterised by a fast biodegradation, which induces temporary instability in the test medium (this would also be observed for natural materials).

In this chapter some preliminary tests will be executed both on the final products and on residuals obtained after biodegradation.

**Table 1. Overview of ecotoxicity criteria of different labelling systems and standards.**

| Labelling system / standard | Ecotoxicity criteria |
|---|---|
| ISO 15380 | Bacterial inhibition (3 h): Min. $EC_{50}$ = 100 mg/l<br>Acute Daphnia toxicity (48 h): Min. $EC_{50}$ = 100 mg/l<br>Acute fish toxicity (96 h): Min. $LC_{50}$ = 100 mg/l[1] |
| Der Blaue Engel: RAL-UZ 178 Biodegradable lubricants and hydraulic fluids[2] - all categories | Final product may not contain substances that are classified as "Hazardous to the Aquatic Environment – Acute Hazard" Category 1 (CLP hazard warning H400) and as "Hazardous to the Aquatic Environment – Chronic Hazard" Category 1 (CLP hazard warning H410). Impurities in the components with substances that correspond to the above mentioned criteria and present above the limit values that would result in classification according to the above mentioned hazard classes are not permitted.<br><br>Substances that would lead to classification in hazard class "Hazardous to the Aquatic Environment – Chronic Hazard" Category 2, 3 or 4 may only be contained in the final product up to a maximum total concentration smaller than the concentration that would lead to classification in hazard class "Chronic toxicity" Category 3 and 4.<br><br>Substances on the OSPAR list, on the EU list of priority substances according to the Water Framework Directive and substances with a water hazard class 2 or 3 are excluded from use in final products (except for substances in water hazard class 2 for production of hydraulic fluids, gear lubricants and greases).<br><br>Organic halogen compounds, nitrite compounds, metal compounds (except Na, K, Mg, Ca and Li and Al for thickening agents), mineral oils for use in release agents for asphalt paving work and mineral oils for use in chain lubricants for motor saws are not permitted. (Exemption: chain lubricants for motor saws may contain 5% mineral oil content due to addition of additives) |

[1] Fluids with low water solubility shall be tested using water-accommodated fractions, prepared according to ASTM D6081.

[2] In deliverable 6.1, three different environmental labels of Der Blaue Engel were mentioned: (1) RAL-UZ 48: Readily Biodegradable Chain Lubricants for Power Saws, (2) RAL-UZ 64: Readily Biodegradable Lubricants and Forming Oils and (3) RAL-UZ 79: Readily Biodegradable Hydraulic Fluids. These documents have been revised and replaced by RAL-UZ 178. The new criteria were published in 2013.

| Labelling system / standard | Ecotoxicity criteria |
|---|---|
| Der Blaue Engel: RAL-UZ 178 - Category 1: Lubricants for areas in which lubricant loss occurs during their intended use | Option 1: final lubricant<br>Algae + daphnia +fish (acute or chronic)<br>> 1000 mg/l (acute) - > 100 mg/l (chronic)<br><br>Option 2: components<br>Daphina + fish (chronic) OR Algae + daphnia + fish (acute)<br>Unlimited “not toxic” (acute > 100 mg/l // chronic > 10 ml/l)<br>Max. 25% “harmful” (acute: 10-100 mg/l // chronic: 1-10 mg/l)<br>Max. 1% “toxic” (acute: 1-10 mg/l // chronic: 0.1-1 mg/l)<br>Max. 0.1% “very toxic” (chronic: <0.1 mg/l) |
| Der Blaue Engel: RAL-UZ 178 – Category 2: Hydraulic fluids | Option 1: final lubricant<br>Algae + daphnia +fish (acute or chronic)<br>> 100 mg/l (acute) - > 10 mg/l (chronic)<br><br>Option 2: components<br>Daphina + fish (chronic) OR Algae + daphnia + fish (acute)<br>Unlimited “not toxic” (acute > 100 mg/l // chronic > 10 ml/l)<br>Max. 20% “harmful” (acute: 10-100 mg/l // chronic: 1-10 mg/l)<br>Max. 5% “toxic” (acute: 1-10 mg/l // chronic: 0.1-1 mg/l)<br>Max. 0.1% “very toxic” (chronic: <0.1 mg/l) |
| Der Blaue Engel: RAL-UZ 178 - Category 3: Chain lubricants | Option 1: final lubricant<br>Algae + daphnia +fish (acute or chronic)<br>> 1000 mg/l (acute) - > 100 mg/l (chronic)<br><br>Option 2: components<br>Daphina + fish (chronic) OR Algae + daphnia + fish (acute)<br>Unlimited “not toxic” (acute > 100 mg/l // chronic > 10 ml/l)<br>Max. 5% “harmful” (acute: 10-100 mg/l // chronic: 1-10 mg/l)<br>Max. 0.5% “toxic” (acute: 1-10 mg/l // chronic: 0.1-1 mg/l)<br>Max. 0.1% “very toxic” (chronic: <0.1 mg/l) |
| Der Blaue Engel: RAL-UZ 178 - Category 4: Gear lubricants | Option 1: final lubricant<br>Algae + daphnia +fish (acute or chronic)<br>> 100 mg/l (acute) - > 10 mg/l (chronic)<br><br>Option 2: components<br>Daphina + fish (chronic) OR Algae + daphnia + fish (acute)<br>Unlimited “not toxic” (acute > 100 mg/l // chronic > 10 ml/l)<br>Max. 20% “harmful” (acute: 10-100 mg/l // chronic: 1-10 mg/l)<br>Max. 5% “toxic” (acute: 1-10 mg/l // chronic: 0.1-1 mg/l)<br>Max. 1% “very toxic” (chronic: <0.1 mg/l) |

| Labelling system / standard | Ecotoxicity criteria |
|---|---|
| Der Blaue Engel: RAL-UZ 178 - Category 5: Greases | Option 1: final lubricant<br>Algae + daphnia +fish (acute or chronic)<br>> 1000 mg/l (acute) - > 100 mg/l (chronic)<br><br>Option 2: components<br>Daphina + fish (chronic) OR Algae + daphnia + fish (acute)<br>Unlimited “not toxic” (acute > 100 mg/l // chronic > 10 ml/l)<br>Max. 25% “harmful” (acute: 10-100 mg/l // chronic: 1-10 mg/l)<br>Max. 1% “toxic” (acute: 1-10 mg/l // chronic: 0.1-1 mg/l)<br>Max. 0.1% “very toxic” (chronic: <0.1 mg/l) |
| EU Ecolabel – all categories | Final product may not contain substances that are classified with CLP hazard warning H400, H410, H411, H412 or H413. Concentration limits for classified substances shall not exceed 0.010% or in case specific concentration limits are referred the concentration should be one tenth of the lowest concentration unless this value is below 0.010%. No derogation from exclusion may be given to substances of very high concern.<br><br>Substances on the OSPAR list, on the EU list of priority substances according to the Water Framework Directive, organic halogen compounds, nitrite compounds and metal compounds (except Na, K, Mg, Ca and Li and Al for thickening agents) are excluded from use in final products in quantities exceeding 0.010%. |
| EU Ecolabel – Category 1 Hydraulic fluids and tractor transmission oils | Option 1: lubricant & main components[3]<br>Main component: > 100 mg/l (algae and daphnia - acute)<br>Lubricant: > 100 mg/l (algae, daphnia and fish - acute)<br><br>Option 2: each substance > 0.1%<br>(Daphnia + fish: chronic OR algae + daphnia: acute)<br>Not toxic[4]: Not limited<br>Harmful[5]: ≤ 20%<br>Toxic[6]: ≤ 5%<br>Very toxic[7]: ≤ 0.1% (M=10) |

[3] Main component: substance accounting for more than 5%.
[4] Not toxic: Acute toxicity > 100 mg/l or NOEC > 10 mg/l.
[5] Harmful: 10 mg/l < acute toxicity ≤ 100 mg/l or 1 mg/l < NOEC ≤ 10 mg/l.
[6] Toxic: 1 mg/l < acute toxicity ≤ 10 mg/l or 0.1 mg/l < NOEC ≤ 1 mg/l.
[7] Very toxic: Acute toxicity ≤ 1 mg/l or NOEC ≤ 0.1 mg/l.

| Labelling system / standard | Ecotoxicity criteria |
|---|---|
| EU Ecolabel – Category 2<br>Greases and stern tube greases | Option 1: lubricant & main components<br>Main component: > 100 mg/l (algae and daphnia - acute)<br>Lubricant: > 1000 mg/l (algae, daphnia and fish - acute)<br><br>Option 2: each substance > 0.1%<br>(Daphnia + fish: chronic OR algae + daphnia: acute)<br>Not toxic: Not limited<br>Harmful: ≤ 25%<br>Toxic: ≤ 1%<br>Very toxic: ≤ 0.1% (M=10) |
| EU Ecolabel – Category 3<br>Chainsaw oils, concrete release agents, wire rope lubricants, stern tube oils and other total loss lubricants | Option 1: lubricant & main components<br>Main component: > 100 mg/l (algae and daphnia - acute)<br>Lubricant: > 1000 mg/l (algae, daphnia and fish - acute)<br><br>Option 2: each substance > 0.1%<br>(Daphnia + fish: chronic OR algae + daphnia: acute)<br>Not toxic: Not limited<br>Harmful: ≤ 5%<br>Toxic: ≤ 0.5%<br>Very toxic: ≤ 0.1% (M=10) |
| EU Ecolabel – Category 4<br>Two-stroke oils | Option 1: lubricant & main components<br>Main component: > 100 mg/l (algae and daphnia - acute)<br>Lubricant: > 1000 mg/l (algae, daphnia and fish - acute)<br><br>Option 2: each substance > 0.1%<br>(Daphnia + fish: chronic OR algae + daphnia: acute)<br>Not toxic: Not limited<br>Harmful: ≤ 25%<br>Toxic: ≤ 1%<br>Very toxic: ≤ 0.1% (M=10) |
| EU Ecolabel – Category 5<br>Industrial and marine gear oils | Option 1: lubricant & main components<br>Main component: > 100 mg/l (algae and daphnia - acute)<br>Lubricant: > 100 mg/l (algae, daphnia and fish - acute)<br><br>Option 2: each substance > 0.1%<br>(Daphnia + fish: chronic OR algae + daphnia: acute)<br>Not toxic: Not limited<br>Harmful: ≤ 20%<br>Toxic: ≤ 5%<br>Very toxic: ≤ 1% (M=10) |

## 3.2 Relevant studies from literature

Cecutti et al. (2008) compared the toxicity of 3 biolubricants and 1 mineral lubricants (new and used after 1000 h in logging machine) towards different aquatic organisms (algae, daphnia and fish) (summary results: Table 2).

Algae were the most sensitive organisms for the biolubricants, while fish were most affected due to mineral oils (Mobilfluide). Moreover it was noticed that toxicity increased after use of the lubricant.

**Table 2. Summary of the ecotoxicity results of Cecutti et al. (2008).**

| Lubricant | Algae $EC_{50}$ – 72 h | Daphnia $EC_{50}$ – 48 h | Fish $LC_{50}$ – 48 h |
|---|---|---|---|
| Biolube – new | 5400 | > 10000 | > 10000 |
| Biolube – used | 5600 | 5900 | > 10000 |
| Biohydran – new | 2800 | > 10000 | > 10000 |
| Biohydran – used | 1800 | > 10000 | > 10000 |
| Helianthe – new | 4800 | > 9900 | > 9900 |
| Helianthe – used | 100 | 2500 | > 10000 |
| Mobilfluide – new | 1300 | 5400 | 390 |
| Mobilfluide – used | 790 | 2450 | 380 |

Tamada et al. (2012) investigated the effect of biodegradation on toxicity. Therefore toxicity levels of lubricant oils (mineral lubricant oil, synthetic lubricant oil and used lubricant oil collected in oil changing places) were compared after different biodegradation periods (0 days, 60 days, 120 days and 180 days) using earthworm (Eisenia andrei), arugula seeds (Eruca sativa) and lettuce seeds (Lactuca sativa).

For the synthetic oil the toxicity decreased when the biodegradation phase increased. For the mineral oil results were contradictory (for Eruca sativa and Eisenia andrei a decrease in toxicity was measured while an increase was measured for the Latuca sativa when the biodegradation period increased). The used oil did not show a reduced toxicity when the biodegradation period increased. This was explained by the high level of heavy metals (from vehicles engine usage), which do not suffer degradation.

## 3.3 Preliminary test 1: Daphnia sp. – Acute toxicity test

### 3.3.1 Objective

A preliminary acute immobilisation test using Daphnia sp. was executed in order to evaluate the toxicity of a mineral chainsaw oil (Huile chaine of DOLMAR – available on the Belgian market) and a ready biodegradable gear oil from Fuchs Europe Schmierstoffe GMBH (ACR1307/01_A MFA 111028078).

The test was executed in line with OECD 202 (adopted 13 April 2004) '*Daphnia* sp., Acute Immobilisation Test'. In this test young daphnids were exposed to the test substances at a range of concentrations (1 mg/l, 10 mg/l and 100 mg/l) for a period of 48 hours. Immobilisation was recorded after 24 hours and 48 hours and compared with control values. The $EC_{50}$ after 48 hours was calculated.

OECD 202 prescribes that the test substance in the test solutions should not exceed the limit of solubility in the dilution water. In order to evaluate this a preliminary test in order to evaluate the water solubility of the test substances was executed (based on OECD 105 'Water Solubility – Preliminary test').

The Daphnia acute immobilisation test was started on Sep-16-2013. The immobilisation was recorded after 24 hours (Sep-17-2013) and after 48 hours (Sep-18-2013).

### 3.3.2 Determination of the water solubility

Different amounts of water were added at room temperature to approximately 0.1 g of the sample. The mixtures were shaken for approximately 17 hours and they were visually checked for any undissolved parts of the sample. A visual presentation of the cylinders with test substances mineral chainsaw oil and ACR1307/01_A MFA 111028078 is given in Figure 1 and Figure 2, respectively.

For test item mineral chainsaw oil it was noticed that the liquid became white and turbid in the high concentrations. The mixture with the lowest concentration (200 mg/l) was not yet completely clear, but the turbidity was already considerable lower when compared to the other concentration series.

For test item ACR1307/01_A MFA 111028078 the liquid was rather clear in the lowest concentration (200 mg/l). Only after a careful inspection still some tiny oil drops were observed at the surface of the liquid.

| 0.1 g – 10 ml<br>10000 mg/l | 0.1 g – 100 ml<br>1000 mg/l | 0.1 g – 250 ml<br>400 mg/l | 0.1 g - 500 ml<br>200 mg/l |
|---|---|---|---|
|  |  |  |  |

**Figure 1. Visual presentation of solubility test on test item mineral chainsaw oil (Huile chaine of DOLMAR).**

| 0.1 g – 10 ml<br>10000 mg/l | 0.1 g – 100 ml<br>1000 mg/l | 0.1 g – 250 ml<br>400 mg/l | 0.1 g - 500 ml<br>200 mg/l |
|---|---|---|---|
|  |  |  |  |

**Figure 2. Visual presentation of solubility test on test item ACR1307/01_A MFA 111028078 (Fuchs Europe Schmierstoffe GMBH).**

### 3.3.3 Test set-up

The different test concentrations for test item Huile chaine of DOLMAR were prepared in following way:

- Test series 100 mg/l
  - 0.0507 g sample was added to 500.01 ml water
  - Test item concentration = 101 mg/l
  - Solution was stirred with a stir bar overnight in order to obtain a homogenous solution before following step was executed
- Test series 10 mg/l
  - 10 ml of test series 100 mg/l was added to an Erlenmeyer
  - Water was added until 100 ml
  - Test item concentration = 1.01 mg / 100 ml = 10 mg/l
  - Solution was stirred with a stir bar in order to obtain a homogenous solution before following step was executed
- Test series 1 mg/l
  - 10 ml of test series 10 mg/l was added to an Erlenmeyer
  - Water was added until 100 ml
  - Test item concentration = 0.1 mg / 100 ml = 1 mg/l

The same approach was followed in order to prepare the different test concentrations for test item ACR1307/01_A MFA 111028078.

The test was executed in a multiwell plate (manufactured by MicroBioTests Inc., Industrie-zone 'De Prijkels', Venecoweg 19, 9810 Nazareth, Belgium). A visual presentation of this plate is given in Figure 3. Each multiwell plate is provided with 4 test wells for the controls (= row X in Figure 3) and 4 test wells for each toxicant concentration (rows 1, 2 and 3 for test series 1 mg/l, 10 mg/l and 100 mg/l, respectively). On the left side the multiwell plates are provided with a column of rinsing wells. These wells serve to prevent dilution of the toxicant in the multiwell cups during the transfer of the organisms. 10 ml dilution water was trans-ferred into each well of the control row, while 10 ml of the respective toxicant concentrations were transferred into each well of the corresponding rows. At least 20 actively swimming Daphnids were transferred from the hatching petri dish into the rinsing wells and consequent-ly exactly 5 Daphnids were transferred from the rinsing wells to the 4 test wells of the same rows. The multiwell plate was placed in the incubator at 20°C in darkness.

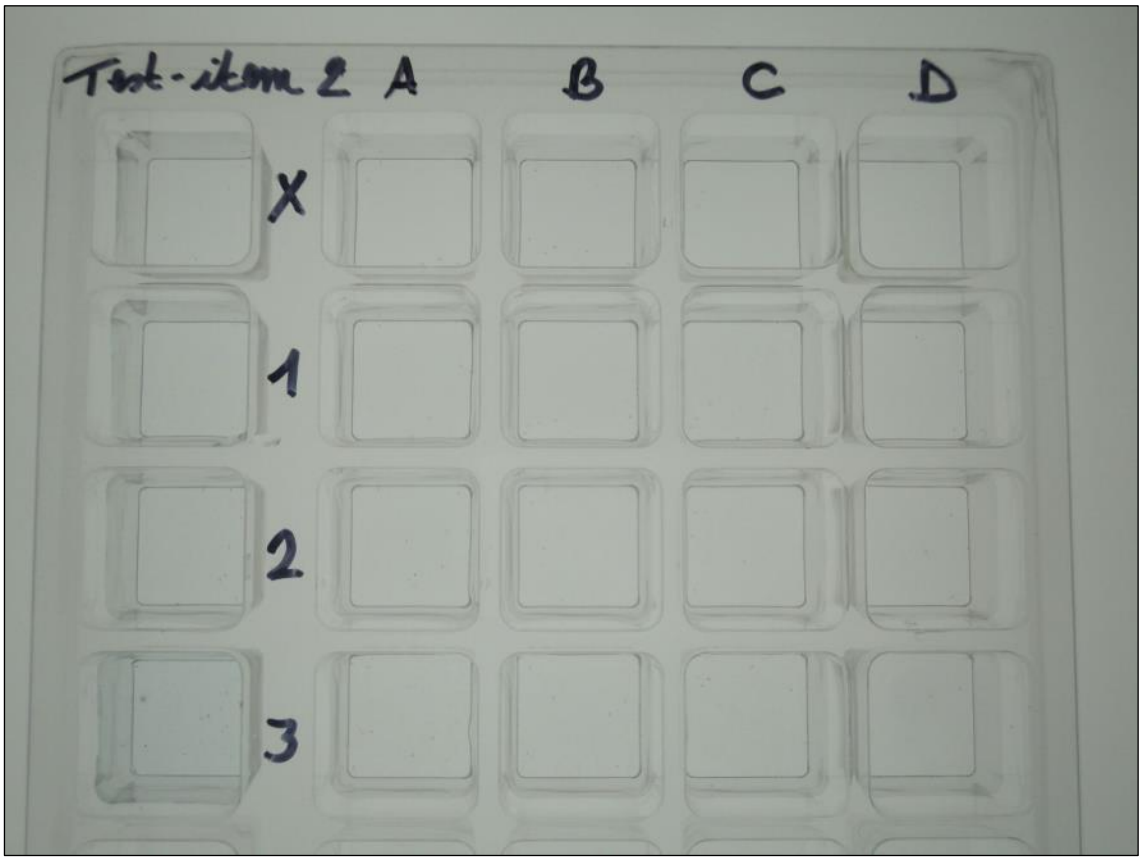



**Figure 3. Overview of the test set-up of the Daphnia sp., Acute Immobilisation Test.**

### 3.3.4 Results

Figure 4 and Figure 5 show the percentage mobile Daphnia's after 48 hours in 3 concentrations (1 mg/l, 10 mg/l and 100 mg/l) of mineral chainsaw oil (Huile chaine of DOLMAR – available on the Belgian market) and a ready biodegradable gear oil (ACR1307/01_A MFA 111028078), respectively.

Based on these figures it can be concluded that the results of this test were not reliable. Mobility should increase when the concentration decreases. This was not observed. For both samples the highest mobility (= lowest effect) was observed in the 10 mg/l concentration series, while the mobility in the 1 mg/l series was significantly lower. A considerable amount of the Daphnia that remained mobile after 48 hours showed abnormal rotating.

➔ **CONCLUSION:** The preparation of the concentration series (1 mg/l, 10 mg/l and 100 mg/l) with a dilution series is not appropriate for this type of test materials (= lubricants) as poorly water soluble substances are not distributed homogenously in an aqueous medium.

Therefore, the test will be repeated:

- The different concentrations will not be prepared with a dilution series, but per test item concentration an exact amount of sample will be added. In this way the test item concentration per bottle is clearly determined and mistakes related to the dilution will be excluded.
- The test will also be executed with a reference material in order to validate that the test conditions are reliable. OECD 202 (Adopted 13 April 2004) 'Daphnia sp., Acute Immobilisation Test' that a reference substance may be tested for $EC_{50}$ as a means of assuring that the test conditions are reliable. Toxicants used in international ring-tests (ISO 6341 'Water quality – Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) – Acute toxicity test') are recommended by OECD 202 for this purpose. Potassium dichromate will be used as a reference material. According to ISO 6341 (2012) the 24 h $EC_{50}$ needs to fall inside the range 0.6 mg/l and 2.1 mg/l.

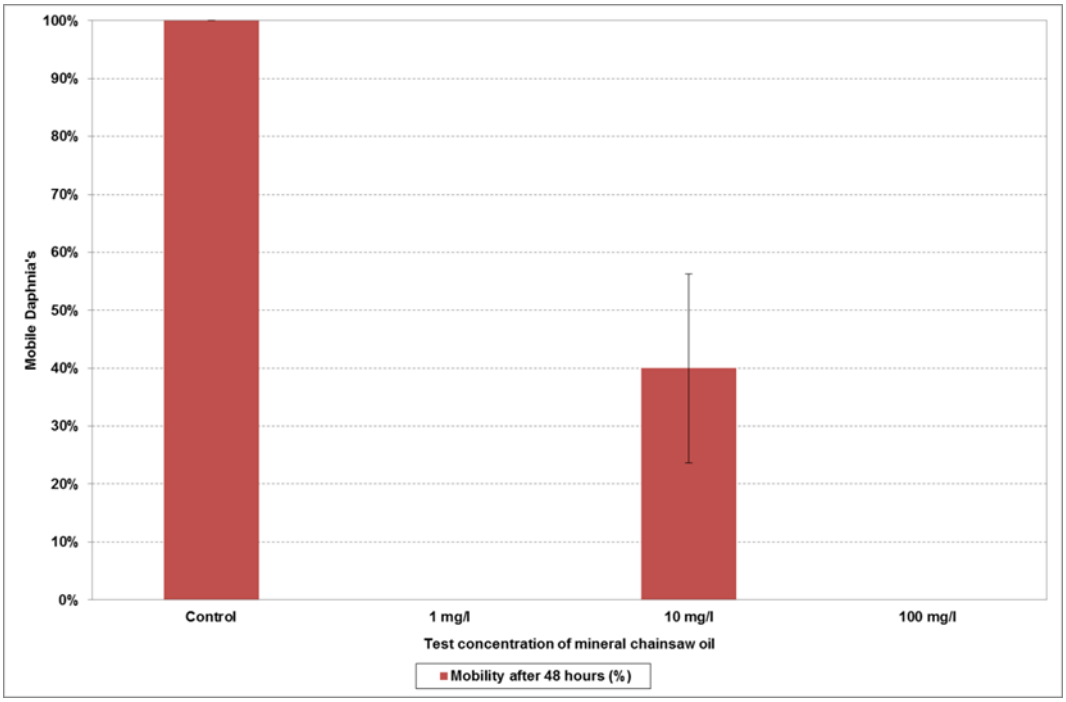



**Figure 4. Percentage mobile Daphnia's after 48 hours in 3 concentrations (1 mg/l, 10 mg/l and 100 mg/l) of mineral chainsaw oil (Huile chaine of DOLMAR).**

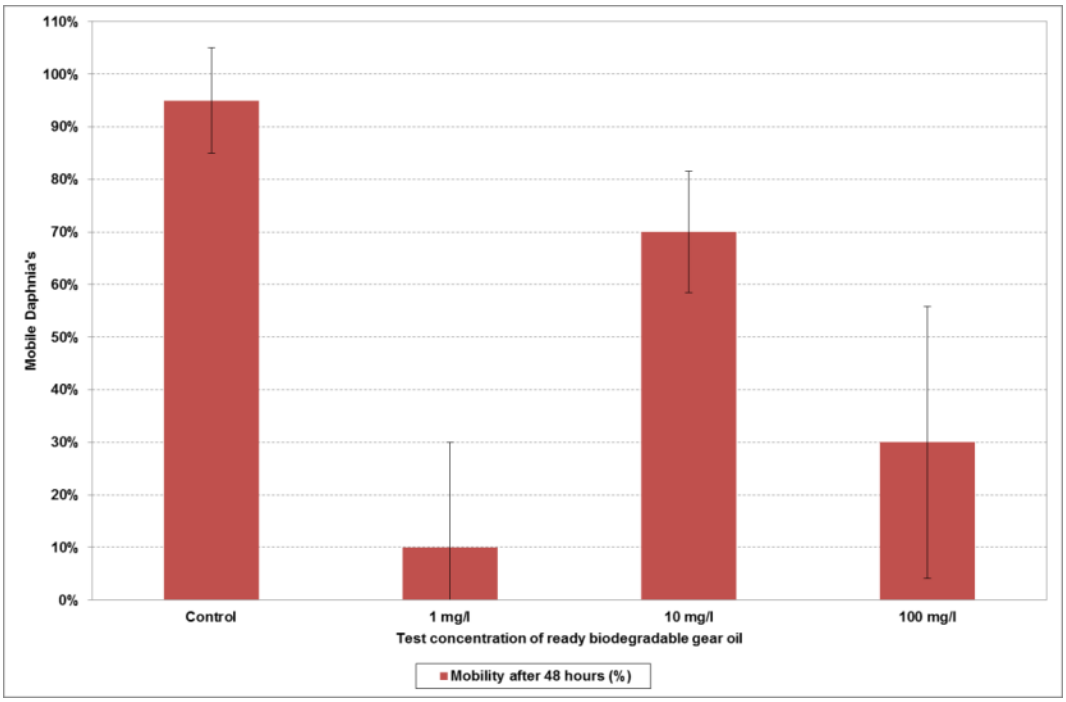



**Figure 5. Percentage mobile Daphnia's after 48 hours in 3 concentrations (1 mg/l, 10 mg/l and 100 mg/l) of ready biodegradable gear oil (ACR1307/01_A MFA 111028078).**

### 3.3.5 Modified test set-up

Due to the problems in the first test set-up, another preparation method for the concentration series was applied.

In a first phase it was tried to homogenise a solution containing 100 mg test item/l by means of an ultrasonic bath (Fisher Scientific – FB15054). Approximately 0.05 g of both test items were added to an Erlenmeyer flask and 500 ml distilled water was added in order to obtain a concentration of 100 mg/l. The Erlenmeyer flask was placed in the ultrasonic bath during 0.5 hour. After 0.5 hour the test item was not homogenised. A droplet of sample remained present on the bottom of the Erlenmeyer flask (Figure 6; blue circle indicates the droplet).


**Figure 6. Visual presentation of an Erlenmeyer with 100 mg/l test item after 0.5 hour in an ultrasonic bath (blue circle: sample).**

As preparing concentrations series of a lubricant by means of dilutions series and homogenisation in an ultrasonic bath did not result in homogenous test series, another approach was followed in a second phase.

The American standard practice for aquatic toxicity testing of lubricants ASTM D6081 – 98 (Reapproved 2009) was used. This practice is suitable for use of fully-formulated lubricants or their components that are not completely soluble at the intended test treat rates. Samples prepared in accordance with this practice may be used in aquatic toxicity tests conducted in fresh water or salt water with algae, large invertebrates and fish.

When the test material is not completely soluble at the test treat rates, toxicity is expressed by means of loading rates (ELXX (Effect Load XX) instead of ECXX (Effect Concentration XX), ILXX (Inhibition Load XX) instead of ICXX (Inhibition Concentration XX) or as LLXX (Lethal Load XX) instead of LCXX (Lethal Concentration XX)). The loading rate refers to the ratio of test material to aqueous medium used in the preparation of WAF (Water Accommodated Fraction), WSF (Water Soluble Fraction) or mechanical dispersion.

As the international standard ISO 15380 (2011) “Lubricants, industrial oils and related products (class L) – Family H (Hydraulic systems) – Specifications for categories HETG, HEPG, HEES and HEPR”, which gives specifications for environmentally acceptable hydraulic fluids, mentions that fluids with low water solubility shall be tested using water accommodated fractions, we will focus on the preparation of water accommodated fractions in order to execute the toxicity tests.

According to ASTM D6081 the water accommodated fraction is the predominantly aqueous portion of a mixture of water and a material poorly soluble in water, which separates in a specified period of time after the mixture has undergone a specified degree of mixing and which includes water, dissolved components and dispersed droplets of the poorly water soluble material.

In order to prepare the exposure matrix the test material should be added directly to the dilution water. Individual WAFs must be generated for each test exposure loading. Serial dilutions of a single WAF are not appropriate due to differential solubility of constituents at low exposure loads.

Height to diameter ratio for the WAF preparation at initiation should be between 1:1 and 2:1 and solutions should not exceed 20 l per individual preparation.

Vessels for WAF preparation should be filled with the appropriate volume of dilution water. Consequently the test material should be slowly added to the top of each vessel after dilution water addition. The test vessel should be capped with foil or a non-reactive covering and stirred at test temperature. The stir plates should be run at a sufficient speed to ensure a vortex depth of 10 to 35 % of the test solution height in the WAF preparation vessel. The test material may not be pulled down to the bottom of the vessel and the rate of stirring may not be so vigorous as to promote emulsification. A standard 20 to 24 hour mixing and 1 to 4 hours settling period should be used. After the mixing and settling period, the aqueous solution should be drained or decanted from below the surface of each preparation vessel. Non-dispersed test material may not be collected with the WAF. The first 5 to 10 ml of solution decanted from the vessel should be discarded. The remaining solution should be used undiluted in the exposure vessels. Test organisms should be placed in the test solutions within 60 min after preparation at study initiation.

The exposure loads used in the toxicity test are based on the loading rate of test material into the preparation vessels.
Following test series were prepared:

- Reference material = Potassium dichromate ($K_2Cr_2O_7$)
  - 0.5 mg/l
  - 1 mg/l
  - 2 mg/l
  - 2.5 mg/l
- Test item 1 = Huile chaine of DOLMAR
  - 0.595 ml test item (= 0.50 g) was added to 5 l standard freshwater in order to prepare the WAF (Visual presentation test set-up: Figure 7)
- Test item 2 = ACR1307/01_A MFA 111028078
  - 0.575 ml test item (= 0.50 g) was added to 5 l standard freshwater in order to prepare the WAF

Remark: As the test guide with regard to the preparation of the WAF prescribes that the test item needs to be added to the dilution water (5 l), it was not possible to place the entire vessel on the analytical balance. Consequently, the volume corresponding to approximately 0.5 g was determined with an automatic pipette.

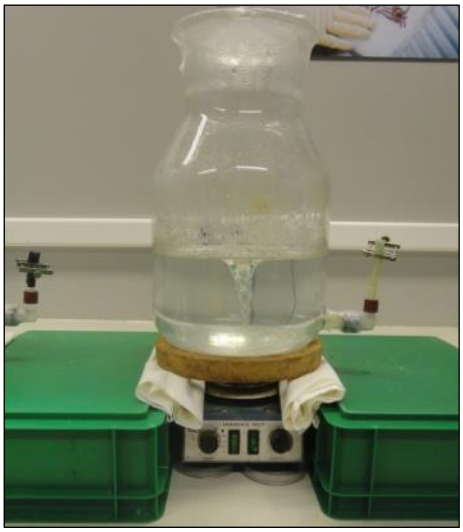


**Figure 7. Visual presentation of the test set-up in order to prepare the WAF.**

The test was executed in a multiwell plate (manufactured by MicroBioTests Inc., Industriezone 'De Prijkels', Venecoweg 19, 9810 Nazareth, Belgium). The multiwell plate was placed in the incubator at 20°C in darkness.

### 3.3.6 Results

Figure 8 shows the percentage mobile Daphnia's after 24 hours and after 48 hours in 4 concentrations (0.5 mg/l, 1 mg/l, 2 mg/l and 2.5 mg/l) of reference material $K_2Cr_2O_7$. According to ISO 6341 (2012) the 24 h $EC_{50}$ of potassium dichromate needs to fall within the range 0.6 mg/l up to 2.1 mg/l. In concentration series 0.5 mg/l no immobility was observed, while 95% immobile Daphnia's were observed in concentration series 1 mg/l and 2 mg/l. This indicates that the $EC_{50}$ of $K_2Cr_2O_7$ falls between 0.5 mg/l and 1 mg/l and that the test is valid.

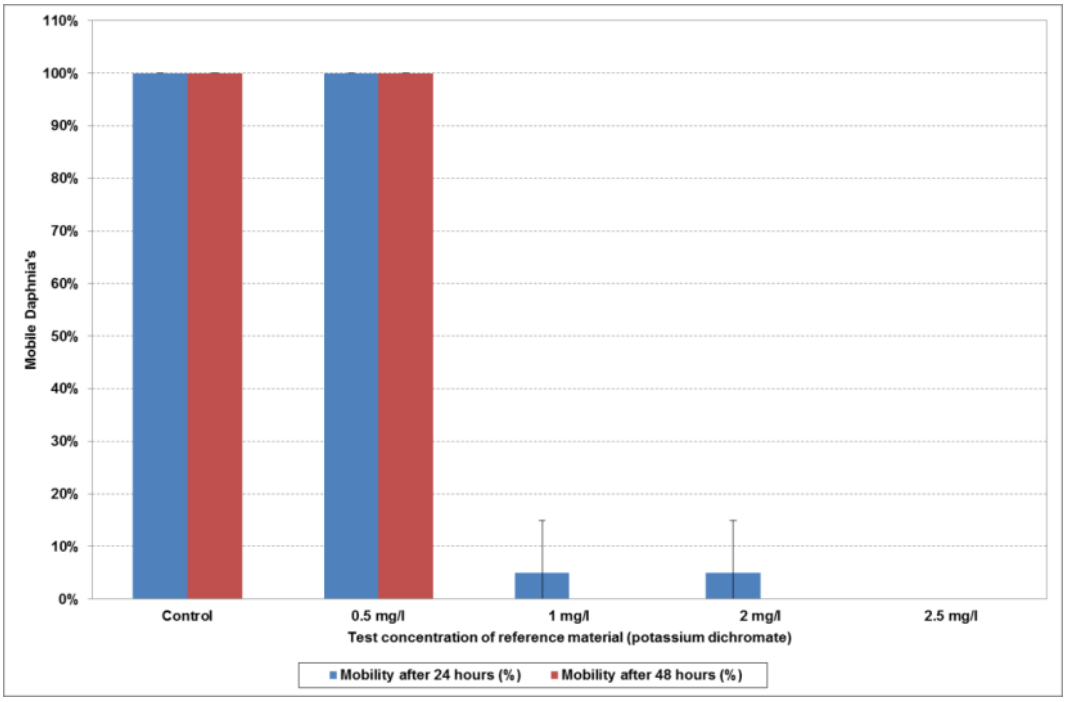



**Figure 8. Percentage mobile Daphnia's after 24 hours and after 48 hours in 4 concentrations (0.5 mg/l, 1 mg/l, 2 mg/l and 2.5 mg/l) of reference material ($K_2Cr_2O_7$).**

Figure 9 shows a visual presentation of the percentage mobile Daphnia's after 24 hours and after 48 hours in 100 mg/l (WAF) mineral chainsaw oil (Huile chaine of DOLMAR – available on the Belgian market) and in 100 mg/l (WAF) ready biodegradable gear oil (ACR1307/01_A MFA 111028078).

The $EL_{50}$ of mineral chainsaw oil is lower than 100 mg/l, while the $EL_{50}$ of the ready biodegradable gear oil is higher than 100 mg/l. It was noticed that all Daphnia that remained mobile in the mineral chainsaw oil after 24/48 hours showed abnormal rotating.

➔ **CONCLUSION:** The executed tests demonstrate that the WAF principle should be used in order to evaluate the ecotoxicological impact of lubricants on aquatic organisms. This should be mentioned in labelling schemes and specifications.

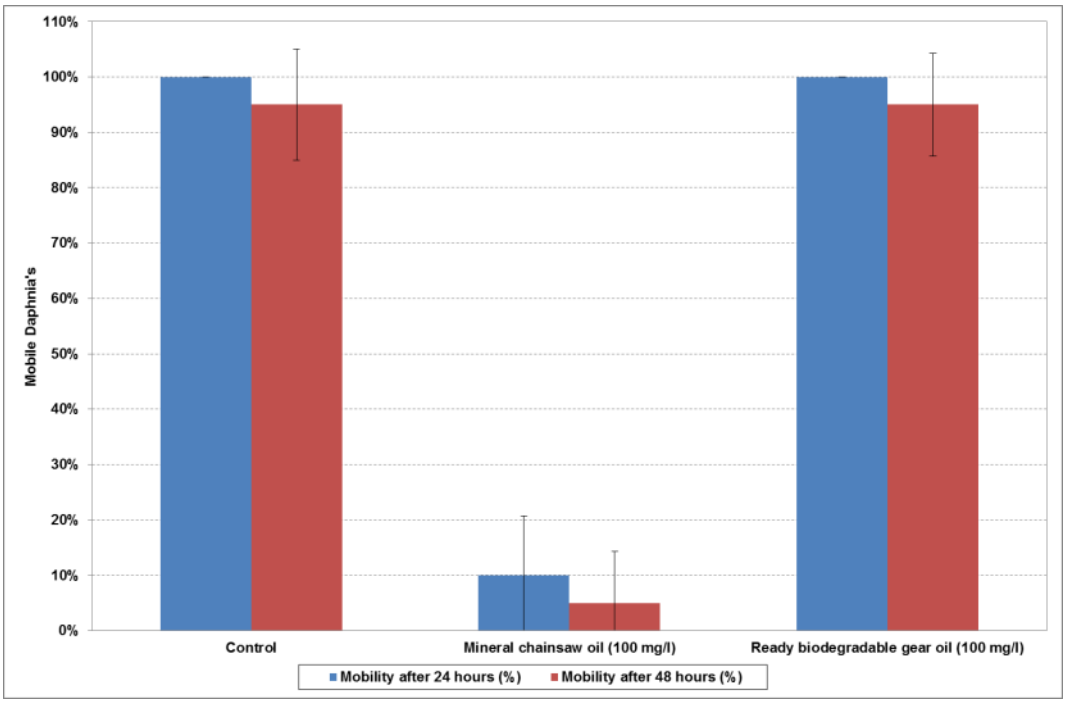



**Figure 9. Percentage mobile Daphnia's after 24 hours and after 48 hours in 100 mg/l (WAF) mineral chainsaw oil (Huile chaine of DOLMAR) and 100 mg/l ready biodegradable gear oil (ACR1307/01_A MFA 111028078) (WAF).**

## 3.4 Preliminary test 2: Daphnia sp. – Acute toxicity test on residuals after biodegradation

### 3.4.1 Objective

A biodegradation test in freshwater (OECD 301F) was executed on positive reference material rapeseed oil, biolubricants L1323/1 (hydraulic oil), L1323/2 (gear oil) and ACR1307/01_A MFA 111028078 (gear oil). The samples were added to the medium with an inert plastic carrier. At start of the test approximately 10 mg sample was added to 250 ml (245 ml mineral medium and 5 ml activated sludge). Consequently the concentration at start was 40 mg/l. The duration of the test was 63 days. The detailed results of the biodegradation test are reported in deliverable 6.2. (Chapter 5.6.10 & Chapter 5.6.11) and the evolution of the biodegradation is given in Figure 10.

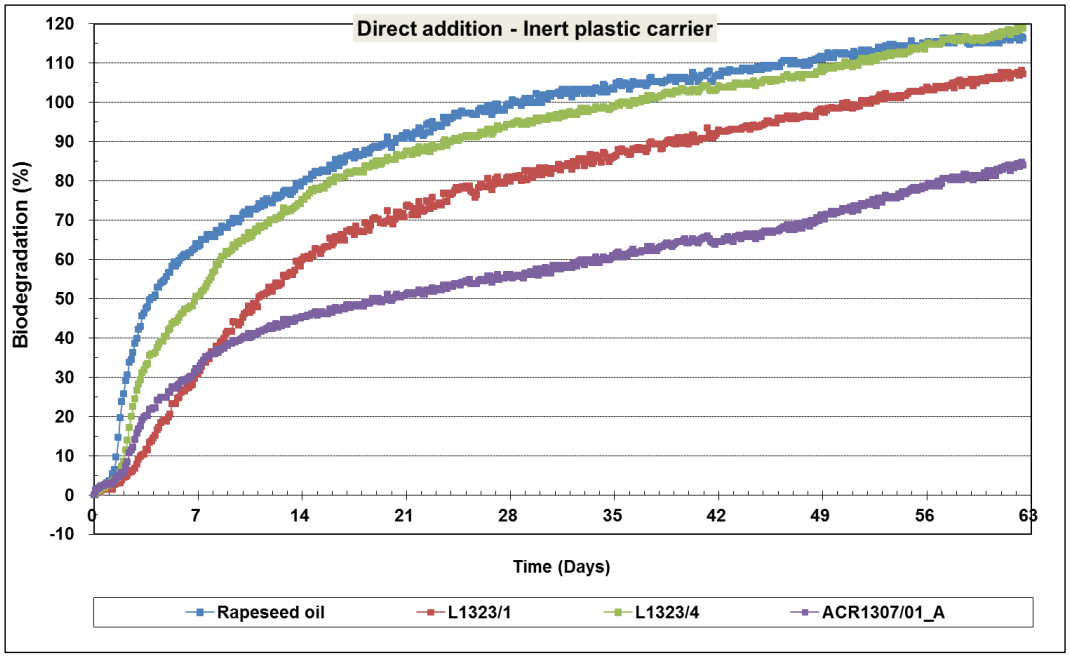



**Figure 10. Evolution of the biodegradation of rapeseed oil, L1323/1, L1323/2 and ACR1307/01_A MFA 111028078 based on the oxygen consumption (addition method: plastic) up to 63 days.**

The test was executed in line with OECD 202 (adopted 13 April 2004) '*Daphnia* sp., Acute Immobilisation Test'. In this test young daphnids were exposed to the residuals obtained after the biodegradation test for a period of 48 hours. Immobilisation was recorded at 24 hours and 48 hours and compared with control values. The $EC_{50}$ at 48 hours was calculated.

The Daphnia acute immobilisation test was started on May-19-2014. The immobilisation was recorded after 24 hours (May-20-2014) and after 48 hours (May-21-2014). Between the end of the biodegradation test and the start-up of the toxicity test, the samples were stored in the freezer.

### 3.4.2 Test set-up

The test set-up is given in Table 3. The water obtained at the end of the biodegradation test was homogenized and 10 ml was added per vial. Four replicates were evaluated per test series.

**Table 3. Test set-up of the daphnia acute toxicity test of water residuals obtained at the end of KBBPPS-27.**

| Test series | Content |
|---|---|
| Control water | Mixture of reactors 13, 14 & 15 |
| Rapeseed oil water | Content of reactor 16 |
| L1323/1 water | Mixture of reactors 17 & 18 |
| L1323/4 water | Mixture of reactors 19, 20 & 21 |
| ACR 1307/01_A water | Mixture of reactors 22, 23 & 24 |

The test was executed in a multiwell plate (manufactured by MicroBioTests Inc., Industriezone 'De Prijkels', Venecoweg 19, 9810 Nazareth, Belgium). At least 20 actively swimming Daphnids were transferred from the hatching petri dish into the rinsing wells and consequently exactly 5 Daphnids were transferred from the rinsing wells to the 4 test wells of the same rows. The multiwell plate was placed in the incubator at 20°C in darkness.

### 3.4.3 Results

Figure 11 shows a visual presentation of the percentage mobile Daphnia's after 24 hours and after 48 hours in control water, Rapeseed oil water, L1323/1 water, L1323/4 water and ACR 1307/01_A water obtained at the end of the aquatic biodegradation test KBBPPS-27 (see deliverable 6.2).

After 24 hours at least 90 % of the daphnia remained mobile in all test series. After 48 hours a decrease in the mobility was observed for the different test series (except for the standard freshwater and the control water obtained at the end of the biodegradation test). The mobility of the daphnia in the series to which lubricants were added at start of the test varied between 55 % and 80 %.

As can be seen in Figure 10, the highest biodegradation percentage was observed for reference material rapeseed oil and lubricant L1323/4 (complete biodegradation: approximately 115 % due to priming effect). The lowest biodegradation was observed for ACR1307/01_A (approximately 85 % biodegradation after 63 days) and an intermediate biodegradation was observed for lubricant L1323/1 (approximately 110 % due to priming effect).

The lubricant sample with the lowest biodegradation was characterised by the lowest effect on the mobility of the daphnia, which was not in line with the expectations. It was expected that more non degraded residuals, which could influence daphnia negatively, would remain present for this sample.

➔ **CONCLUSION:** From this first preliminary test a limited effect (mobility of dapnia was still higher than 50 % in all test series after 48 hours) on mobility of daphnia is observed although the initial concentration at start of the biodegradation test was only 40 mg/l.

Taken into account that the concentration of ARC1307/01_A at start of the biodegradation test was lower when compared to the WAF evaluated in preliminary test 1 (Chapter 5.3), it remains difficult to draw clear conclusions. The influence of the biodegradation on the toxicity will be further evaluated in the interlaboratory test (see Chapter 7).

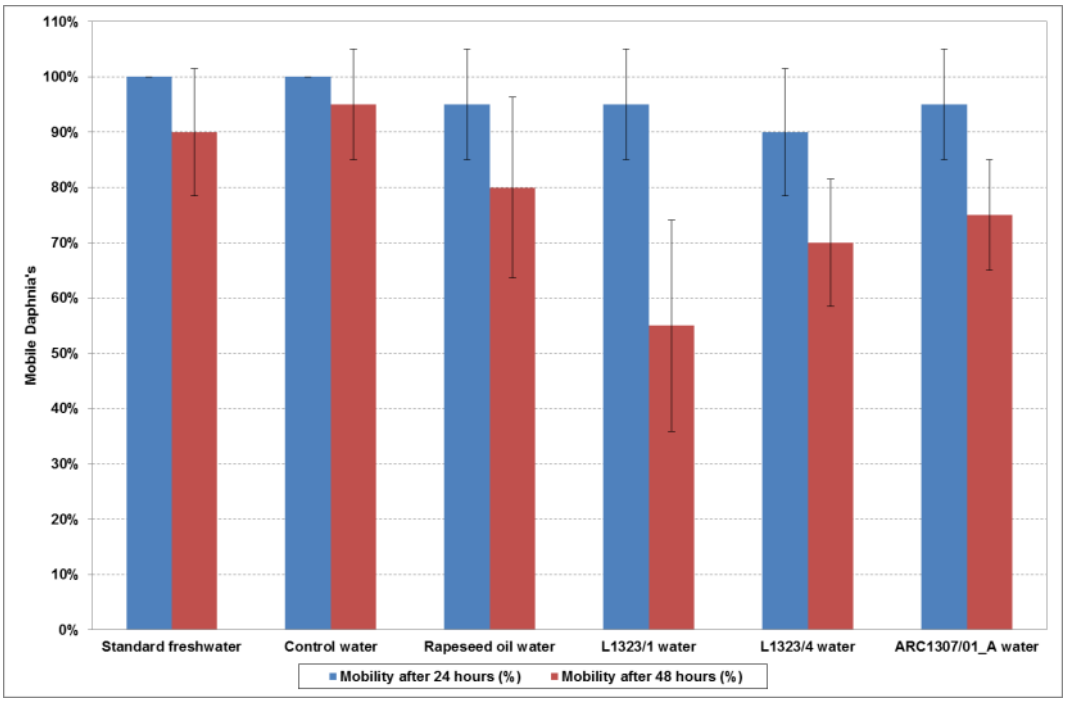



**Figure 11. Percentage mobile Daphnia's after 24 hours and after 48 hours in control water, Rapeseed oil water, L1323/1 water, L1323/4 water and ACR 1307/01_A water obtained at the end of the aquatic biodegradation test KBBPPS-27.**

# 4 Evaluation of environmental safety in soil environment

## 4.1 Evaluation of environmental safety in current labelling systems

An overview of the existing requirements with regard to environmental safety of the international specification ISO 15380, the German Blue Angel ecolabel and the European ecolabel is given in Table 1 (Chapter 3.1).

These labelling systems and specifications for environmentally friendly lubricants evaluate toxicity by means of aquatic test organisms on different trophic levels (bacteria, algae, daphnia and/or fish). In spite of the fact that lubricants are in some cases (unavoidable or accidently) released in a soil environment, toxicity by means of terrestrial organisms (soil bacteria, earthworms or plants) is not prescribed.

Terrestrial toxicity was not considered in de European ecolabel as the Dangerous Substance Directive did not set out criteria for classification of substances for non-aquatic environment. Moreover, toxicity of substances present in lubricants to terrestrial organisms is generally lower than the toxicity to aquatic organisms. This is generally expected for organic substances due to their adsorption to organic material in soil (Theodori et al., 2004).

## 4.2 Preliminary test 1: Earthworm acute toxicity test

### 4.2.1 Objective

The immediate toxicity (= not after a biodegradation phase) of a mineral chainsaw oil (Huile chaine of DOLMAR – available on the Belgian market) was evaluated. Also the toxicity of a ready biodegradable gear oil (ACR1307/01_A MFA 111028078) was evaluated.

### 4.2.2 Test set-up

Both test items were tested in 3 concentrations (100 mg/kg dry artificial soil, 1000 mg/kg dry artificial soil and 10 000 mg/kg dry artificial soil).

**Mineral chainsaw oil (Huile chaine of DOLMAR)**

In total, 12 glass jars were used. Approximately 750 g artificial soil or test soil was added to each jar. The test set-up is given in Table 4.

**Table 4. Test set-up of the earthworm toxicity test on mineral chainsaw soil (weight per jar).**

| Test series | Artificial soil (g wet weight) | Artificial soil (g dry weight) | Mineral chainsaw oil (mg wet weight) |
|---|---|---|---|
| **4 × Artificial soil** | 750 | 564 | - |
| **2 × Test series 1 (100 mg/kg)** | 750 | 564 | 56.4 |
| **3 × Test series 2 (1000 mg/kg)** | 750 | 564 | 564 |
| **3 × Test series 3 (10000 mg/kg)** | 750 | 564 | 5640 |

The artificial soil consists of a mixture of 9 % peat, 27 % kaolin clay and 64 % industrial sand (on dry weight basis). According to ASTM E 1676-04 '*Standard Guide for Conducting Laboratory Soil Toxicity or Bioaccumulation tests with the Lumbricid Earthworm Eisenia Fetida and the Enchytraeid Potworm Enchytraeus albidus*' the artificial soil should have a pH of 7.0 ± 0.5. A pH of 7.1 was measured, while a moisture content of 33.0 % on dry weight basis was measured. The used worm species is Eisenia fetida fetida. The worms are reared at OWS N.V., Dok Noord 5, B-9000 Gent, Belgium.

**Ready biodegradable gear oil (Fuchs Europe Schmierstoffe GMBH)**

In total, 13 glass jars were used. Approximately 750 g artificial soil or test soil was added to each jar. The test set-up is given in Table 5.

**Table 5. Test set-up of the earthworm toxicity test on ready biodegradable gear oil (weight per jar).**

| Test series | Artificial soil (g wet weight) | Artificial soil (g dry weight) | Biodegradable gear oil (mg wet weight) |
|---|---|---|---|
| **4 × Artificial soil** | 750 | 557 | - |
| **3 × Test series 1 (100 mg/kg)** | 750 | 557 | 55.7 |
| **3 × Test series 2 (1000 mg/kg)** | 750 | 557 | 557 |
| **3 × Test series 3 (10000 mg/kg)** | 750 | 557 | 5570 |

The artificial soil was characterised by a pH of 6.9, while a moisture content of 34.8 % on dry weight basis was measured.

In order to homogenize the sample with the artificial soil, the sample was weighted in a petri dish together with 50 g artificial soil. This was first thorough mixed and subsequently this mixture was added to the rest of the artificial soil. The obtained mixtures were thoroughly mixed before they are used for the earthworm test. This procedure is clearly less cumbersome when compared to the aquatic toxicity testing. The earthworm, acute toxicity test was done in glass jars of 1000 ml, containing a mixture of artificial soil and test item. Also the pure artificial soil was tested.

One day before start-up of the test, the worms were conditioned in the artificial soil. At the start of the test, each glass jar was filled with 750 g of test soil or artificial soil. Subsequently, 10 viable earthworms were put on top of the mixture (Figure 12). The weight of the worms was determined at start.

**Figure 12. Visual presentation of the start-up of the earthworm toxicity tests.**

After all glass jars were filled, they were closed and put at room temperature (20°C ± 2°C) and with continuous lighting. The test on the mineral chainsaw oil was started on Aug-07-2013, while the test on the ready biodegradable gear oil was started on Oct-03-2013. The test was finished after 14 days. At the end of the test the survival and the live weight of the earthworms was determined for each jar separately. Any behavioural or pathological symptoms were also noted. The toxicity of possible residuals of the test item was evaluated by comparing the survival and mean weight in the test soil to that in the artificial soil.

### 4.2.3 Results

**Mineral chainsaw oil (Huile chaine of DOLMAR)**

The test was stopped after 14 days. Table 6 shows the average percentage of survival and mortality at the end of the test. Also the live weight per worm and the average preservation of the weight at start of the surviving worms are given. The survival percentages and the mean weight percentages (as a percentage of weight at start) are also shown in Figure 13 and Figure 14. Also an overview of the earthworms retrieved at the end of the test for the different concentrations of the test mixture is given in Figure 15.

100 % survival was measured for the artificial soil, which means that the pass level of 90 % survival is easily reached and that the test is valid.

No mortality was observed in the different test series. This implies that the $LC_{50}$ of the mineral chainsaw oil is > 10 000 mg/kg dry artificial soil.

The average weight of the earthworms in the test series with 10 000 mg mineral chainsaw oil per kg dry artificial soil was somewhat lower when compared to the other series. For the artificial soil and the 100 mg/kg and 1000 mg/kg series an increase in the mean weight was observed, while a decrease was observed for the 10 000 mg/kg series (86 % compared to the weight at start).

When comparing the results of the aquatic toxicity tests with Daphnia with this test, it can be concluded that Daphnia are more sensitive when compared to earthworms.

**Table 6. Average and standard deviation of percentage survival, mortality and live weight yield for each test series.**

| Test series | Survival (%) | | Mortality (%) | Live weight yield (g per worm) | | Live weight yield (% of start) | |
|---|---|---|---|---|---|---|---|
| | AVG | STD | AVG | AVG | STD | AVG | STD |
| Artificial soil | 100 | 0 | 0 | 0.34 | 0.01 | 110 | 2 |
| 100 mg test item/kg | 100 | 0 | 0 | 0.38 | 0.03 | 107 | 3 |
| 1000 mg test item/kg | 100 | 0 | 0 | 0.39 | 0.01 | 108 | 3 |
| 10000 mg test item/kg | 100 | 0 | 0 | 0.31 | 0.01 | 86 | 1 |

With AVG = average and STD = standard deviation.

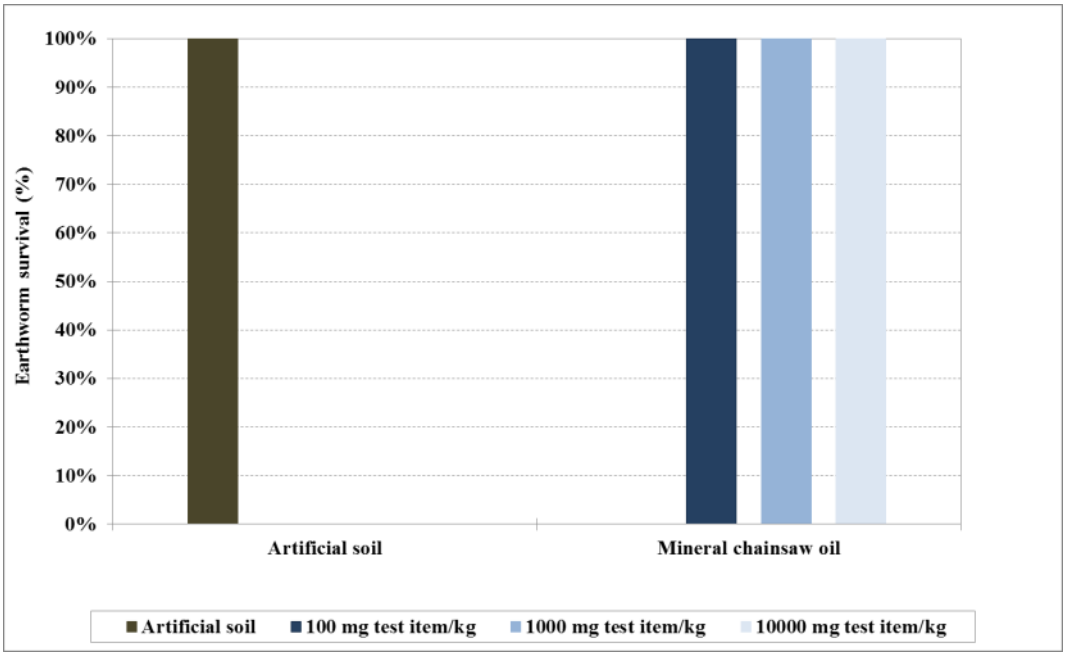



**Figure 13. Average survival of earthworms in the artificial soil and the different concentration series (Mineral chainsaw oil).**

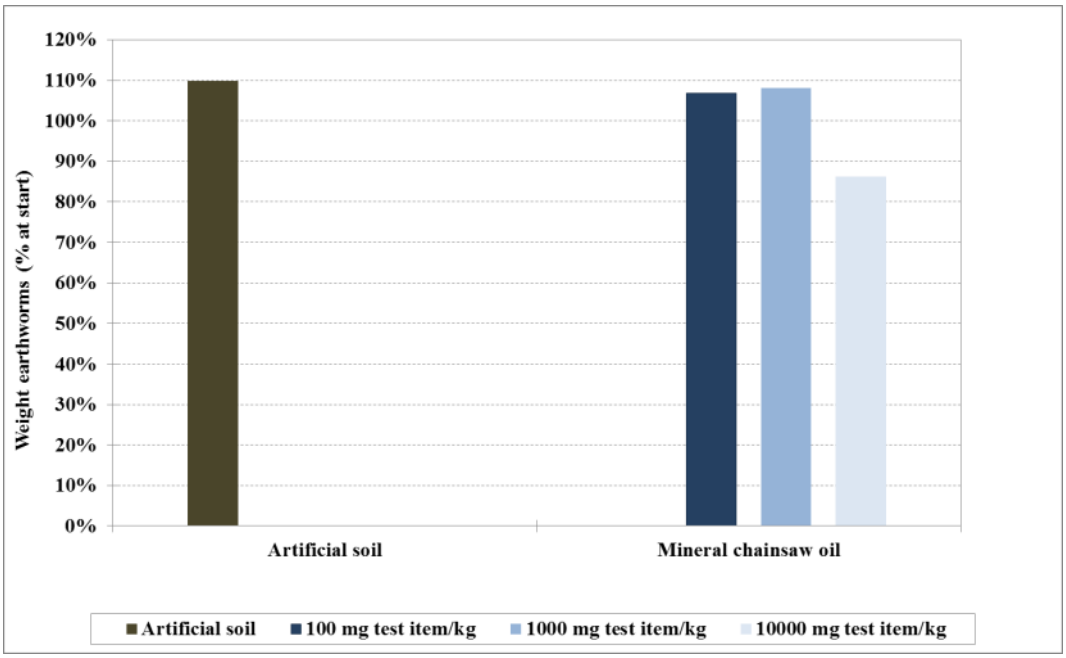



**Figure 14. Average weight of earthworms (as % of weight at start) in the artificial soil and the different concentration series (Mineral chainsaw oil).**

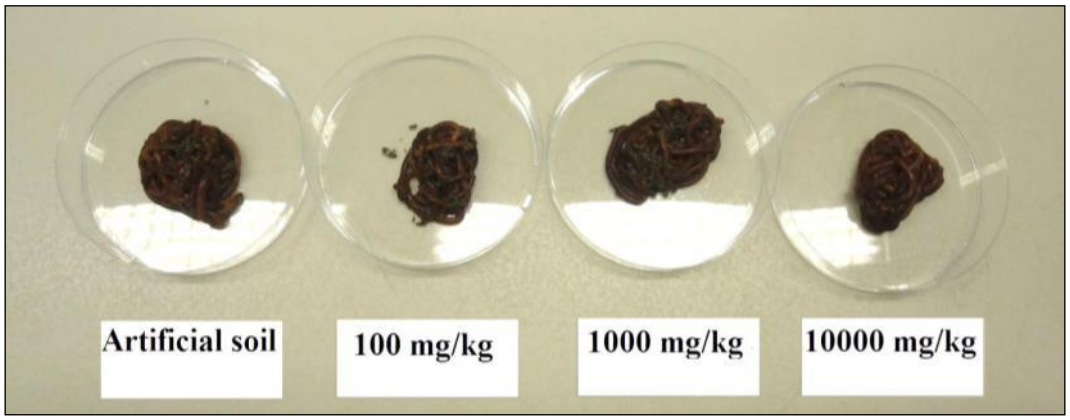



**Figure 15. Overview of the earthworms retrieved at the end of the test in the different concentration series (Mineral chainsaw oil).**

At the end of the earthworm, acute toxicity test, the artificial soil and the different test series were analysed for pH and salt content. The results of all these analyses are given in Table 7. For all test series rather neutral pH levels were measured (6.7 – 7.1). A low salt content was measured for the different test series.

**Table 7. Chemical analysis on the artificial soil and the different test series (Mineral chainsaw oil).**

| Test series | pH | EC (µS/cm) |
|---|---|---|
| **Artificial soil** | 6.7 | 140 |
| **Test series 1 (100 mg/kg)** | 6.9 | 150 |
| **Test series 2 (1000 mg/kg)** | 7.0 | 140 |
| **Test series 3 (10000 mg/kg)** | 7.1 | 150 |

**Ready biodegradable gear oil (Fuchs Europe Schmierstoffe GMBH)**

The test was stopped after 14 days. Table 8 shows the average percentage of survival and mortality at the end of the test. Also the live weight per worm and the average preservation of the weight at start of the surviving worms are given. The survival percentages and the mean weight percentages (as a percentage of weight at start) are also shown in Figure 16 and Figure 17 Also an overview of the earthworms retrieved at the end of the test for the different concentrations of the test mixture is given in Figure 18.

100 % survival was measured for the artificial soil, which means that the pass level of 90 % survival is easily reached and that the test is valid.

No mortality was observed in the different test series. This implies that the $LC_{50}$ of ready biodegradable gear oil (ACR1307/01_A MFA 111028078) is > 10 000 mg/kg dry artificial soil.

The average weight of the earthworms in the test series was higher when compared to the average weight in the artificial soil series. Consequently, no toxic effect was observed.

**Table 8. Average and standard deviation of percentage survival, mortality and live weight yield for each test series.**

| Test series | Survival (%) | | Mortality (%) | Live Weight Yield (g per worm) | | (% of start) | |
|---|---|---|---|---|---|---|---|
| | AVG | STD | AVG | AVG | STD | AVG | STD |
| Artificial soil | 100 | 0 | 0 | 0.34 | 0.01 | 109 | 4 |
| | | | | | | | |
| 100 mg test item/kg | 100 | 0 | 0 | 0.38 | 0.03 | 121 | 5 |
| 1000 mg test item/kg | 100 | 0 | 0 | 0.39 | 0.02 | 126 | 5 |
| 10000 mg test item/kg | 100 | 0 | 0 | 0.39 | 0.01 | 126 | 5 |

With AVG = average and STD = standard deviation.

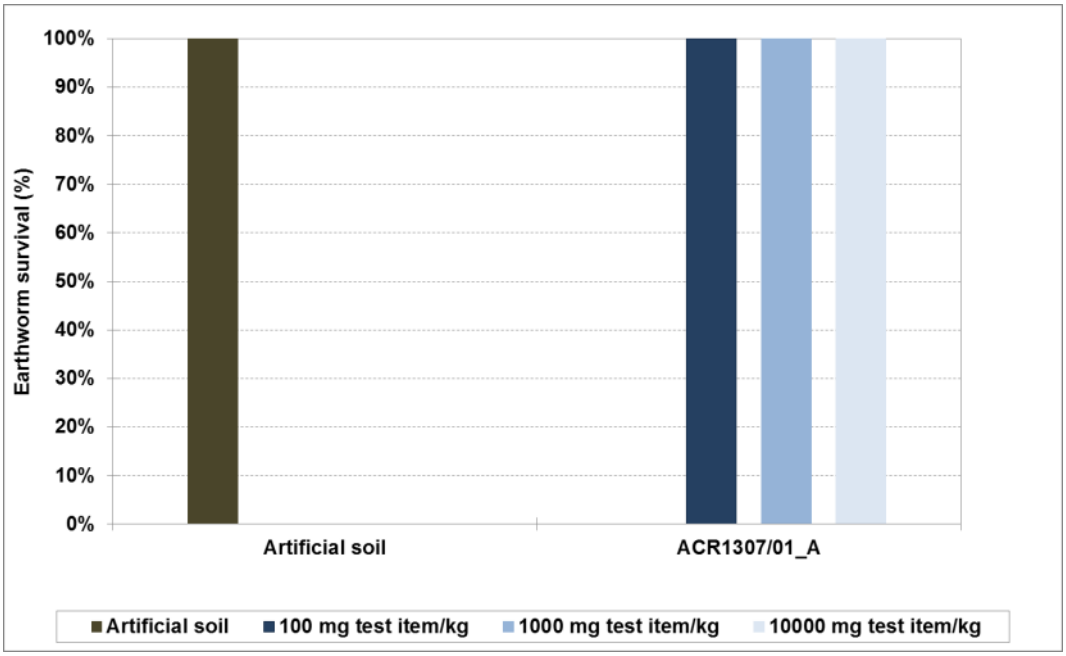



**Figure 16. Average survival of earthworms in the artificial soil and the different concentration series (ACR1307/01_A MFA 111028078).**

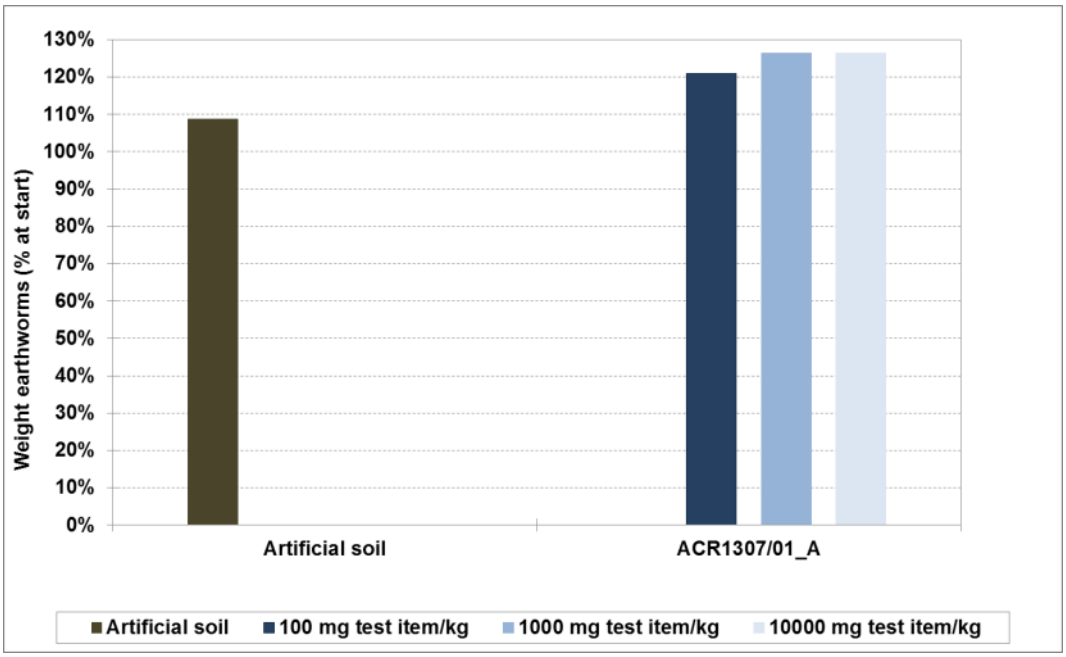



**Figure 17. Average weight of earthworms (as % of weight at start) in the artificial soil and the different concentration series (ACR1307/01_A MFA 111028078).**

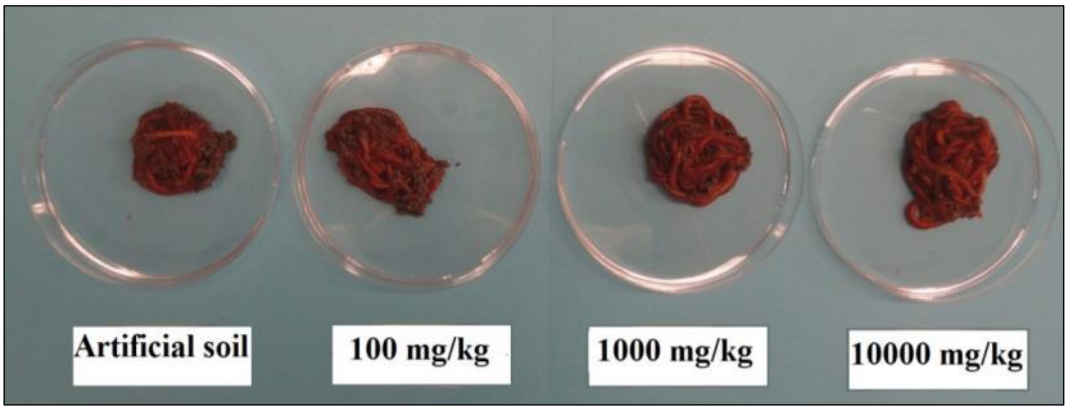



**Figure 18. Overview of the earthworms retrieved at the end of the test in the different concentration series (ACR1307/01_A MFA 111028078).**

At the end of the earthworm, acute toxicity test, the artificial soil and the different test series were analysed for pH and salt content. The results of all these analyses are given in Table 9. For all test series rather neutral pH levels were measured (7.0 – 7.4). A low salt content was measured for the different test series.

**Table 9. Chemical analysis on the artificial soil and the different test series (ACR1307/01_A MFA 111028078).**

| Test series | pH | EC (µS/cm) |
|---|---|---|
| **Artificial soil** | 7.4 | 180 |
| **Test series 1 (100 mg/kg)** | 7.3 | 160 |
| **Test series 2 (1000 mg/kg)** | 7.0 | 140 |
| **Test series 3 (10000 mg/kg)** | 7.1 | 150 |

➔ **CONCLUSION:** From the preliminary tests on Mineral chainsaw oil and ACR1307/01_A MFA 111028078 it was observed that the acute toxicity of these samples towards earthworms was rather limited. Daphnia seem to be significantly more sensitive (especially towards the mineral chainsaw oil). The $EL_{50}$ of the mineral chainsaw oil towards daphnia was < 100 mg/l, while in the current test only a slight effect on the mean weight (and no effect on the survival) was observed even in highest concentration of 10 000 mg/kg.

The test set-up will be repeated on some additional samples in order to double-check this effect.

## 4.3 Preliminary test 2: Earthworm acute toxicity test

### 4.3.1 Objective

Previous test set-up was repeated on 3 additional samples. The immediate toxicity (= not after a biodegradation period) of biolubricants BIOMULTIUSOS 130536BM and ACR1307/01_C MFA 111028080 and mineral oil “Race oil - Road - Friction technology” was evaluated.

### 4.3.2 Test set-up

The test items were tested in 3 concentrations (100 mg/kg dry artificial soil, 1000 mg/kg dry artificial soil and 10 000 mg/kg dry artificial soil).

In total, 30 glass jars were used. Approximately 600 g artificial soil or test soil was added to each jar. The test set-up is given in Table 10.

**Table 10. Test set-up of the earthworm toxicity test on biolubricants BIOMULTIUSOS 130536BM and ACR1307/01_C MFA 111028080 and mineral oil “Race oil - Road - Friction technology” (weight per jar).**

| Test series | Artificial soil (g wet weight) | Artificial soil (g dry weight) | Test item (mg wet weight) |
|---|---|---|---|
| **3 × Artificial soil** | 600 | 446 | - |
| **3 × BIOMULTIUSOS soil (100 mg/kg)** | 600 | 446 | 0.0447 |
| **3 × BIOMULTIUSOS soil (1000 mg/kg)** | 600 | 446 | 0.4471 |
| **3 × BIOMULTIUSOS soil (10000 mg/kg)** | 600 | 446 | 4.4709 |
| **3 × ACR1307/01_C soil (100 mg/kg)** | 600 | 446 | 0.0446 |
| **3 × ACR1307/01_C soil (1000 mg/kg)** | 600 | 446 | 0.4460 |
| **3 × ACR1307/01_C soil (10000 mg/kg)** | 600 | 446 | 4.4602 |
| **3 × Race oil soil (100 mg/kg)** | 600 | 446 | 0.0679 |
| **3 × Race oil soil (1000 mg/kg)** | 600 | 446 | 0.6791 |
| **3 × Race oil soil (10000 mg/kg)** | 600 | 446 | 6.7912 |

The artificial soil consists of a mixture of 9 % peat, 27 % kaolin clay and 64 % industrial sand (on dry weight basis). According to ASTM E 1676-04 '*Standard Guide for Conducting Laboratory Soil Toxicity or Bioaccumulation tests with the Lumbricid Earthworm Eisenia Fetida and the Enchytraeid Potworm Enchytraeus albidus*' the artificial soil should have a pH of 7.0 ± 0.5. A pH of 6.9 was measured, while a moisture content of 34.6 % on dry weight basis was measured. The used worm species is Eisenia fetida fetida. The worms are reared at OWS N.V., Dok Noord 5, B-9000 Gent, Belgium.

After all glass jars were filled, they were closed and put at room temperature (20°C ± 2°C) and with continuous lighting. The tests were started on Jan-08-2014. The test was finished after 14 days. At the end of the test the survival and the live weight of the earthworms was determined for each jar separately. Any behavioural or pathological symptoms were also noted. The toxicity of possible residuals of the test item was evaluated by comparing the survival and mean weight in the test soil to that in the artificial soil.

### 4.3.3 Results

The test was stopped after 14 days. Table 11 shows the average percentage of survival and mortality at the end of the test. Also the live weight per worm and the average preservation of the weight at start of the surviving worms are given. The survival percentages and the mean weight percentages (as a percentage of weight at start) are also shown in Figure 19 and Figure 20.

100 % survival was measured for the artificial soil, which means that the pass level of 90 % survival is easily reached and that the test is valid.

No mortality was observed in the different test series. This implies that the $LC_{50}$ of the different samples is > 10 000 mg/kg dry artificial soil.

➔ **CONCLUSION:** In total (previous test and current test) two mineral and 3 bio-based lubricants were evaluated on toxicity toward earthworms in high concentrations (10 000 mg per kg dry soil). For all lubricants the $EC_{50}$ (based on survival and mean weight) was higher than 10 000 mg/kg in spite of the fact that some of the samples (race oil) were labelled as dangerous for the environment. Consequently earthworms are less sensitive that aquatic organisms towards the evaluated samples.

**Table 11. Average and standard deviation of percentage survival, mortality and live weight yield for each test series.**

| Test series | Survival (%) | | Mortality (%) | Live weight yield (g per worm) | | Live weight yield (% of start) | |
|---|---|---|---|---|---|---|---|
| | AVG | STD | AVG | AVG | STD | AVG | STD |
| Artificial soil | 100 | 0 | 0 | 0.34 | 0.00 | 90 | 6 |
| Biomultiusos (100 mg/kg) | 100 | 0 | 0 | 0.33 | 0.04 | 87 | 1 |
| Biomultiusos (1000 mg/kg) | 100 | 0 | 0 | 0.37 | 0.02 | 100 | 2 |
| Biomultiusos (10000 mg/kg) | 100 | 0 | 0 | 0.38 | 0.02 | 101 | 2 |
| ACR1307/01_C (100 mg/kg) | 100 | 0 | 0 | 0.35 | 0.03 | 92 | 3 |
| ACR1307/01_C (1000 mg/kg) | 100 | 0 | 0 | 0.39 | 0.04 | 105 | 2 |
| ACR1307/01_C (10000 mg/kg) | 100 | 0 | 0 | 0.39 | 0.02 | 101 | 5 |
| Race oil (100 mg/kg) | 100 | 0 | 0 | 0.34 | 0.05 | 90 | 3 |
| Race oil (1000 mg/kg) | 100 | 0 | 0 | 0.34 | 0.01 | 91 | 3 |
| Race oil (10000 mg/kg) | 100 | 0 | 0 | 0.36 | 0.04 | 92 | 1 |

With AVG = average and STD = standard deviation.

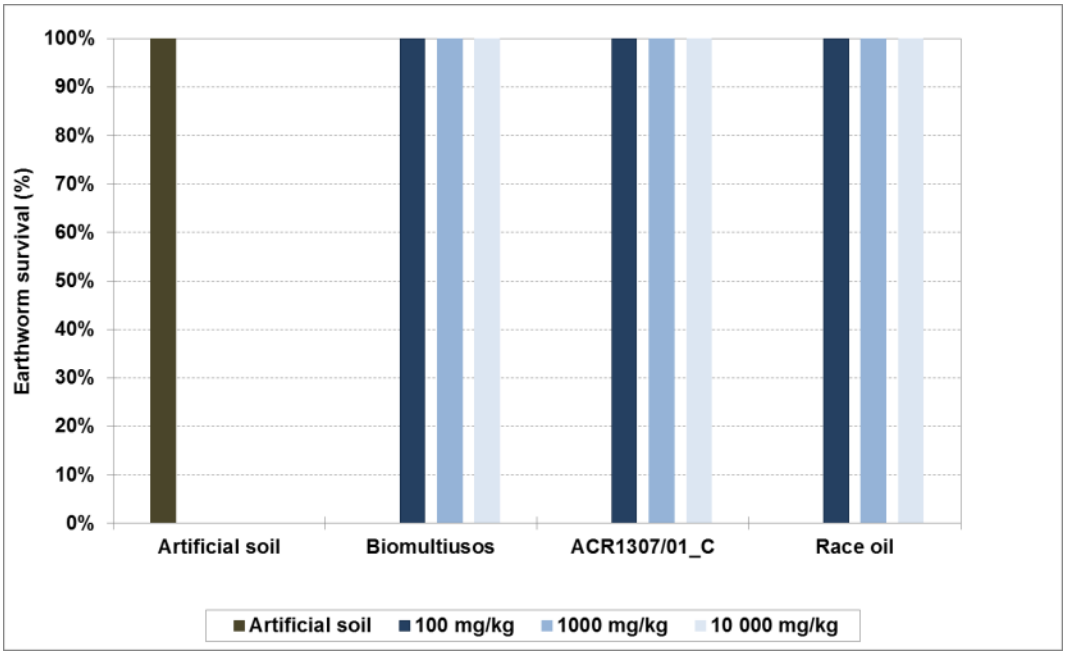



**Figure 19. Average survival of earthworms in the artificial soil and the different concentration series of biolubricants BIOMULTIUSOS 130536BM and ACR1307/01_C MFA 111028080 and mineral oil "Race oil - Road - Friction technology".**

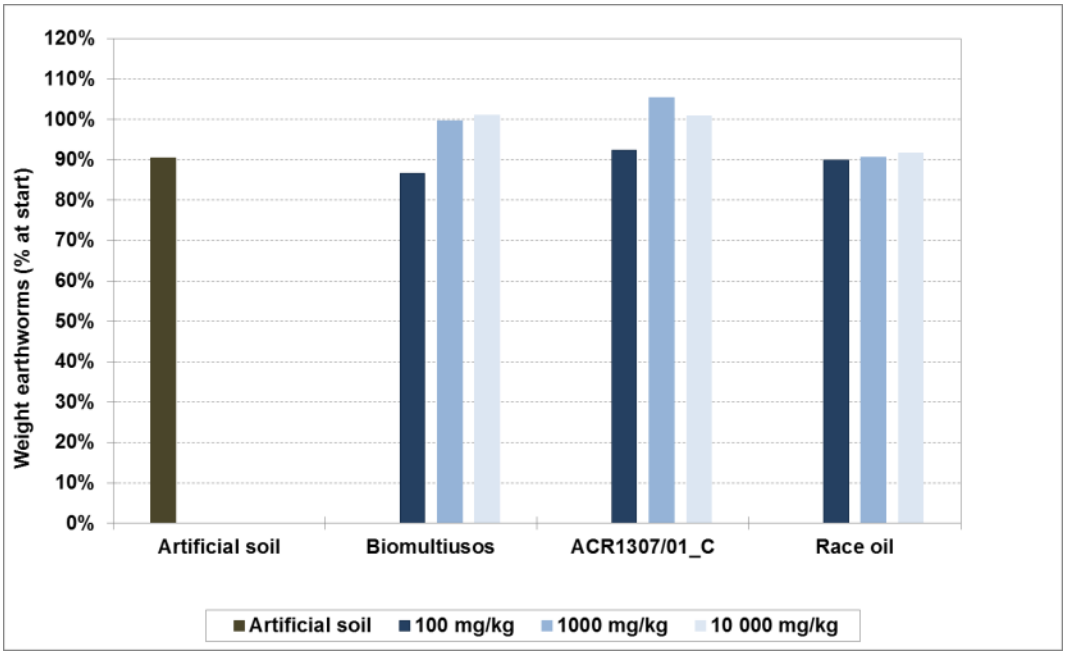



**Figure 20. Average weight of earthworms (as % of weight at start) in the artificial soil and the different concentration series of biolubricants BIOMULTIUSOS 130536BM and ACR1307/01_C MFA 111028080 and mineral oil "Race oil - Road - Friction technology".**

At the end of the earthworm, acute toxicity test, the artificial soil and the different test series were analysed for pH and salt content. The results of all these analyses are given in Table 12. For all test series rather neutral pH levels were measured (6.5 – 7.5). A low salt content was measured for the different test series.

**Table 12. Chemical analysis on the artificial soil and the different test series.**

| Test series | pH | EC (µS/cm) |
|---|---|---|
| Artificial soil | 7.3 | 177 |
| Biomultiusos (100 mg/kg) | 7.5 | 197 |
| Biomultiusos (1000 mg/kg) | 7.2 | 147 |
| Biomultiusos (10000 mg/kg) | 6.9 | 118 |
| ACR1307/01_C (100 mg/kg) | 7.4 | 173 |
| ACR1307/01_C (1000 mg/kg) | 6.6 | 133 |
| ACR1307/01_C (10000 mg/kg) | 6.5 | 94 |
| Race oil (100 mg/kg) | 6.8 | 140 |
| Race oil (1000 mg/kg) | 7.3 | 173 |
| Race oil (10000 mg/kg) | 6.8 | 125 |

## 4.4 Preliminary test 3: Earthworm, acute toxicity test on residuals after biodegradation

### 4.4.1 Objective

A biodegradation test in soil (standard soil as prescribed by ISO 17556) was executed on positive reference material rapeseed oil, biolubricants L1323/3 and ACR 1307/01_A and mineral chainsaw oil (Dolmar). At start of the test approximately 6.25 g sample was added to 500 g standard soil (concentration: 1.25 %). The duration of the test was 210 days. The detailed results of the biodegradation test are reported in deliverable 6.2. (Chapter 6.4.2 & Chapter 6.4.3) and the evolution of the biodegradation is given in Figure 21.

The biodegradation of these samples was also evaluated in natural soil, but toxicity towards earthworms was only evaluated in the series that used standard soil due to the fact that:

(1) the test item concentration was higher in the standard soil (1.25 %) when compared to the natural soil (0.2 %);
(2) the composition of the standard soil as prescribed in soil biodegradation method ISO 17556 (70 % sand + 10 % clay + 16 % soil + 4 % compost) is rather similar when compared to the artificial soil that is used in order to evaluate toxicity towards earthworms (70 % sand + 20 % clay + 10 % peat).

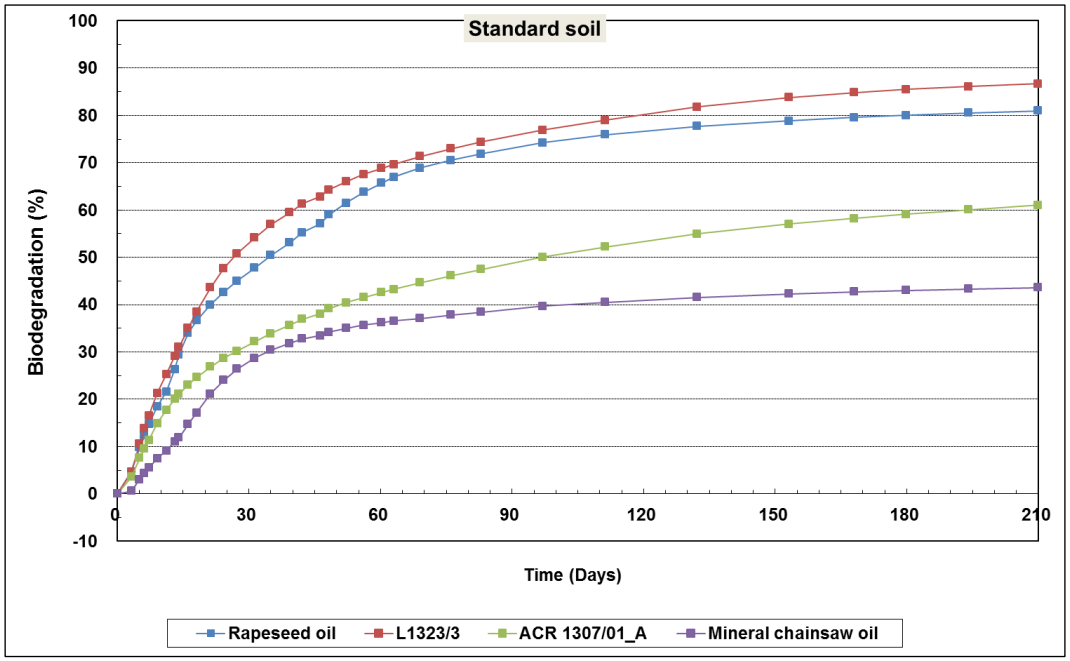



**Figure 21. Evolution of the biodegradation of rapeseed oil, L1323/3, ACR1307/01_A MFA 111028078 and mineral chainsaw oil in standard soil (ISO 17556).**

The toxicity of possible metabolites, undegraded components and inorganic components that remain present after the biodegradation were evaluated by means of an earthworm, acute toxicity test with a duration of 14 days.

### 4.4.2 Test set-up

The test set-up is given in Table 13. The soil obtained at the end of the biodegradation test was divided into 2 equal parts and the exact weight per replicate was determined. The control was only tested in one replicate.

**Table 13. Test set-up of the earthworm, acute toxicity test of soil residuals obtained at the end of a biodegradation test with a duration of 210 days.**

| Test series | Replicate 1 (g wet weight) | Replicate 2 (g wet weight) |
|---|---|---|
| Control soil (= mixture of reactors 1, 6 & 11) | 497.43 | |
| Rapeseed oil soil (= mixture of reactors 2, 7 & 12) | 600.08 | 600.05 |
| L1323/3 soil (= mixture of reactors 3, 8 & 13) | 600.09 | 600.03 |
| ACR 1307/01_A soil (= mixture of reactors 4, 9 & 14) | 600.03 | 600.08 |
| Mineral chainsaw oil soil (= mixture of reactors 5, 10 & 15) | 600.02 | 600.04 |

One day before start-up of the test, the worms were conditioned in the artificial soil. At the start of the test, each glass jar was filled with the soil. Subsequently, 10 viable earthworms were put on top of the soil. The weight of the worms was determined at start.

After all glass jars were filled, they were closed and put at room temperature (20°C ± 2°C) and with continuous lighting.

### 4.4.3 Results

The test was stopped after 14 days. Table 14 shows the average percentage of survival and mortality at the end of the test. Also the live weight per worm and the average preservation of the weight at start of the surviving worms are given. The survival percentages and the mean weight percentages (as a percentage of weight at start) are also shown in Figure 22 and Figure 23.

No survival was measured in the control soil after 14 days, while complete survival was measured in the positive reference soil and the test soils. For positive reference material rapeseed oil and biolubricants L1323/3 and ACR 1307/01_A no significant decrease in the mean weight of the earthworms was observed after 14 days, while the weight of the earthworms in the mineral chainsaw oil soil was significantly reduced up to 53.3 % of the initial weight. The results of the test soils were in line with the observations in Chapter 4.2, in which the direct toxicity of mineral chainsaw oil and ACR 1307/01_A was evaluated in a concentration up to 10 000 mg/kg (= 1 %). In this test also no toxic effects were observed for ACR 1307/01_A, while a weight decrease was observed for the mineral chainsaw oil.

The results in the control soil were in contrast with the expectations. It is unusual that complete survival is observed in the series with the test soils, which contain control soil and 1.25 % sample, while no survival is measured in the control soil as such. It might be possible that no sufficient nutrients remained present in the control soil for the earthworms after a biodegradation phase of 210 days, while more nutrients remained present in the soils to which 1.25 % sample was added.

Based on this first preliminary test, it is difficult to draw clear conclusions and therefore this will be further investigated in the next phase of the project.

**Table 14. Average and standard deviation of percentage survival, mortality and live weight yield for each test series.**

| Test series | Survival (%) | | Mortality (%) | Live weight yield (g per worm) | | Live weight yield (% of start) | |
|---|---|---|---|---|---|---|---|
| | AVG | STD | AVG | AVG | STD | AVG | STD |
| Control soil | 0 | - | 100 | - | - | - | - |
| Rapeseed oil soil | 100 | 0 | 0 | 0.32 | 0.02 | 96.5 | 1.3 |
| L1323/3 soil | 100 | 0 | 0 | 0.31 | 0.01 | 97.2 | 1.3 |
| ACR 1307/01_A soil | 100 | 0 | 0 | 0.31 | 0.02 | 91.7 | 3.0 |
| Mineral chainsaw oil soil | 100 | 0 | 0 | 0.18 | 0.00 | 53.3 | 0.6 |

With AVG = average and STD = standard deviation.

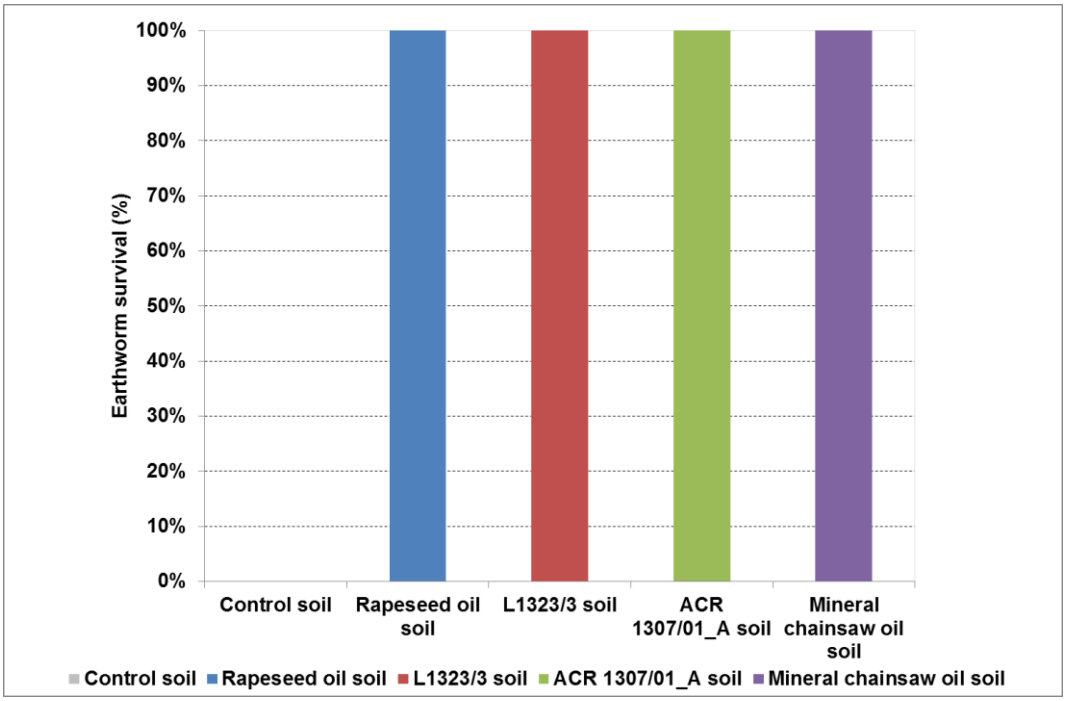



**Figure 22. Average survival of earthworms in the control soil, rapeseed oil soil, L1323/3 soil, ACR 1307/01_A soil and mineral chainsaw oil soil.**

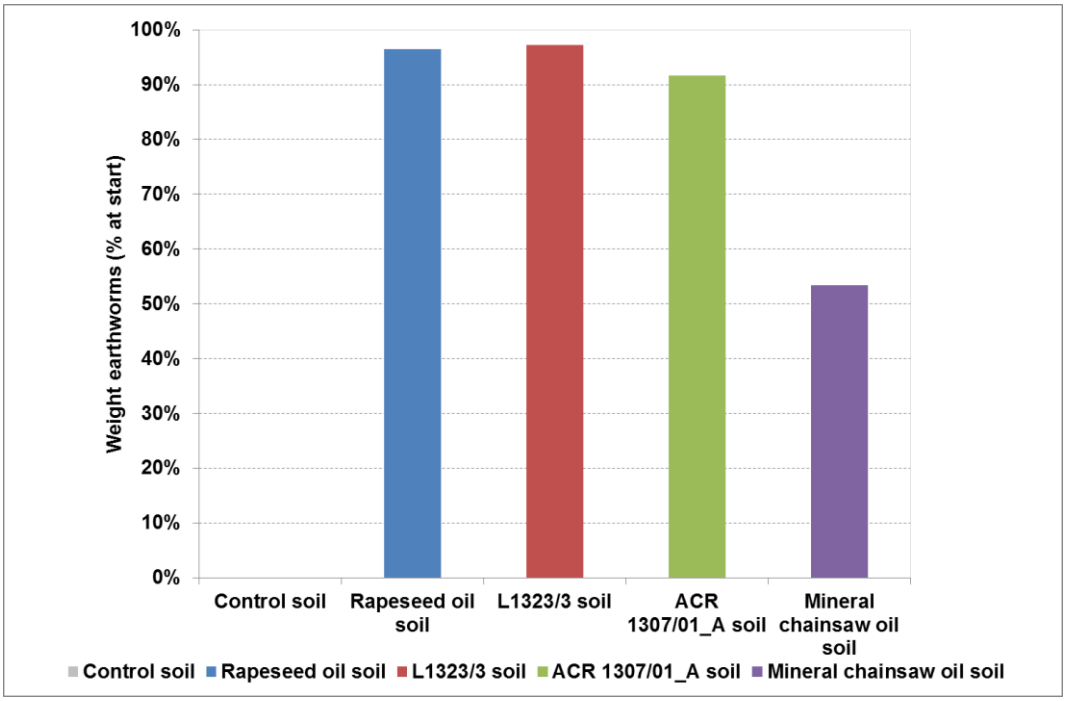



**Figure 23. Average weight of earthworms (as % of weight at start) in the control soil, rapeseed oil soil, L1323/3 soil, ACR 1307/01_A soil and mineral chainsaw oil soil.**

## 4.5 Preliminary test 4: Terrestrial plant test

### 4.5.1 Objective

The test was executed in line with OECD 208 (adopted 19 July 2006) 'Terrestrial Plant Test: Seedling Emergence and Seedling Growth test'. The test assesses effects on seedling emergence and early growth of higher plants following exposure to the test substance in the soil. Seeds were placed in contact with soil treated with the test substance and evaluated effects following usually 14 to 21 days after 50 % emergence of the seedlings in the control group. Endpoints measured are seedling emergence and dry shoot weight. Garden cress (*Lepidium sativum*) was chosen as a representative of the dicotyledonae, while barley (*Hordeum vulgare*) was chosen as a representative of the monocotyledonae.

### 4.5.2 Test set-up

The toxicity of a mineral chainsaw oil (Huile chaine of DOLMAR) on terrestrial plants was evaluated in test set-up 1.

The soil inoculum was a mixture of natural soils collected from the surface layer of a sandy soil derived from a field in Lokeren, of a forest soil from Zwijnaarde and of 2 forest soils from Moerbeke (Belgium). The soil was sieved over a 2 mm sieve to remove stones, recognizable roots and plant debris, and other impurities. A mixture of the different soils (90 %) and mineral wool (10 %) was used as inoculum. The mineral wool was added in order to avoid the compaction of the soil. An inorganic nutrient solution, containing $K_2HPO_4$, $MgSO_4.7H_2O$, $NaNO_3$, urea and $NH_4Cl$, was added to the soil mixture in order to ensure that plants are not stressed through nutrient deficiencies. The characteristics of the soil mixture are given in Table 15.

**Table 15. Characteristics of the soil inoculum.**

| Characteristics | Soil inoculum |
|---|---|
| Total solids (%) | 73.2 |
| Moisture content (%) | 26.8 |
| Volatile solids (% on total solids) | 9.8 |
| Ash content (% on total solids) | 90.2 |
| pH | 7.5 |
| E.C. (µS/cm) | 678 |
| Total N (g/kg TS) | 6.6 |
| C/N | 7 |

OECD 208 prescribes that the maximum plant density would be around 3 – 10 seeds per 100 cm² depending on the size of the seeds. This is a small amount in comparison with the amount of seeds as prescribed in Annex E of EN 13432 (2000) (100 seeds per pot) or when compared to the amount of seeds mentioned in Methodenbuch 1998 (Kapitel II: 5. Pflanzenverträglichkeit – Bundesgütegemeinschaft Kompost e.V.) (50 barley seeds per pot). If only 10 seeds are added per pot, this could lead to a higher variability.

Ten seeds were added per pot (as prescribed by OECD 208). Two additional test series were also started in order to evaluate the influence of the amount of seeds per pot. For barley plants 50 seeds (Methodenbuch 1998) were added per pot in the additional test series, while for cress plants 100 seeds (EN 13432 – Annex E) were added per pot in the additional test series.

The barley seeds are barley seeds 'Barke non treated' and are derived from AVEVE, Tiensestraat 300, B-3400 Landen, Belgium. The seeds were examined for their germinative capacity. The germinative capacity was 98 %, which is above the recommended value of 90 %.

The seeds are cress seeds type 'large-leaved' and are derived from AVEVE, Pantserschipstraat 6, B-9000 Gent, Belgium. The cress seeds were examined for their germinative capacity. The germinative capacity was 97 %, which is well above the recommended value of 90 %.

Two test concentrations were tested: 100 mg/kg dry soil and 2000 mg/kg dry soil[8]. Test soil 1 was prepared in a batch of 1500.6 g soil and 0.11 g mineral chainsaw oil, while for test soil 2 an exact amount of mineral chainsaw oil was added per pot. It was not possible to prepare test soil 1 per pot as the amount of test material per pot was too low in order to weight is accurately. In total, 12 plant pots were used per plant species. Approximately 250 g was added to each plant pot. The test set-up is given in Table 16.

**Table 16. Test set-up of the plant toxicity test on mineral chainsaw soil (weight per pot).**

| Test series | Control soil (g wet weight) | Control soil (g dry weight) | Mineral chainsaw oil (mg wet weight) |
|---|---|---|---|
| **3 × Control soil** | 250 | 183 | - |
| **3 × Test soil 1 (100 mg/kg)** | 250 | 183 | 18.3 |
| **3 × Test soil 2 (2000 mg/kg)*** | 250 | 183 | 366 |
| **3 × Test soil 2 (2000 mg/kg)**** | 250 | 183 | 366 |

* 10 seeds per pot (OECD 208)
** 50 barley seeds per pot (Methodenbuch 1998) or 100 cress seeds per pot (EN 13432)

[8] Normally 1000 mg/kg dry solids would be tested, but due to a mistake, the double concentration was added to the soil.

### 4.5.3 Results

The barley test was stopped after 10 days, while the cress test was stopped after 14 days. At the end of the test the amount of plants per pot was determined, the fresh weight was measured per pot and after a drying period of 2 days the dry weight of the plants was measured.

Figure 24 shows a visual presentation of the germination rate of the barley plants and the cress plants in the different soils.

The addition of mineral chainsaw oil (up to 2000 mg/kg dry soil) exerted no significant negative effect on the germination rate of barley plants. Moreover, it was also observed that the standard deviation was indeed lower in the test soil with 50 seeds (= 2000 mg/kg in line with Methodenbuch 1998) when compared to the test soil with only 10 seeds as prescribed by OECD 208.

Also for cress plants no significant negative effect was observed on the germination rate when adding test material mineral chainsaw oil, but the growth of the cress plants in test soil 2 with 100 seeds was very variable. During the test some plants chlorosis and necrosis was observed (Figure 25), while this was not the case in the test series to which only 10 seeds were added. At the end of the test some death plants were already completely wilted and it was not possible anymore to count them. This resulted in a low germination rate. Possibly this is caused by the fact that these particular seeds were in contact with the oil (the chance on direct contact is smaller when less seeds are present).

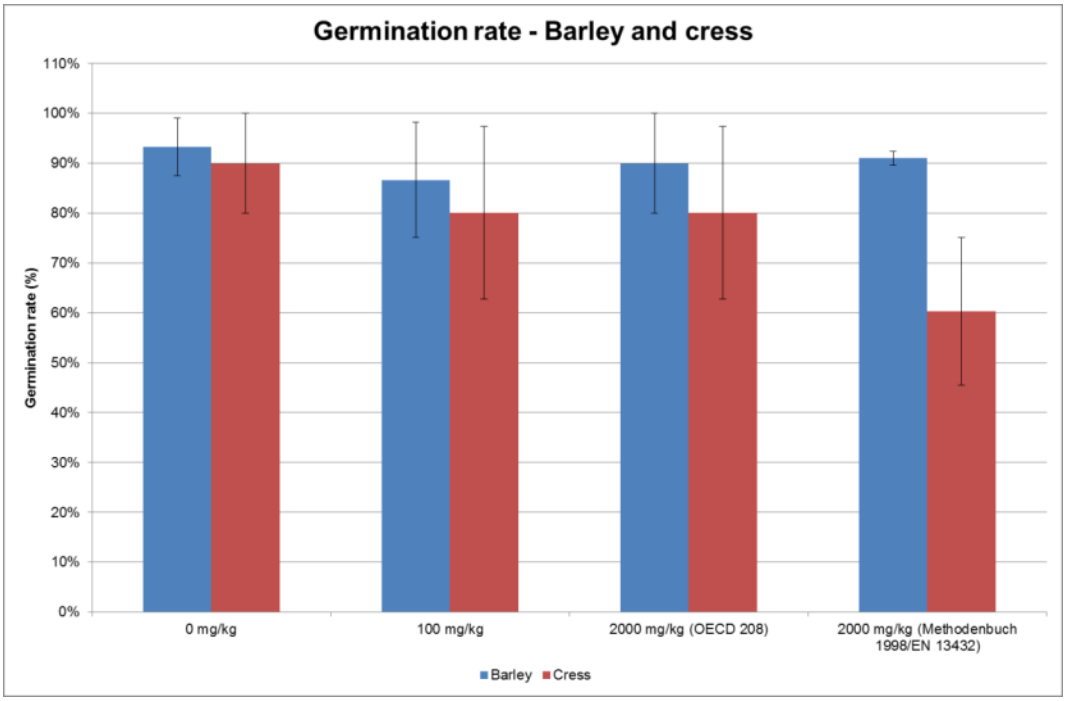



**Figure 24. Germination rate of barley plants and cress plants in control soil, test soil 1 (100 mg mineral chainsaw oil/kg) and test soil 2 (2000 mg mineral chainsaw oil/kg).**

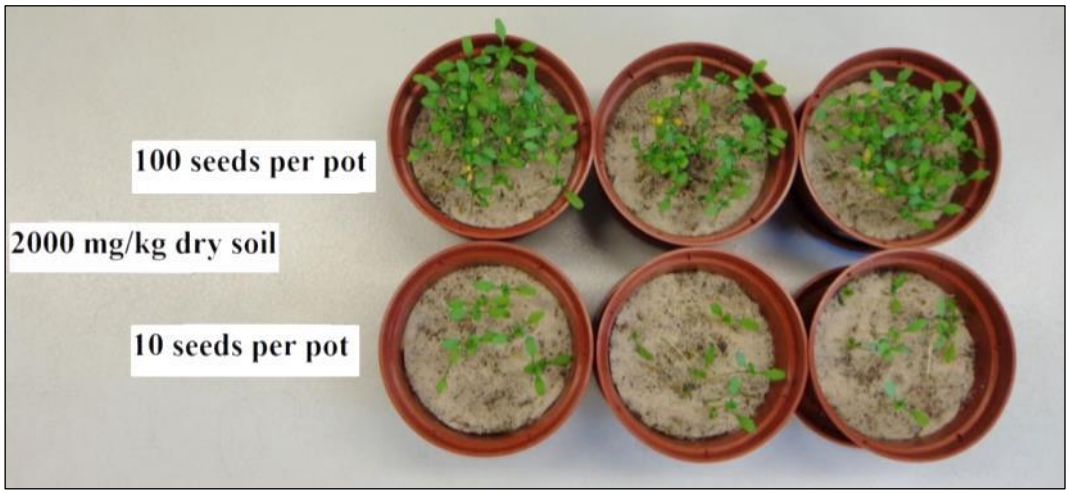



**Figure 25. Visual comparison between the cress plants in the test series with 10 seeds per pot and the test series with 100 seeds per pot (test soil 2 = 2000 mg mineral chainsaw oil/kg dry soil).**

Figure 26 and Figure 27 show a visual presentation of the average dry weight plant yield per pot in the different soils for barley plants and cress plants, respectively. No negative effect was observed caused by the addition of mineral chainsaw oil in a concentration up to 2000 mg/kg dry weight.

In order to evaluate the difference between the test series (2000 mg/kg) with 10 seeds per pot and the test series with 50 barley seeds or 100 cress seeds per pot, the dry weight per plant was calculated in the different series (Figure 28 and Figure 29). It was noticed that the dry weight plant yield per plant was significantly lower in the test series with the higher amount of seeds when compared to the 10 seeds of OECD. This might be caused by the fact that plants are more limited in space and/or in nutrients.

It was also observed that the standard deviation of the dry weight plant yield per plant in the test series with a higher amount of seeds was significantly lower, which is advantageous for the reproducibility of the test.

➔ **CONCLUSION:** Standard deviation is lower when higher amount of plants is present per pot. Therefore, in case plant toxicity is used in order to evaluate the toxicological effect, a higher amount of seeds is recommended when compared to the amount as prescribed by OECD 208.

Plants are less sensitive when compared to the evaluated aquatic organisms (daphnia). For daphnia a concentration of 100 mg/l already induced a significant reduction of the mobility ($EC_{50}$ of mineral chainsaw oil < 100 mg/l), while no decrease in weight was observed for 2000 mg/l.

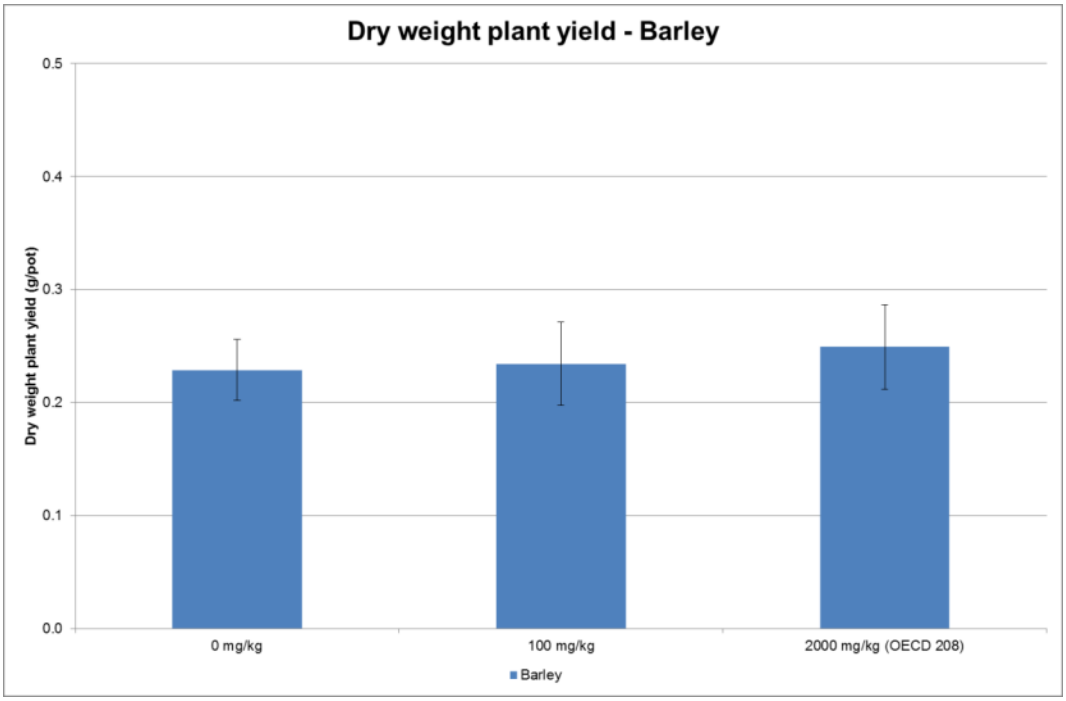



**Figure 26. Dry weight plant yield of barley plants per pot in control soil, test soil 1 (100 mg mineral chainsaw oil/kg) and test soil 2 (2000 mg mineral chainsaw oil/kg).**


**Figure 27. Dry weight plant yield of cress plants per pot in control soil, test soil 1 (100 mg mineral chainsaw oil/kg) and test soil 2 (2000 mg mineral chainsaw oil/kg).**

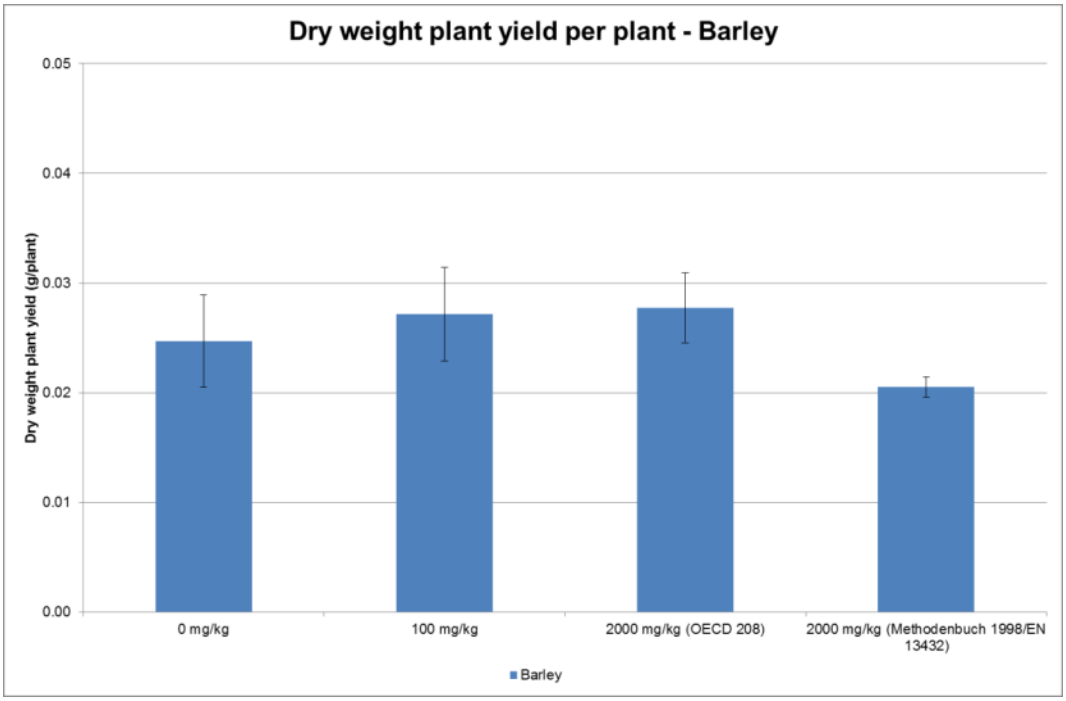



**Figure 28. Dry weight plant yield of barley plants per plant in control soil, test soil 1 (100 mg mineral chainsaw oil/kg) and test soil 2 (2000 mg mineral chainsaw oil/kg).**


**Figure 29. Dry weight plant yield of cress plants per plant in control soil, test soil 1 (100 mg mineral chainsaw oil/kg) and test soil 2 (2000 mg mineral chainsaw oil/kg).**

## 4.6 Preliminary test 5: Terrestrial plant test on residuals of bio-based chain-saw oil after biodegradation

### 4.6.1 Objective

The ecotoxicity related to biodegradation residuals or metabolites of bio-based lubricants in soil was investigated by the Agricultural University of Athens employing at a first stage reference bio-based lubricants (i.e. bio-based chainsaw oil (Oregon)), which is characterised by a fast biodegradation. The soil type used was natural Clay-Loam (described in deliverable 6.2 as soil D). Various quantities of bio-based lubricant were used to test ecotoxicity. Following a biodegradation period that varied from one month (first set of experiments) up to three months, ecotoxicity tests were performed according to OECD and EN standards in order to evaluate the toxicity of possible residuals or metabolites obtained after the applied biodegradation phase of 1 month or 3 months. At a second stage, the soil media used to test biodegradation of other types of bio-based lubricants will be used for testing ecotoxicity and the results will be presented in an updated version of the deliverable due to the very long time needed for these biodegradation tests.

### 4.6.2 Test set-up for bio-based chain oil

Two separate tests were performed in order to evaluate the ecotoxicity after the biodegradation of the bio chain oil Oregon with super adhesion additive to cress plants. The first test was performed according to the specifications of OECD 208, whereas the second test was based on the specifications of EN 13432 (2000). Both tests followed the same preparation procedure as there were no different requirements according to the guidelines.

In each test 12 non-porous plastic pots were used in total. Each pot has internal diameter of 9 cm. Inside the pots a plastic net with low permeability was placed to hold the test soil. A quantity of approximately 320 g of test soil D (Clay Loam) was added to each pot. Soil water content was adjusted to 80 % of the WHC of soil D, in each pot.

Four series with three replicates of pots were created: 5, 10 and 20 g of bio-based chain oil were added to the soil and mixed in the test series 1, 2 and 3, respectively. The fourth series was the control soil set with no bio-lubricant. The pots were kept in a room without light in order to allow biodegradation of the oil for a period of one month. Every two or three days a small quantity of water was added in the pots to replace losses from evaporation. The content of the reactors was not mixed. The test set-up is given in Table 17.

**Table 17. Test set-up of the plant ecotoxicity test (weight per pot).**

| Test series | Test soil (wet weight, g) | Test soil (dry weight, g) | BIO CHAIN OIL (g) |
|---|---|---|---|
| **3 × Control series** | 364 | 320 | - |
| **3 × Test series 1 (5 g oil)** | 364 | 320 | 5 |
| **3 × Test series 2 (10 g oil)** | 364 | 320 | 10 |
| **3 × Test series 3 (20 g oil)** | 364 | 320 | 20 |

The natural soil D consists of clay 28.9 %, sand 43.1 % and silt 28 % (on dry weight basis) with pH of 7.9.

After the addition of the seeds and during the whole period of the tests, in both cases (OECD 208 and EN 13432) the pots were placed inside a controlled environment room to preserve appropriate conditions for the germination. The temperature hovered around 20$^{o}$C and the artificial lighting conditions were programmed to provide 10000 lx for 14 hours per day.

### 4.6.3 Performance of the test according to EN 13432

Following the provisions of EN 13432, 100 seeds of cress were added to each pot with the test soil to examine possible ecotoxicity effects at the germination and the growth of the plants (Figure 30). The addition of the seeds of the third repetition of test series 2 has been performed by incorporating the seeds into the soil. However, this practice led to infection of the plants by fungi, so the specific pot was disregarded in the experiment.

**Figure 30. Visual presentation of a pot to which 100 seeds were added (EN 13432).**

The germination of the majority of the seeds was almost completed after a period of two days. The plants that have emerged were kept under good cultivation conditions and observed every two or three days for a period of two weeks.

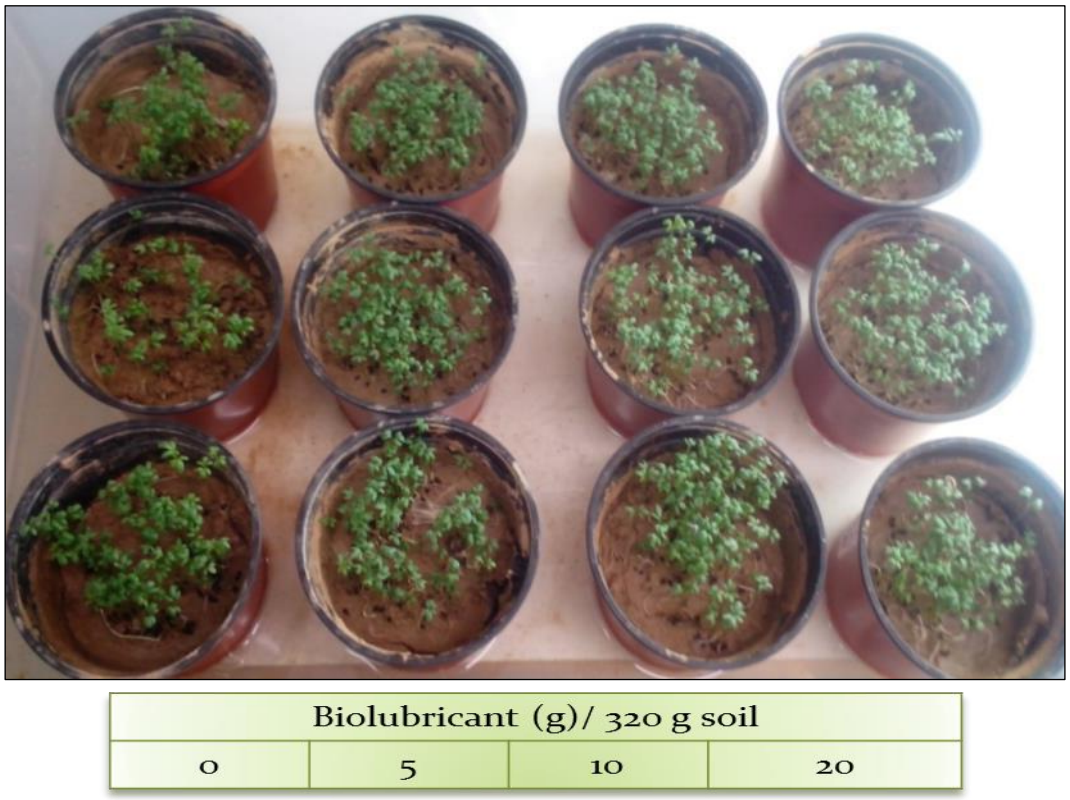



**Figure 31. Ecotoxicity test EN 13432 (test day 12).**

### 4.6.4 Results (EN 13432)

A deviation from EN 13432 was made because the germination of the seeds in the case of EN 13432 was not evaluated quantitatively. The relevant information on the germination rate may be estimated through the ecotoxicity test based on OECD 208 conducted at the same time and under the same conditions (Table 19). Figure 31 also indicates an average germination rate for the pots used for the ecotoxicity test according to EN 13432 of approximately 90%.

After a period of 14 days the plants were harvested and weighed. The results are presented in Table 18 and Figure 32.

**Table 18. Fresh weight of shoot (EN 13432).**

| Test series | Biolubricant (g) | Fresh weight of shoot (g) | | | | |
|---|---|---|---|---|---|---|
| | | Repetition 1 | Repetition 2 | Repetition 3 | Average | SD |
| **Control** | 0 | 4,33 | n/a (the plants were infected) | 4,17 | **4.25** | **0,10** |
| **1** | 5 | 2,39 | 2,38 | 2,34 | **2,37** | **0,03** |
| **2** | 10 | 2,21 | 1,94 | n/a | **2,08** | **0,19** |
| **3** | 20 | 1,41 | 1,81 | 1,61 | **1,61** | **0,20** |

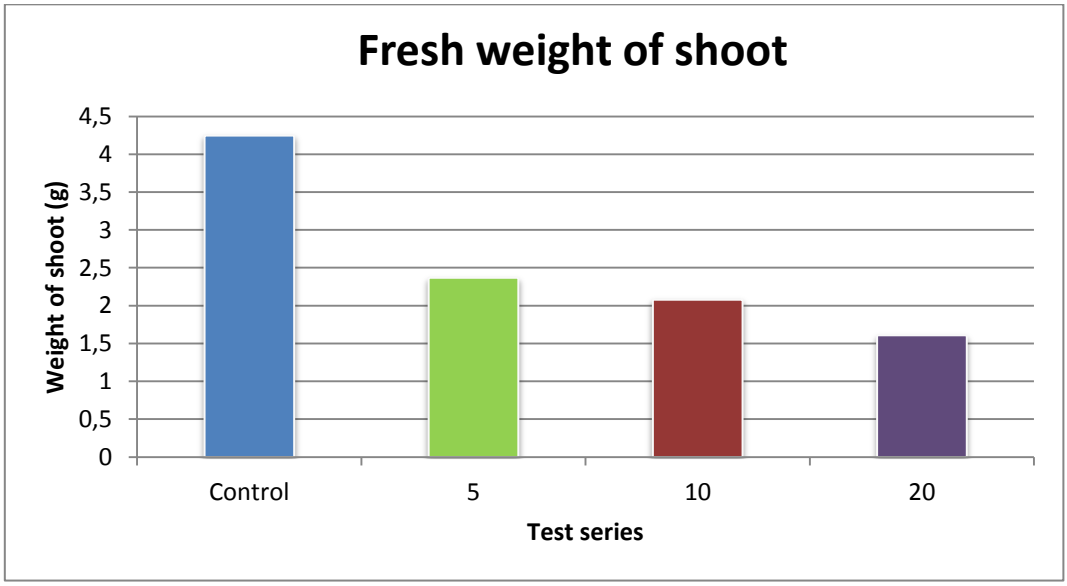



**Figure 32. Fresh weight of shoot of cress seedlings after two weeks of growth (EN 13432).**

### 4.6.5 Performance of the test according to OECD 208

Following the provisions of OECD 208, 10 seeds of cress were added to each pot with the test soil to examine any ecotoxicity effects at the germination and the growth of the plants. The germination of the majority of the seeds was almost completed after a period of two days. The plants that have emerged were kept under good cultivation conditions (information about the conditions was mentioned before) and observed every two or three days for a period of two weeks (Figure 33).

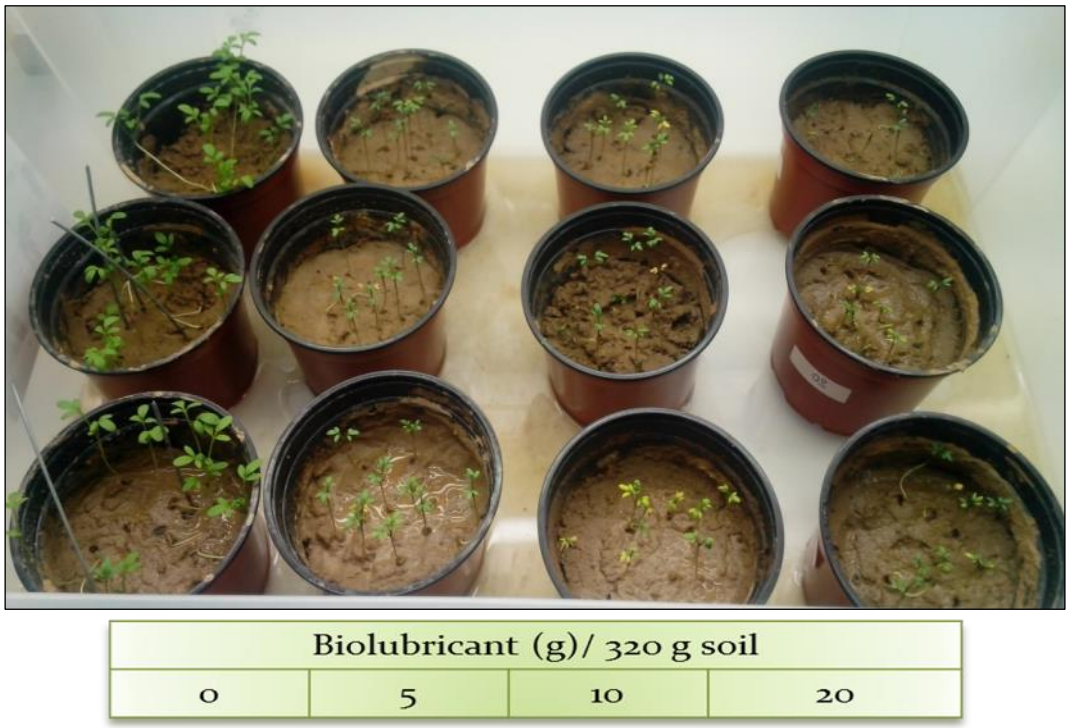



**Figure 33. Last day (two weeks of growth) of the ecotoxicity test OECD 208.**

### 4.6.6 Results (OECD 208)

According to OECD 208 several parameters were evaluated. The percentage of plant emergence is presented in Table 19.

**Table 19. Germination of seeds (OECD 208).**

| Test series | Seeds that germinated | | | |
|---|---|---|---|---|
| | | | | Average (%) |
| **Control groups** | 9 | 9 | 10 | 93,33 |
| **Test series 1 (5 g oil)** | 10 | 10 | 9 | 96,67 |
| **Test series 2 (10 g oil)** | 9 | 8 | 10 | 90,00 |
| **Test series 3 (20 g oil)** | 8 | 8 | 8 | 80,00 |

The plants were inspected every two or three days for any visual detrimental effects on different parts of the plant. During the first week of the test, it was observed that the plants in the pots with the highest amount of bio-lubricant (test series 3 where 20 g of oil had been incorporated), produced chlorosis. Furthermore, in the same plants, stem and leaf deformations could be distinguished. During the second week the symptoms became more evident. Another observation made was that the development of the plants of every test series was different. The higher amount of bio-lubricant used in the soil the poorer the development

of the plants as compared to the control group. During the progress of the tests the symptoms of chlorosis and stem and leaf deformations of test series 3 exacerbated and were also observed in plants of the series 2. During the last days of the experiment minor symptoms of chlorosis began to appear in the plants of the series (Figure 34 - Figure 36).

**Figure 34. Chlorosis in plants – Series 1 (Ecotoxicity test OECD 208).**

**Figure 35. Chlorosis and stem and leaf deformation - Series 2 (last day of the test - two weeks of growth) (Ecotoxicity test OECD 208).**


**Figure 36. Chlorosis and stem and leaf deformation- Series 3 (last day of the test- two weeks of growth) (Ecotoxicity test OECD 208).**

After a period of 14 days the plants were harvested and the height of the shoot was measured. Furthermore, the plants of each pot were weighed. The results are presented in Table 20 and Table 21, respectively, and Figure 37 and Figure 38, respectively.

**Table 20. Height of shoot (OECD 208).**

| Test series | Biolubricant (g) | Height of shoot (cm) | | | | |
|---|---|---|---|---|---|---|
| | | Repetition 1 | Repetition 2 | Repetition 3 | Average | SD |
| **Control** | 0 | 6,00 | 7,00 | 6,50 | **6,50** | **0,50** |
| **1** | 5 | 4,50 | 4,00 | 4,00 | **4,17** | **0,29** |
| **2** | 10 | 3,50 | 3,50 | 4,00 | **3,67** | **0,29** |
| **3** | 20 | 1,50 | 1,50 | 1,50 | **1,50** | **0,00** |

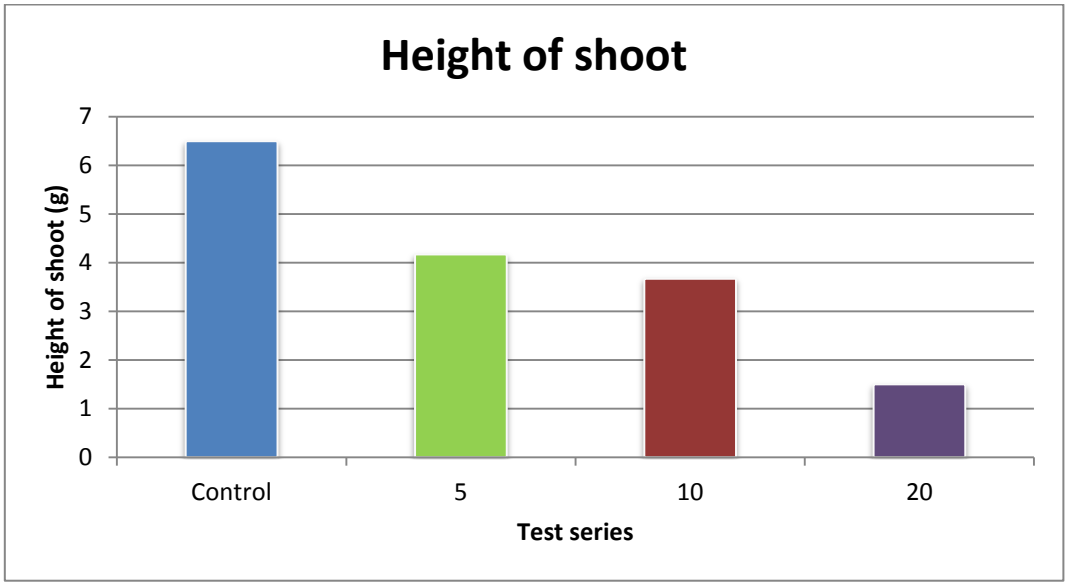



**Figure 37. Height of shoot of cress seedlings after two weeks of growth (OECD 208).**

**Table 21. Fresh weight of shoot (OECD 208).**

| Test series | Biolubricant (g) | Fresh weight of shoot (g) | | | | |
|---|---|---|---|---|---|---|
| | | Repetition 1 | Repetition 2 | Repetition 3 | Average | SD |
| **Control** | 0 | 0,60 | 0,63 | 0,90 | **0,71** | **0,17** |
| **1** | 5 | 0,25 | 0,22 | 0,19 | **0,22** | **0,03** |
| **2** | 10 | 0,16 | 0,16 | 0,17 | **0,16** | **0,01** |
| **3** | 20 | 0,12 | 0,13 | 0,10 | **0,12** | **0,02** |

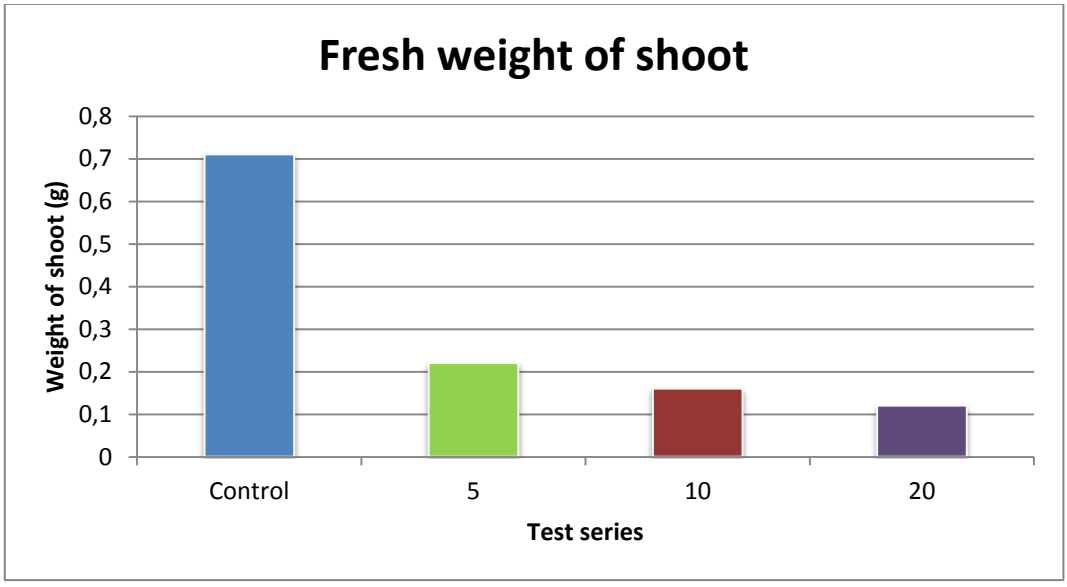



**Figure 38. Fresh weight of shoot of cress seedlings after two weeks of growth (OECD 208).**

## 4.7 Preliminary test 6: Terrestrial plant test on residuals of sunflower oil after biodegradation

### 4.7.1 Objective

The ecotoxicity related to biodegradation residuals or metabolites of bio-based lubricants in soil was investigated by the Agricultural University of Athens employing sunflower oil, which are characterised by a fast biodegradation. The soil type used was natural Clay-Loam (described in deliverable 6.2 as soil D). Various quantities of sunflower oil were used to test ecotoxicity. Following a biodegradation period that varied from one month (first set of experiments) up to three months, ecotoxicity tests were performed according to OECD and EN standards in order to evaluate the toxicity of possible residuals or metabolites obtained after the applied biodegradation phase of 1 month or 3 months. At a second stage, the soil media used to test biodegradation of other types of bio-based lubricants will be used for testing ecotoxicity and the results will be presented in an updated version of the deliverable due to the very long time needed for these biodegradation tests.

The same procedure was followed to evaluate the ecotoxicity of sunflower oil after biodegradation.

The tests were performed in order to evaluate the possible ecotoxicity after the biodegradation of the sunflower oil to cress plants. According to the initial set-up of the test the same quantities of sunflower oil as the quantities of bio-based chain oil with super adhesion additive (OREGON) were added in the test soil. However, due to several problems that are described in detail below, a second test set up with lower quantities of sunflower oil was applied. The tests were performed according to the specifications of OECD 208 and EN 13432 (2000). All tests followed the same preparation procedure as there were no different requirements according to the guidelines.

### 4.7.2 Test set-up 1

Two series of tests were prepared in order to examine the ecotoxicity effects after two different periods. The first test was scheduled to start after a period of one month of biodegradation in soil and the second test to start after a period of three months of biodegradation in soil. In each test two series of 12 non-porous plastic pots were used. Each pot has internal diameter of 9 cm. Inside the pots a plastic net with low permeability was placed to hold the test soil. A quantity of approximately 320 g of test soil D (Clay Loam) was added to each pot. To soil water content was adjusted to 80 % of the WHC of soil D in each pot. Four series with three replicates of pots were created.

According to the initial planning for the experiment, three series 1, 2 and 3, were set-up in addition to control samples: 5, 10 and 20 g respectively of sunflower oil were added to the soil in each pot and mixed thoroughly. After several days, at the surface of the test soil a black formation became visible (Figure 39).


**Figure 39. Growth of black fungus on the surface of the test soil in pots with sunflower oil added – after a period of one month from the test set-up.**

Microscopic examination showed that it was a black fungus (Figure 40).

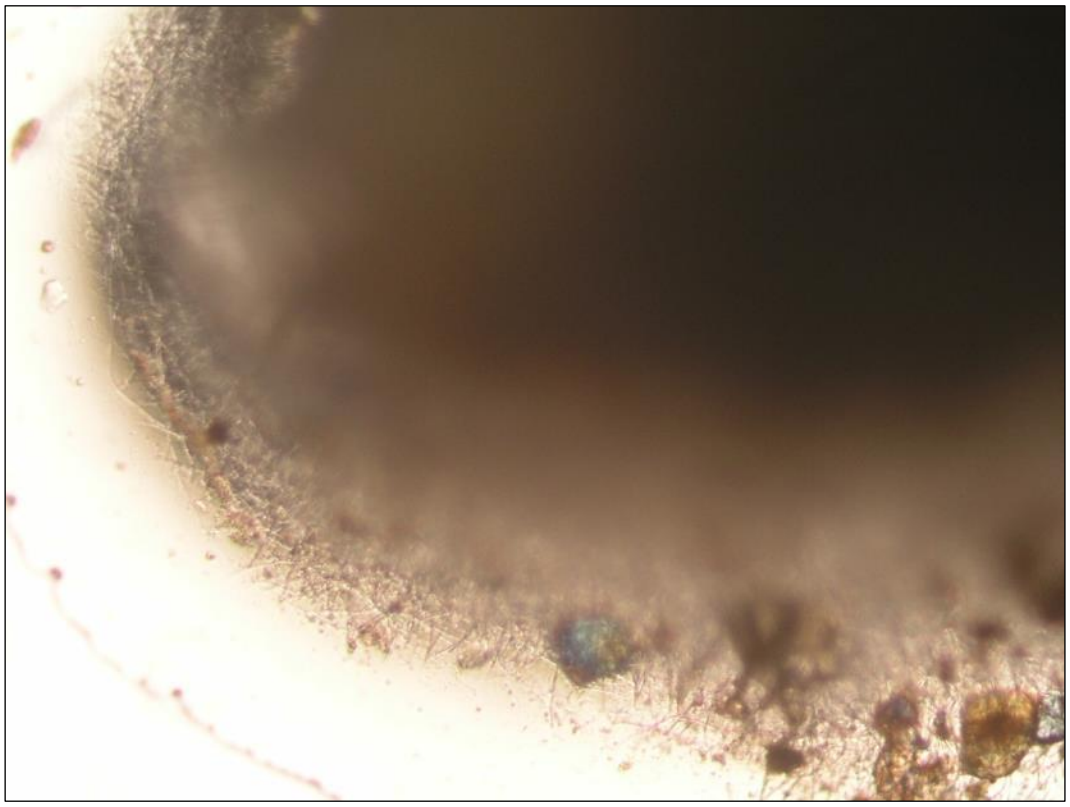


**Figure 40. Microscopic photo of the black fungus.**

The presence of the fungus indicated that the biodegradation of the sunflower oil had not been completed. For that reason it was decided to mix thoroughly the soil and to extend the biodegradation period and to incubate the pots for one additional month before adding the seeds.

Two months after the addition of the sunflower oil to the soil, two sets of the ecotoxicity tests were performed. According to the specifications of OECD 208, 10 seeds of cress were added to each pot with the test soil. In the second test based on the specifications of EN 13432 (2000), 100 seeds of cress were added to each pot. Following the corresponding testing procedures the same preparation procedure was followed as described earlier.

However, the seeds in the pots with 10 and 20 g of sunflower oil didn't germinate at all. For that reason this series of tests was terminated.

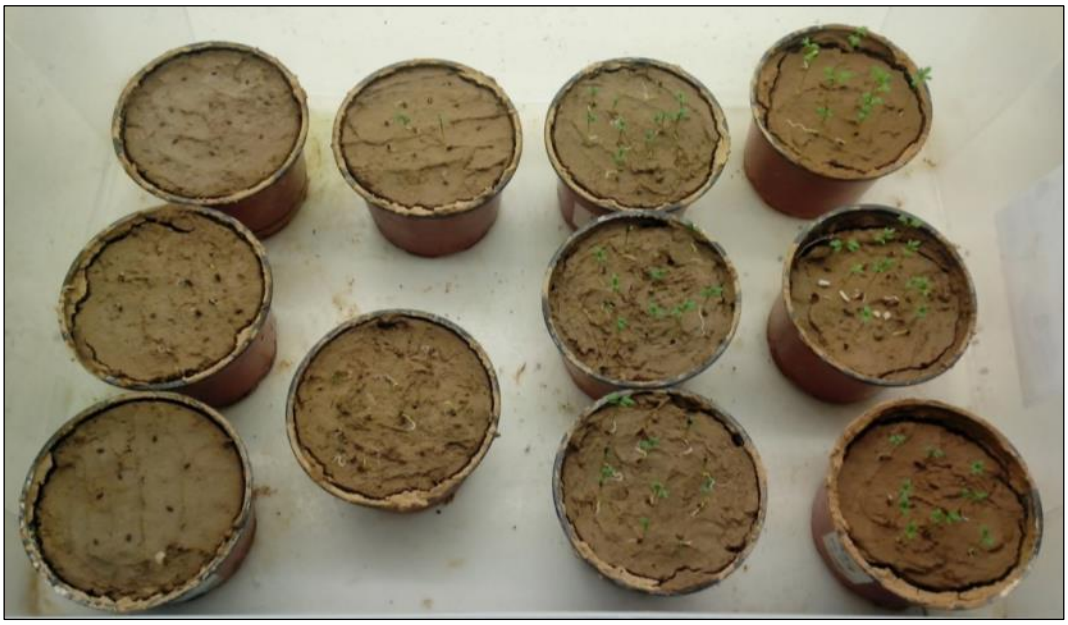
In order to examine the progress of the biodegradation in this series of tests, small quantities of soil – approximately 15 g from each pot – were sent for laboratory tests. The amount of the total organic carbon (TOC) and the nitrogen (N) were measured. The analysis for the total organic substance and TOC was performed according to the Walkey Black method. For the analysis of the total Nitrogen the Bucchi method was followed. The initial nitrogen value of the soil was 0,13 % wt/dry wt and that of TOC was 0.98 % wt/dry wt. The results from the analysis presented in Table 22 shows a rather uniform reduction of the TOC (in the range of 22-32 %) indicating that the biodegradation had not sufficiently advanced in 3 months. The results of Table 22 also demonstrate that there was no significant decrease in the availability of nitrogen.

**Table 22. Laboratory results for the soil from the pots with sunflower oil – first test set-up.**

| Test soil Sample | Organic C (% wt/dry wt) | Total N (% wt/ dry wt) |
|---|---|---|
| **0 g sunflower oil** | 0,78 | 0,108 |
| **5 g sunflower oil** | 1,64 | 0,122 |
| **10 g sunflower oil** | 2,27 | 0,122 |
| **20 g sunflower oil** | 4,49 | 0,112 |

Because of the problems encountered the same pots were kept for another month to allow biodegradation to proceed and then the test was repeated.

After the period of the three months of biodegradation, the seeds were again planted as described before. In both tests, the germination of the seeds was almost completed after a period of two days. However, in some pots the seeds didn't germinate at all. The plants that were emerged were kept under good cultivation conditions and observed every two or three days for a period of two weeks. The overall picture of the cress plants after one week and 10-days (Figure 41, Figure 42) is very similar to the one observed in the case of chainsaw oil (Oregon) (Figure 33).



| Biolubricant (g)/ 320 g soil | | | |
|---|---|---|---|
| 20 | 10 | 5 | 0 |

**Figure 41. Growth of cress plants (OECD 208) after 7 days in pots with sunflower oil (three month from the test set-up).**

| Biolubricant (g)/ 320 g soil | | | |
|---|---|---|---|
| 20 | 10 | 5 | 0 |

**Figure 42. Growth of cress plants (OECD 208) after 10 days in pots with sunflower oil (three month from the test set-up).**

However, gradually the plants that had emerged were infected with fungi (Figure 43 and Figure 44) and the second series of tests was terminated.

**Figure 43. Cress plants infected with fungi (EN 13432).**

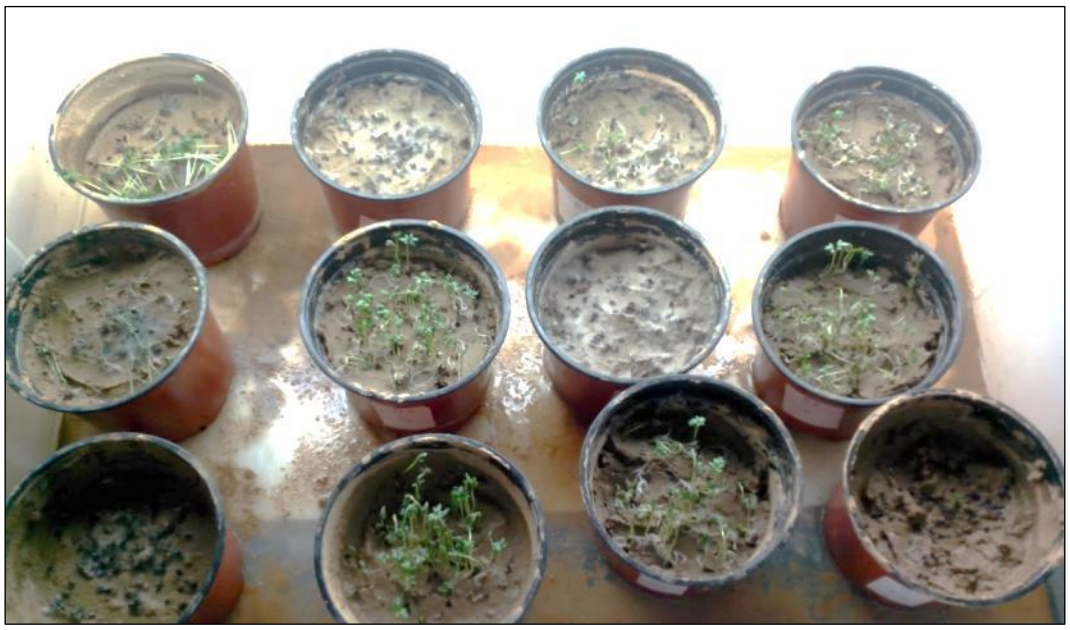


**Figure 44. Cress plants infected with fungi (EN 13432).**

A third series of tests was carried out with the same soil by removing the surface of the soil and mixing the rest soil thoroughly. The pots were kept for two days in order to stabilize the soil conditions. This time the two tests were performed according to the specifications of OECD 208: 10 seeds were planted in each pot of the two repetitions. The germination of the seeds differed at each series of pots. The percentage of seed germination rate was significantly higher at the pots with no sunflower oil and with 5 g of sunflower oil than the relevant

percentage at the series of the pots with 10 and 20 g of sunflower oil. Furthermore, once again, the growth of the plants was more intensive at the series with no sunflower oil and was declining respectively at each series of pots with more quantity of added sunflower oil. In one of the pots, at the fifth day of the test, scattered fungi infections were again observed and the next days the plants started to wither and die and the test was stopped (Figure 45).

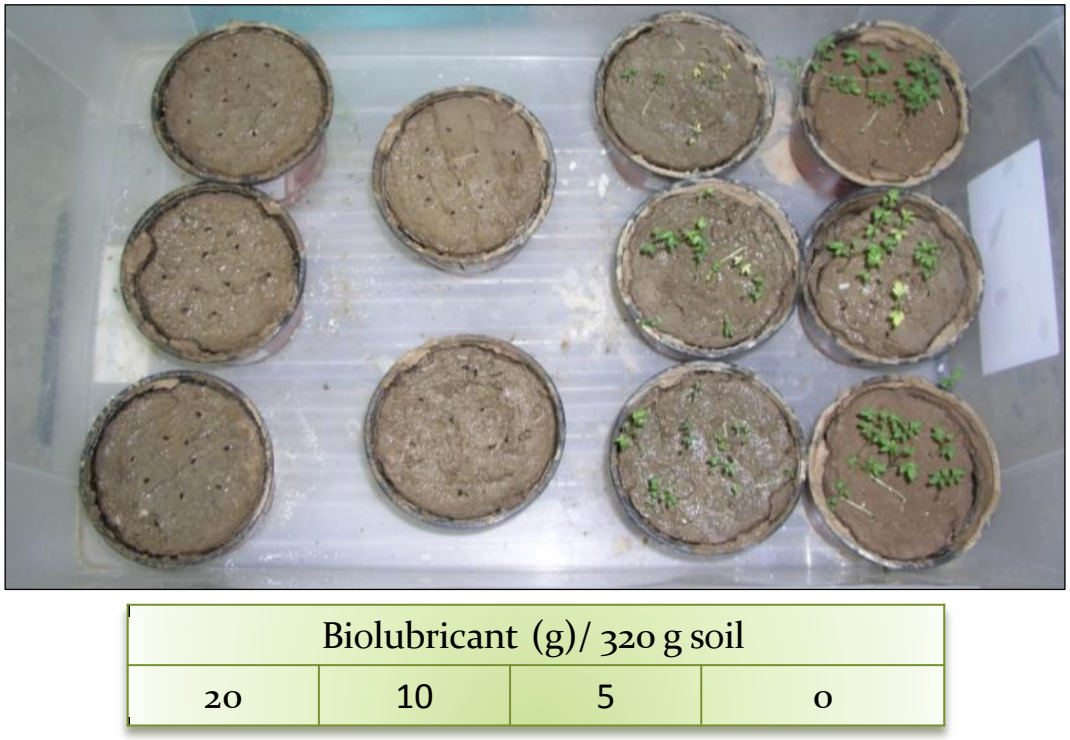



**Figure 45. Growth of cress plants (OECD 208) after 14 days in pots with sunflower oil (three and a half months from the test set-up).**

### 4.7.3 Test set-up 2

Due to the problems encountered during the above mentioned series of tests with sunflower oil an alternative test set-up with lower quantities of sunflower oil was prepared. 24 replicates of the same non-porous plastic pots with internal diameter of 9 cm were prepared. Inside the pots a layer of gravel and over it a plastic net with low permeability were placed to hold the test soil. A quantity of approximately 330 g of test soil A (Loam) was added to each pot. To achieve a water content of 80 % of the WHC of soil A, 56,7 g of water was also added to each pot. Furthermore, in order to speed up the biodegradation of the sunflower oil, a quantity of fertilizer (13-2-44 Potassium Nitrate) corresponding to the quantity of the sunflower oil was added in each pot. Four series with three replicates of pots were created.

In the new test series, lower quantities of 1, 2 and 5 g of sunflower oil were added to the test soil A (series 1, 2 and 3 respectively) and mixed thoroughly. The natural soil A consists of clay 22,0 %, sand 25,7 % and silt 52,3 % (on dry weight basis) with pH of 7,9. The biodegra-

dation of the sunflower oil was estimated that will be more effective and quicker due to the lower quantities of added oil and the addition of the fertilizer. Every two or three days a small quantity of water was added in the pots to replace losses from evaporation. The test set-up is given in Table 23.

**Table 23. Test set-up of the plant ecotoxicity test (weight per pot).**

| Test series | Added nutrients N* (g) | Test soil (dry weight, g) | Sunflower oil (g) |
|---|---|---|---|
| **3 × Control series** | 0,077 | 330 | - |
| **3 × Test series 1 (1 g sunflower oil)** | 0,077 | 330 | 1 |
| **3 × Test series 2 (2 g sunflower oil)** | 0,154 | 330 | 2 |
| **3 × Test series 3 (5 g sunflower oil)** | 0,385 | 330 | 5 |

(*) In the form of NPK 13-2-44 . Nitrogen is nitric form (-$NO_3$)

**The test is in Progress**

## 4.8 Preliminary test 7: Earthworm, acute toxicity test on residuals of sunflower oil after biodegradation

### 4.8.1 Objective

The ecotoxicity test was performed according to the specifications of OECD 207 by the Agricultural University of Athens.

### 4.8.2 Test set-up

According to OECD 207 the recommended worm species for the ecotoxicity tests is *Eisenia fetida*. A culture of these worms was installed at the laboratory of AUA in order to breed the test worms and have the necessary number of them for the tests (Figure 46).

**Figure 46. Culture of Eisenia fetida at the laboratory of AUA.**

To examine any possible ecotoxicity effects of the biodegradation of the sunflower oil on earthworms, following the provisions of OECD 207 three different quantities of sunflower oil were tested. Two sets of four series of 4 non-porous small plastic containers were used. Inside each container a quantity of approximately 750 g of test soil D (Clay Loam) was added. Water content of the soil was adjusted to 80 % of the WHC of soil D, in each container. One series of 3 containers contained only soil without sunflower oil and served as the control samples. The other three series were set-up by adding 1, 2 and 3 g of sunflower oil, respectively, and mixing thoroughly. All containers were kept for a period of one month to allow biodegradation of the sunflower oil. Every two or three days the containers were weighted to check if there were any losses from evaporation. If there was a reduction of the weight an equivalent quantity of water was added in the container. The test set-up is presented in Table 24.

**Table 24. Test set-up of the worms ecotoxicity test (weight per pot).**

| Test series | Test soil (wet weight, g) | Test soil (dry weight, g) | Sunflower oil (g) |
|---|---|---|---|
| **3 × Control series** | 882.45 | 750 | - |
| **3 × Test series 1 (1 g oil)** | 882.45 | 750 | 1 |
| **3 × Test series 2 (2 g oil)** | 882.45 | 750 | 2 |
| **3 × Test series 3 (3 g oil)** | 882.45 | 750 | 3 |

After a period of one month 160 adult worms (3-6 mg weight) selected from the laboratory cultivation were weighted and washed. In each container of the one set 10 of these worms were placed on the surface of the test soil (Figure 47). The containers were kept for a period of two weeks in a room with continuous light to ensure that the worms enter and remain inside the test medium throughout the duration of the test. The room's temperature was approximately 20°C.

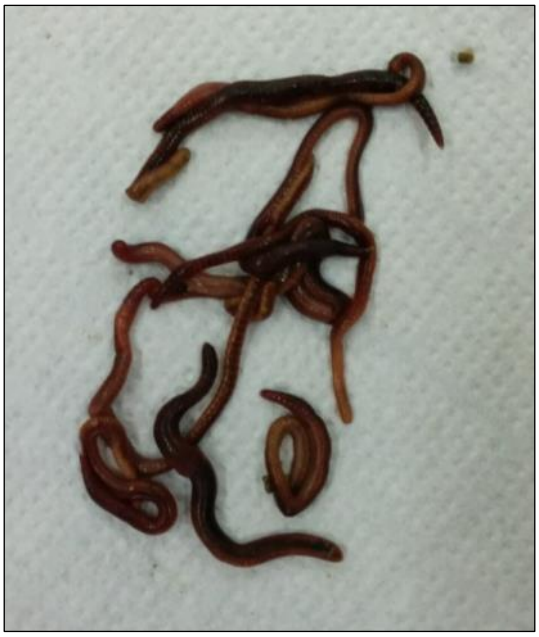


**Figure 47. 10 worms (Eisenia Fetida) prepared for ecotoxicity test.**

### 4.8.3 Results

The seventh day of the test the mortality of the worms was assessed. All the worms were alive and reacted to mechanical stimulus at the front end (Figure 48 & Figure 49). After the first 7-day assessment, the worms were placed in the corresponding jars of the second 14-day set of containers.

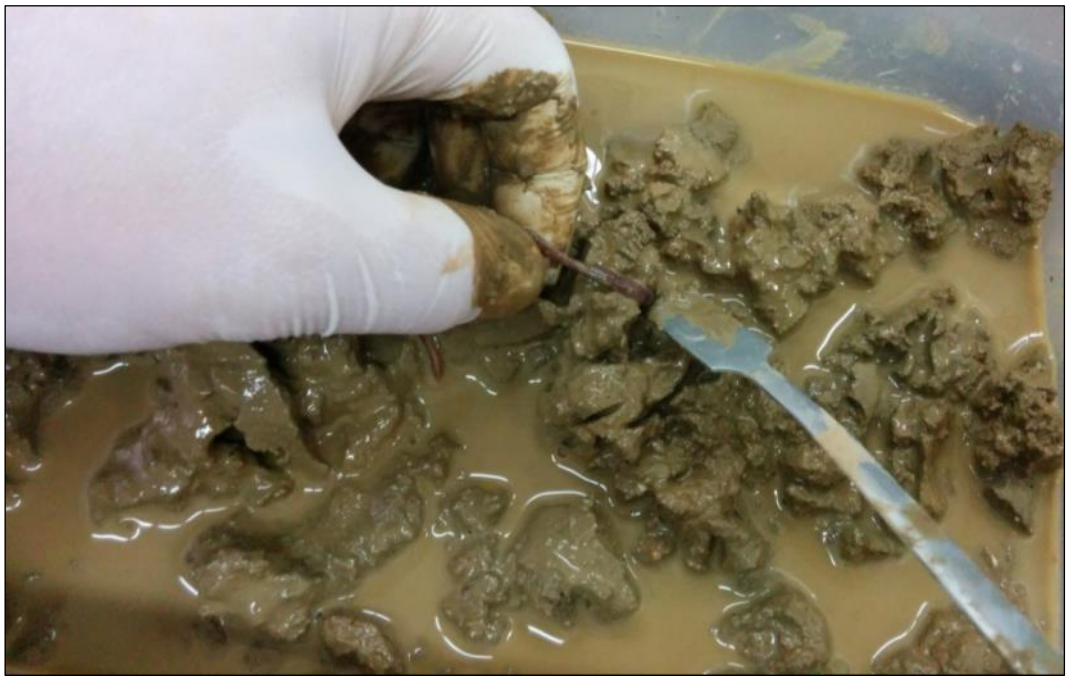


**Figure 48. Removing carefully the worms from the test soil – Reacting to mechanical stimulus at the front end.**

**Figure 49. Worms removed from test soil in order to assess mortality and other behavioural or pathological symptoms.**

The same results were observed on the fourteenth day. All the worms were still alive and they reacted to mechanical stimulus at the front end. No other behavioural or pathological symptoms were observed. The results of the test are presented in Table 25.

**Table 25. Mortality and behavioural or pathological symptoms on worms from the test on eco-toxicity effects – the case of sunflower oil.**

| Test series | Mortality | Behavioural or pathological symptoms |
|---|---|---|
| **3 × Control series** | No | No |
| **3 × Test series 1 (1 g oil)** | No | No |
| **3 × Test series 2 (2 g oil)** | No | No |
| **3 × Test series 3 (3 g oil)** | No | No |

The worms were again weighed, but no significant differences in their average weight were observed (Figure 50). The average live weight of the worms is presented in Table 26.

**Table 26. Results of the test on eco toxicity effects on worms – sunflower oil.**

| Test series | Live average worm weight (mg) | |
|---|---|---|
| | 1st Day | 14th Day |
| **3 × Control series** | 0.43 | 0.46 |
| **3 × Test series 1 (1 g oil)** | 0.42 | 0.43 |
| **3 × Test series 2 (2 g oil)** | 0.46 | 0.44 |
| **3 × Test series 3 (3 g oil)** | 0.41 | 0.44 |

**Figure 50. Worms prepared to be weighed.**

# 5 Proposed test methodology: environmental safety in water

In the next phase of the project the samples that will be used for the interlaboratory biodegradation test (Table 27) will be used in order to evaluate if toxicity can increase during the biodegradation phase.

**Table 27. Overview of the samples for interlaboratory test.**

| Sample | MFA.-Nr. | Description |
|---|---|---|
| **JA1405/01** | 111033888 | ISO VG 10 hydraulic oil |
| **JA1405/02** | 111033889 | ISO VG 46 hydraulic oil |
| **JA1405/03** | 111033890 | ISO VG 320 gear oil |
| **JA1405/04** | 111033891 | Grease |
| **JA1405/05** | 111033892 | Grease |
| **JA1405/06** | 111033896 | High oleic sunflower oil (HOSO) |

Therefore, the above mentioned samples will be added in a 1000 mg/l concentration to freshwater and the toxicity of the water accommodated fraction will be evaluated. Consequently, an inoculum (activated sludge) will be added in order to initiate the biodegradation and at the end of the biodegradation phase (28 days) the toxicity will be evaluated again. Possibly also toxicity at an intermediate moment will be evaluated. Toxicity will be evaluated by means of algae or daphnia.

# 6 Proposed test methodology: environmental safety in soil

Additional ecotoxicity tests are planned to be performed by the Agricultural University of Athens on natural soil to investigate the effect of biodegradation of various bio-based lubricants as compared to the reference bio-lubricants presented in this deliverable.



# 7 Conclusion

## 7.1 Tasks and achievements

Before initiating the development of the eco-toxicological impact study, a questionnaire was developed and several companies, associations (contacts provided by NEN) and the technical committees CEN TC 19/WG 33 Biolubricants and CEN TC 411/WG 2 Bio-based solvents were contacted in order to map the needs with regard to biodegradability testing, environmental safety and labelling schemes. The results of these questionnaires are discussed in detail in Deliverable 6.2 “Draft biodegradability standard”.

The results of the questionnaires and the contacts with the CEN committees revealed that no further needs exist with regard to environmental safety of bio-based solvents. For bio-based lubricants needs exist with regard to biodegradability, but no specific issues with regard to environmental safety were mentioned.

In a first phase the criteria with regard to environmental safety of the Blue Angel ecolabel and the European ecolabel for lubricants were investigated. It can be concluded that these labelling systems for environmentally friendly lubricants evaluate their toxicity already satisfactorily by means of mainly aquatic test organisms on different trophic levels (bacteria, algae, daphnia and/or fish). Moreover also the European legislation is taken into account in order to ensure that substances labelled as harmful for the environment (according the CLP (Classification, Labelling and Packaging) regulation) are not present or only present at very limited concentration in the labelled lubricants.

This approach differs from the approach used for biodegradable plastics (e.g. compostable plastics or plastics biodegradable in soil). For such materials, environmental safety by means of ecotoxicity tests is evaluated on possible residuals or metabolites obtained at the end of the biodegradation phase. This can be explained by the fact that the different constituents of plastic materials are normally not available in the product as such. However, during the biodegradation certain constituents can be released in the environment and toxicity can increase. For lubricants (fluid or paste), this is less possible since the different constituents are probably already available in the product as such and consequently evaluation of the product before biodegradation can probably be considered as the most stringent approach.

In spite of the fact that lubricants are in some cases (intentionally or accidently) released in a soil environment, toxicity by means of terrestrial organisms (soil bacteria, earthworms or plants) is not prescribed due to the fact that toxicity of substances present in lubricants to terrestrial organisms is generally lower than the toxicity to aquatic organisms. This is generally expected for organic substances due to adsorption to organic material in soil as mentioned in the background document with regard to the European Ecolabel for lubricants.



In our opinion the procedure used in the labelling systems is very detailed and takes into account the current legislation in order to avoid that toxic compounds enter in the environment.

Therefore only a few preliminary tests were executed in order to (1) evaluate if toxic effects of lubricants towards terrestrial organisms are indeed less significant when compared to aquatic organisms and to (2) compare toxicity before and after the biodegradation phase. From the first preliminary results it can indeed be concluded that terrestrial organisms (earthworms and plants) are less sensitive to lubricants when compared to aquatic organisms.

The plant ecotoxicity tests performed by the Agricultural University of Athens on natural soil where reference bio-lubricants (e.g. sunflower oil) at various concentrations were biodegraded indicate a special sensitivity of the cress plants to the presence of non-biodegraded bio-lubricants. The mechanism of the inhibition of the plants growth will be analysed following the completion of the on-going ecotoxicity tests.

The earthworm based ecotoxicity tests executed by the Agricultural University of Athens did not show any negative effect of the biodegradation of reference bio-lubricants in natural soil. Provided that bio-based lubricants other than positive reference are non-toxic (tested for ecotoxicity as materials) no negative effect is expected towards earthworms from their biodegradation in natural soil.

## 7.2 Problems and solutions

From the contacts with CEN TC 411/WG 2 Bio-based solvents, it was concluded that no further research with regard to biodegradability and environmental safety of bio-based solvents was needed.

Therefore, the research focussed mainly on (bio-based) lubricants. In the next phase possibly also other bio-based products will be evaluated in case this would be necessary.

## 7.3 Project planning and status

In the next phase of the project the samples used for the interlaboratory biodegradation test will be added in a 1000 mg/l concentration to freshwater and immediate toxicity and toxicity after the biodegradation phase will be evaluated and compared. Also further research toward ecotoxicity in soil will be executed in order to further investigate difficulties observed in these first preliminary tests.



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