# Ecotoxicological evaluation of Ecodor EC 250

**Confidential**

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# Table of Contents

# Summary

An ecotoxicological survey was performed with the enzyme solution Ecodor EC 250. For this, acute toxicity tests were performed at four trophic levels: bacterium (*Pseudomonas putida*), green algae (*Raphidocelis subcapitata*), crustacea (*Daphnia magna*) and fish (*Brachydanio rerio*). Additionally, the biodegradability and the mutagenicity of Ecodor EC 250 were examined. In the investigation a maximum concentration of 100 mg/l testproduct was used. Whenever toxicity was found, the effectparameter ($EC_n$) was determined, where possible.
In the following table a summary of the results is presented.

**Tabel** Summary of the results of the bioassays with Ecodor EC 250. Sample dilutions are given in italic, as rounded off figures. **" > 1: 10,000" means a dilution higher than 10,000.**

| test with: | **Bacteria** | **Algae** | **Crustacea** | **Fish** | **Biodegradation** | **Mutagenicity** |
|---|---|---|---|---|---|---|
| testorganism: | *Ps. putida* | *Raphidocelis subcapitata* | *Daphnia magna* | *Brachydanio rerio* | activated sludge | Salmonella |
| testduration: | 30 minutes | 72 hour | 48 hour | 96 hour | 28 days | o/n |
| effectparameter: | $EC5_0$ (mg/l | $EC_{50}$ (mg/l)[1] | $EC_{50}$ (mg/l) | $LC_{50}$ (mg/l) | % degradation after 33 days | mutagenic effects (g/l) |
| Ecodor EC 250 | *> 1:10,000* | *A: 1:10,000 µ: > 1:2,500* | *> 1: 10,000* | *1: 20,000* | 61 | *> 1:20* |

[1]: Growth inhibition based on both growth (A) and growth rate (µ).

# 1. Introduction

For legislative reasons an ecotoxicological examination on Ecodor EC 250 has been carried out. For this purpose, the acute toxicity on four trophic levels, the biodegradation and mutagenicity were studied. The choice of the tests were based on the demands of the legislator concerning the potential environmental hazard of the product.

The following tests were carried out:

### Toxicity for bacteria
Growth inhibition of *Pseudomonas putida* carried out according to ISO 10712 (1995);

### Acute toxicity to algae
Test with *Raphidocelis subcapitata* (former *Selenastrum capricornutum*) carried out according to OECD 201 (1984)/ISO 8692 (1989);

- Acute toxicity to crustacea
  Test with the waterflea *Daphnia magna* carried out according to OECD 202 (1984)/ISO 6341 (1996);
- Acute toxicity to fish
  Test with the zebra fish *Brachydanio rerio* carried out according to OECD 203 (1992).
- Mutagenicity
  Ames test carried out with 2 Salmonella strains incl. S9 activation, (performed by KIWA, Nieuwegein , The Netherlands).
- Biodegradation
  Closed Bottle test, carried out according to OECD 301D (1992), method based on oxygen demand.

This report contains all methods, results and raw data of the performed bioassays. Before this investigation was started acute toxicity screeningtests were performed. The results are also included in this report.

# 2. Methods

## 2.1. Testsample

The testsample (1litre enzyme solution) was supplied by Ecodor International B.V. at AquaSense Amsterdam on April 18 2000. The sample was labeled (Ecolims number 309552) and stored at 4°C in the dark. The sample is a dark brown coloured liquid with a characteristic odor. The product is used as a dilution in water (dilution 1: 250 or higher) and sprayed on the target (odor source). The odor is absorbed by the product particles and degraded by the enzymes. This product is widely applicable for various odor problems. The product used in this study was the concentrated form.
Before testing the desired amount of sample was brought at the right temperature before used in any of the tests. The composition of the product is given in Annex 10.

## 2.2. Bioassays

In the following sections a brief description of all testmethods is given. For a more detailed description the specific guidelines have to be referred to.

### 2.2.1. Growth inhibition of *Pseudomonas putida*

The growth inhibition test with the bacterium *Ps. putida* (DSMZ 50026) was carried out according to ISO 10712 (1995). In this test an exponential growing culture is exposed to various concentrations of the test substance in the test medium (growth substrate). The growth of the bacterium is followed by optical density measurements at 660 nm at the start and end of test. The bacteria are incubated at 23 ± 1 °C for 16 hours on a shaking device. For the present test a dilution range was prepared as follows: 100, 40, 16, 6.4 and 2.56 mg/l testsample. Simultaneously a reference test with 3,5 dichlorophenol was carried out to check the sensitivity of the used bacterial strain. Analyses were carried out in duplicate. For each concentration a growth curve was made, and these were used to calculate inhibition of growth as compared to the controles. The effects are expressed as $EC_{50}$ (effect concentration) value. This is defined as the concentration where 50% of the bacterial growth is inhibited compared to the control.

### 2.2.2. Acute toxicity to *Raphidocelis subcapitata*

The test with the green algae *Raphidocelis subcapitata* (formerly known as *Selenastrum capricornutum*) was carried out according to OECD 201 (1984)/ISO 8692 (1989). In this test the inhibition of growth and growth rate was determined after 24, 48 and 72 hours of exposure. The following concentrations were tested : 400, 160, 64, 25.6 en 10.2 mg/l.

The algal density in the test vessels was approximately $10^4$ algae/ml. The test was performed at 20 ± 2 °C, in an illuminated incubator (90 µE/$m^2$/s). The test was performed in triplicate in 250 ml vessels.

Before testing, the following parameters were determined in the highest concentration tested and control.

- Oxygen ;
- Acidity;
- Nitrate;
- Ammonium;
- Conductivity.

At the beginning of the test and after 24, 48, 72 hours, samples were measured spectrophotometrically at 685 nm. The algal densities were used to calculate inhibition of growth (A) and growth rate (µ) compared to the control. $EC_{50}$ values and NOEC[1] and LOEC[2] values were calculated using the software program TOXCALC (1995).

[1] NOEC=No Observed Effect Concentration: Highest concentration where no significant effects could be observed.

## 2.2.3. Acute toxicity to Daphnia magna

The acute toxicity test with the waterflea *Daphnia magna* was carried out according to the OECD 202 (1984) / ISO 6341 guideline (1996). The water fleas belong to clone 4 as defined by Calow & Bradley (1987). As effect parameter the immobility was studied. The Daphnids were observed every 24 hours for immobilisation and other adverse behavioural effects. Daphnids which were not able to swim for 15 seconds after gentle agitation of the test vessel were considered immobile. The water fleas were subjected to the following concentration effluent: 100, 50, 25, 12.5, 6.25 and 3.13 mg/l The dilutions were made using DSW[3]. The test volume was 100 ml per test vessel. 100% DSW was used as control. The test was performed in 4-fold, with 5 organisms (less than 24 hours old) in each vessel. The test temperature was 20 ± 1°C and the light-regime was 16 hours light and 8 hours dark. During the test the organisms were not fed.

Before testing, the following parameters were determined in the highest test concentration and control.

- Oxygen ;
- Acidity;
- Nitrate;
- Ammonium;
- Conductivity.

From the results the effectparameter ($EC_{50}$) was calculated using the ToxCalc-software program (Tidepool, 1995).

## 2.2.4. Acute toxicity to Brachydanio rerio

The test with the zebra-fish *Brachydanio rerio* was carried out according to OECD 203 (1992). The test duration was 96 hours, and after 24, 48, 72 and 96 hours the fish were observed for abnormal behaviour and mortality. The fish were kept in quarantine for at least 14 days before the start of the test. The test was performed using 7 fish per test vessel. All the fish used were 2,0 ± 1,0 cm in length. During the test the fish were not fed. The test temperature was 23 ± 1°C, and the light regime was 16 hours light and 8 hours dark. The test vessels were aerated, and the test medium was not refreshed during the test. The following concentration range was used : 400, 160, 64, 25.6 and 10.2 mg/l Before the testing following parameters were determined in the highest testconcentration and control.

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[2] LOEC=Loest Observed Effect Concentration: Lowest concentration where a significant effect could be observed.

[3] Dutch Standard Water (DSW) is deionized water ammended with some defined minerals.

- Oxygen ;
- Acidity;
- Nitrate;
- Ammonium;
- Conductivity.

## *2.2.5.* Mutagenicity test

The Ames test (Green, *et al*, 1976) is used for the detection of mutagenic effect of compounds. For this, two strains (histidine depended) of Salmonella (type TA 98 en TA 100) were used. For the test with type TA 98 a concentration range of : 1000-320-180- 100 en 56 gram/l of the pure product was tested. Because of toxic effects, the type TA 100 was tested with a concentration range of 50-16-9-5 en 2,8 gram/l of the pure product. Indirect effects of the sample were also tested by addition of a S9 fraction to the samples. The fraction contains enzymes which converts pro-mutagens into mutagene active substances. A sample is designated as mutagenic as the amount of revertants (reverse mutations) is at least two times the amount of spontaneously revertants, and in addition a dose-response relation should be established.

## *2.2.6.* Biodegradation test

The biodegradation of the test sample was investigated by the performance of a biodegradation test. The Closed Bottle test was performed according to the OECD guideline 301D. The test is based on the oxygen consumption of aerobic sludge in a closed vessel over a defined period of time. In the test, vessels were filled with nutrient medium, inoculum (sludge from a WWTP predominantly treating municipal wastewater, Westpoort, Amsterdam, The Netherlands), and testsubtance (6 mg/l COD, chemical oxygen demand). The vessels were air-tight closed and after 7, 14 , 21 and 28 days at 20 ± 1°C the oxygen levels were measured. A decrease in the oxygen concentration is an indirect measure for the biodegradation. To check the activity of the inoculum, a well degradable substance (acetate) was tested simultaneously. The percentages biodegradation are based on the amount of COD of the test substance (determined using a cuvet test, Dr. Lange, Tiel, The Netherlands) in the vessel. A nitrification inhibitor (allylthiourea) was added to avoid disturbances by nitrification. A compound is “readily biodegradable” when more than 60% is biodegraded within 28 days.

# 3. Results and discussion

## 3.1. Validity criteria and quality of testorganisms

Validity criteria are defined in the guidelines used. These are given in Table 3.1.

All toxicity tests were found to be valid, according to these criteria.

The quality of the test organisms used in the acute toxicity tests are periodically tested using reference compounds. The results are checked with the criteria in the guidelines and ringtest results. If the data is not valid the organisms will not be used for further testing.

**Tabel 3.1.** Validity criteria of the bioassays.

| Parameters | criterium | measured values |
|---|---|---|
| **Bacteria** (ISO 10712, 1995) | | |
| Increase density control cultures | > 60 | > 204 |
| $EC_{50}$ reference compound (mg/l) | 10 – 30 | 14.9 |
| **Algae** (ISO 8692, 1989) | | |
| Increase factor density in controls after 72 hours | > 16 | 114.9 |
| Variation of pH in controls | ≤ 1,5 | 0.2 |
| **Crustacea** (ISO 6341, 1996) | | |
| Oxygen concentration at end of test (%) | ≥ 35 | 95 |
| Immobile organisms in controls at end of test (%) | ≤ 10 | 5 |

| | | |
|---|---|---|
| **Fish** (OECD 203, 1992) | | |
| Oxygen concentration in all test vessels (%) | ≥ 60 | 98 – 102 |
| Mortality in controls (%) | ≤ 10 | 0 |
| **Mutagenicity** (Ames test) | | |
| Correlation coefficient of dose effect relation | ≥ 0.70 | n.a. |
| Toxicity of sample | no | no[1] |
| **Biodegradation** (OECD 301D, 1992) | | |
| Degradation of reference substance within 14 days (%) | > 60 | 94 |
| Difference between oxygen concentration duplicates end of test (%) | < 20 | 3 |
| Oxygen concentration at the end of the test (mg/l) | > 0.5 | 2 |
| Decrease oxygen concentration in controls after 28 days (mg/l) | < 1.5 | 1.35 |

[1] = A slight toxicity was found at the highest tested concentrations, but this did not affected the test results.

## 3.2. Physical and chemical parameters acute toxicity tests

A summary of the results of the semi-quantitative measurements of the physical and chemical parameters (confounding factors) is given in the following Annex.

**Annex 2:** acute test with *Raphidocelis subcapitata*

**Annex 3:** acute test with *Daphnia magna*

**Annex 4:** acute test with *Brachydanio rerio*

For the other tests no semi-quantitative measurements were performed, because there were not critical for the test. When the criteria are met, no negative effects of these parameters should occur. The criteria and the measured values as presented confirm that all criteria for the acute toxicity tests were met.

## 3.3. Results screeningtests

Before the definitive acute toxicity tests were initiated, acute toxicity screeningtests were performed. For this, the organisms were exposed to a concentration of 100 mg/l of the test substance and a control. The results are presented in the following table.

**Tabel 3.2.** Results of the screeningtests with Ecodor EC 250.

| test with: | **Algae** | **Crustacea** | **Fish** |
|---|---|---|---|
| testorganism: | *Raphidocelis subcapitata* | *Daphnia magna* | *Brachydanio rerio* |
| testduration: | 72 hours | 48 hours | 96 hours |
| effectparameter: | growth inhibition % | immobility % | mortality % |
| Ecodor EC 250 | 76[1] | 100[2] | 40 |

[1] = Value is based on growth (biomass).
[2] = The test was repeated, due to ambiguous results (see section 3.4).

## 3.4. Results bioassays

De results of the bioassays, as presented in Annex 4 to 9, are summarized in Table 3.3. In this table negative effects are presented $EC_{50}$- of $LC_{50}$-values. NOEC values are presented in brackets. In the table are the results presented in both mg/l and as dilution factor (italic) as rounded off figures.

**The bacterium *Pseudomonas putida*** (Annex 4)
Based on the results as presented no toxicity could be found for *Ps. putida* at the concentrations tested up to 100 mg/l. The test was valid as shown by the test results of the reference compound 3,5-DCP (section 3.1)

**The algae *Raphidocelis subcapitata*** (Annex 5)
In the test with the green algae a significant inhibition of growth was observed at the highest testconcentrations. The effects are expressed as $EC_{50}$ for growt and growth rate. The value for growth rate is usually higher than the value for growth. During the test a correction for the colour of the testsample was performed because sample colour disturbs photometric measurements.

**Tabel 3.3.** Results of the bioassays with Ecodor EC 250. NOEC's are given in brackets when applicable. Sample dilutions (calculated from the concentrations given in mg/l) are given in italic, as rounded off figures. **" > 1: 10,000" means a dilution higher than 1: 10,000.**

| test with: | **Bacteria** | **Algae** | **Crustacea** | **Fish** | **Biodegradation** | **Mutagenicity** |
|---|---|---|---|---|---|---|
| testorganism: | *Ps. putida* | *Raphidocelis subcapitata* | *Daphnia magna* | *Brachydanio rerio* | activated sludge | Salmonella |
| testduration: | 30 minutes | 72 hour | 48 hour | 96 hour | 28 days | o/n |
| effectparameter: | $EC_{20}$ (mg/l | $EC_{50}$ (mg/l) | $EC_{50}$ (mg/l) | $LC_{50}$ (mg/l) | % degradation after 33 days | mutagenic effects (g/l) |
| Ecodor EC 250 | > 100 (100)<br>*> 1:10,000* | A: 102,8 (25,6)<br>µ: > 400 (64)<br>*A: 1:10,000*<br>*µ: > 1:2,500* | > 100 (100)<br>*> 1: 10,000* | 52.5 (<10.2)<br>*1: 20,000* | 61 | > 50 (50)<br>*> 1:20* |

[1]: Growth inhibition based on both growth (A) and growth rate (µ).

**The crustacea *Daphnia magna*** (Annex 6)
In the test with Daphnids no immobilty was observed at the tested concentrations, up to a 100 mg/l. Due to this fact the $EC_n$ value could not be calculated. These results are inconsistent with the results obtained in the screeningstest. The quality of the Daphnids could not be guarantied during the screening tests, therefore the tests were repeated using a concentration range. In this last test no adverse effects were observed.

**The fish *Brachydanio rerio*** (Annex 7)
In the test with zebra fish acute toxic effects were observed at the highest concentrations tested. From the screeningstest it was already clear that effects might be expected. From the results it is clear that the zebra fish is the most sensitive species tested so far. An explanation for the high sensitivity may be given when more data on the test substance is available.

**Mutagenicity** (Annex 8)
A sample is to be designated as mutagene as the amount of revertants (reverse mutations) are at least two times the amount of spontaneously revertants, and there should be a dose-response relation. The testsubstance was tested as 100% pure product and as a 1:20 diluted sample. The 100% sample was acute toxic for the teststrain TA100, and therefore a diluted sample was tested. In both tests no mutagenic effects were found. In the test with TA 100 a slight toxicity was observed at the highest concentrations tested, but this did not affected the test results.

**Biodegradation** (Annex 9)
Biodegradation of the test substance was initiated at day 0 and

continued up until day 21. Thereafter, a plateau was reached and this continued until the end of the test. At day 28 48.3 % was biodegraded, indicating recalcitrant components in the sample. The test substance could not be designated as “readily biodegradable” due to the low degradation percentages found at day 28. To check if the biodegradation process continues after 28 days of incubation a second test was performed. In this test the incubation was continued up to 33 days. From the results (see Annex 9) it is clear that on day 33 a biodegradation percentage of 61 was reached, which indicated that the test substance is biodegradable. The biodegradation curve is comparable with the initial test (54 % after 28 days). The criteria of 60% within 28 days was again not met, and therefore the test substance cannot be designated as “readily” biodegradable, but it is unambiguous that the substance will mineralize in the natural environment. Toxicity of the test substance and/or nitrification inhibitor was not observed as shown by the biodegradation percentages found, and the endogenous respiration, which was not inhibited. The reference test with sodium actetate (see section 3.1) indicated that the activity of the inoculum was in agreement with the criteria.

# 4. Conclusions

An ecotoxicological investigation was performed with the concentrated enzyme solution Ecodor EC 250. For this, ecotoxicity tests on four trophic levels were performed. Additionally, the mutagenicity and the biodegradation were examined.
The test substance was not toxic for the bacterium *Pseudomonas putida* and the crustacea *Daphnia magna* using a dilution of 1:10,000 or higher. The sample was acute toxic for the green algae *R. subcapitata* en de zebra fish *Brachydanio rerio* at dilutions lower or equal to 1:10,000.
The test substance was found to be not mutagenic at dilutions of 1:20 or higher. The test substance is biodegradable (61% after 33 days), but can not be designated as ‘readily” biodegradable, because the criteria of 60% within 28 days was not met.

The product is used as a dilution in water (dilution 1: 250 or higher), and sprayed on the target (odor source). The odor is absorbed by the product particles and degraded by the enzymes. This product is widely applicable for various odor problems. The initial product used in this study was the concentrated form which, as such, is never used for any application.

# 5. Literature

Green, M.H.L., Muriel, W.J., Bridges, B.A. (1976). Use of the simple fluctuation test to detect low levels of mutagens, Mutat. Res. 38:33

ISO 6341 (1996). Water quality - Determination of the inhibition of the mobility of *Daphnia magna* Straus (*Cladocera, Crustacea*) - Acute toxicity test.

ISO 8692 (1989). Water quality - Freshwater algal growth inhibition test with *Scenedesmus subspicatus* and *Selenastrum capricornutum*.

ISO 10712 (1995). Water Quality – Pseudomonas putida growth inhibition test (Pseudomonas cell multiplication inhibition test).

OECD 203 (1992). Fish, acute toxicity test. OECD guideline for testing of chemicals. OECD, Paris, France, 203 adopted 17.07.92.

OECD. (1992). Closed Bottle test. Test Guideline 301D, July 17th, 1992. Guidelines for testing of chemicals, Organisation for Economic Cooperation and Development, Paris

OECD. (1984). *Daphnia sp*., Acute immobilisation test and reproduction test. Test Guideline 202, June 7th, 1984. Guidelines for testing of chemicals, Organisation for Economic Cooperation and Development, Paris

Tidepool (1995). ToxCalc user's guide. Comprehensive toxicity data analysis and database software. Version 5.0 for microsoft Excel under Windows 3.1 or Apple Macintosh. Tidepool Scientific Software.