

(Contract **121762**)

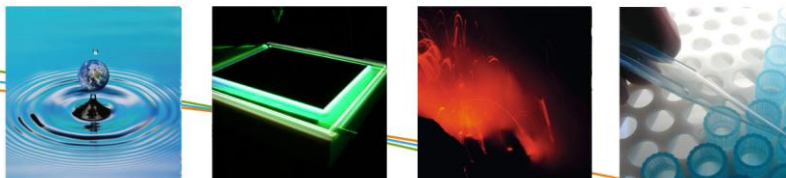
Ecotoxicity and biodegradability of Provifrost KA ECO

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CHAPTER 1: SUMMARY

The test substance PROVIFROST KA ECO (50% solution of potassium-acetate in water with additives) was tested for its acute aquatic ecotoxicity. The test was performed according to OECD guidelines for testing chemicals.

Algal growth inhibition test (OECD 201)
Daphnia immobility test (OECD 202)
Fish mortality test (OECD 203)

Also the biodegradability of the test substance was assessed in the Zahn Wellens test (OECD guideline for testing chemicals 302B). The inoculum for this test was collected at the municipal waste water treatment plant of Geel (Aquafin).

Individual test reports are provided in this report.

The test concentrations were 1 g/l for Daphnia and 500 mg/l for algae and fish (limit test). The LC50 value for Daphnia was > 1000 mg/l (nominal concentration) for an exposure period of 48 hours. Algae and fish did not experience any adverse effect up to the highest test concentrations. No chemical measurements were performed during the tests: stability of the test substance during the test is therefore not documented and results can only be reported as nominal concentrations.

The results show that the test substance PROVIFROST KA ECO is not acute toxic to Daphnia, algae and fish.

The degradation rate of the test substance was very fast: initially 200 mg/l DOC as test substance was added to the test vessels and within 48 hours DOC was depleted to values equal to control values. The test substance is ready (bio)degradable in aerobic conditions.

CHAPTER 2: INDIVIDUAL TEST REPORT

Test report: DAPHNIA ACUTE TOXICITY TEST

Test substance: PROVIFROST KA ECO (50% solution of potassium-acetate in water with additives) – Batch

The test was performed on two batches.

Test substance Codes: 12L001 (batch 20121005GVH) /12J001 (batch 2012060108)

Test protocol

Principles: The test protocol is based on the OECD guideline for testing chemicals N°202: Acute Daphnia immobility test (OECD 202) :

Newborn water fleas (*Daphnia Magna*; less than 24 hours old) are exposed to a dilution series of the test substance. The number of immobile organisms is assessed after 24 and 48 hours of exposure.

Conditions:

- Glass vessels (50 ml) with translucent cover
- Content: 20 ml
- 5 organisms (neonates < 24 h of age) per vessel
- 4 replicates per test condition, 6 replicates for controls
- Location: BIO0060
- Temperature: 22.5°C +/- 0.5*
- Light/dark: 16/8
- Static set up
- Test duration: 48h
- Nominal Test concentrations: 1-0.5-0.25-0.125-0.0625 g/l
- Control and dilution medium: JP4 medium (annex 2)
- Assessment of immobile organisms: after 24h and 48 h

**Temperature during the test is 22 ± 0.5 °C instead of the OECD recommended value of 20 ± 2 °C. This deviation is due on the one hand to the limited possibilities of the individual room temperature regulation system and on the other hand to provide more comfortable room temperature conditions for the personnel that has to work daily with this test system. Since this higher temperature does not affect the survival in controls nor has any effect on reproduction in the breeding containers, we decided to increase the temperature conditions permanently. Temperature remains however within ± 2 °C during individual tests (22.0-22.3 °C in this specific test).*

Test code: DAC12022b

The test was performed from 11-13/12/2012.

Test code: DAC12023

The test was performed from 18-20/12/2012

Physical chemical assessments:

pH (BIO0781) at start and end of the test in all dilutions

Conductivity (BIO 1539) in freshly prepared dilutions
 O2 (BIO0870) at the start and at the end of the test in all dilutions
 No chemical measurements were performed to confirm the stability of the test substance.

Test Facility: VITO BIO – Industriezone Vlasmeer 7 – B2400 MOL

Test results

Physical chemical measurements

Table 1.1: physical chemical measurements in freshly prepared dilutions of Provifrost KA ECO


Dilution (g/l)	pH		Oxygen (%)		Conductivity (µS/cm)		
	test	022b	023	022b	023	022b	023
1		8.25	8.14	96	97	1109	1124
0.5		8.12	8.08	98	98	881	881
0.25		8.07	8.05	96	98	750	742
0.125		8.07	8.05	97	98	682	682
0.0625		8.04	8.06	97	97	660	657
Boundary condition for daphnia		5-9		>60		<7500	

(pH and oxygen were again measured at the end of the test: see raw data)
 pH and oxygen are within acceptable ranges throughout the experiments.

DAC12022b

Raw data

Table 1.2: Raw data of the acute daphnia test DAC 12022b

 vision on technology TDAPE002-Frm4v01	MRG-LABORATORIES Acute toxicity Test with Daphnia Immobilisation
	DAC12022b 12L001 provifrost ECO v2 JP4

recipient N°	concentration g/l	# organisms at start	mobility (# mobile organisms)		end		conductivity (µS/cm)
			24 h	48 h	pH	O ₂ (%)	
1	0,00	5	5	5	8,09	98,0	670
2	0,00	5	5	5			
3	0,00	5	5	5			
4	0,00	5	5	5			
5	0,00	5	5	5			
6	0,00	5	5	5	8,04	97,0	660
7	0,06	5	5	4			
8	0,06	5	5	5			
9	0,06	5	5	5			
10	0,06	5	5	5			
11	0,13	5	5	5	8,07	97,0	682
12	0,13	5	5	5			
13	0,13	5	5	5			
14	0,13	5	5	5			
15	0,25	5	5	5			
16	0,25	5	5	5	8,07	96,0	750
17	0,25	5	5	5			
18	0,25	5	5	5			
19	0,50	5	5	5			
20	0,50	5	5	5			
21	0,50	5	5	5	8,12	98,0	881
22	0,50	5	5	5			
23	1,00	5	5	4			
24	1,00	5	5	5			
25	1,00	5	5	5			
26	1,00	5	5	5	8,26	96,0	1109

Validity of the test:

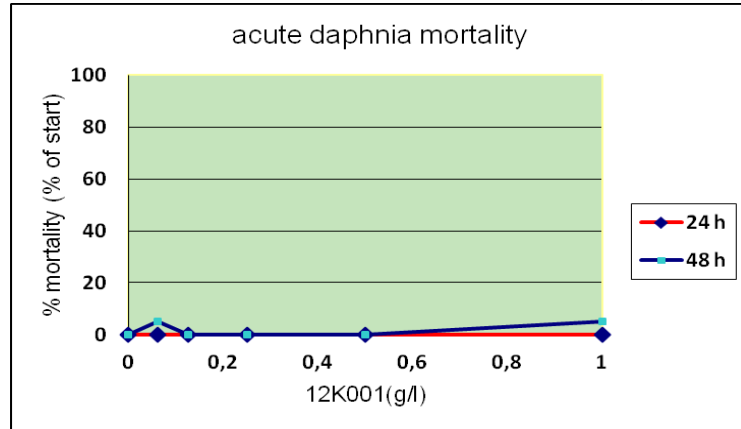
- no mortality was seen in the control organisms
- oxygen concentration was > 60% saturation.

Test results are therefore valid.

Effects:

Figure 1.1 shows the concentration effect curve. There is no adverse effect on the mobility of daphnia in the test range (up to 1000 mg/l).


Fig.1.1: concentration effect curve for Provifrost KA ECO (DAC 12022b)



DAC12023

Raw data

Table 1.3: Raw data of the acute daphnia test DAC 12023

 <small>vision on technology</small>	MRG-LABORATORIES Acute toxicity Test with Daphnia Immobilisation		
	TDAPE002-Frm4v01	DAC12023	12J001

recipiënt N°	concentration µg/l	# organisms at start	mobility (# mobile organisms)		end		conductivity (µS/cm)
			24 h	48 h	pH	O ₂ (%)	
1	0,00	5	5	5	8,11	99,0	655
2	0,00	5	5	5			
3	0,00	5	5	5			
4	0,00	5	5	5			
5	0,00	5	5	5			
6	0,00	5	5	5			
7	0,06	5	5	5	8,06	97,0	657
8	0,06	5	5	5			
9	0,06	5	5	5			
10	0,06	5	5	5			
11	0,13	5	5	5	8,05	98,0	682
12	0,13	5	5	5			
13	0,13	5	5	5			
14	0,13	5	5	5			
15	0,25	5	5	5	8,05	98,0	742
16	0,25	5	5	5			
17	0,25	5	5	5			
18	0,25	5	5	5			
19	0,50	5	5	5	8,08	98,0	881
20	0,50	5	5	5			
21	0,50	5	5	5			
22	0,50	5	5	4			
23	1,00	5	5	5	8,14	97,0	1124
24	1,00	5	5	5			
25	1,00	5	4	4			
26	1,00	5	4	4			

Validity of the test:

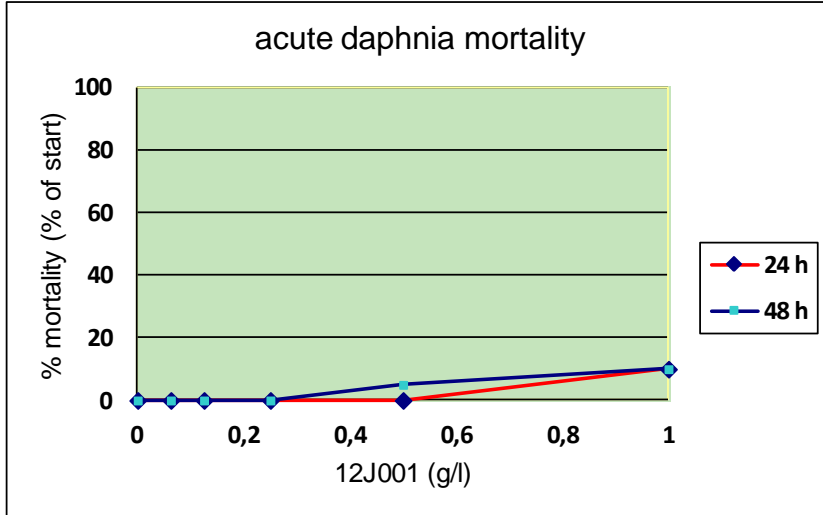
- no mortality was seen in the control organisms
- oxygen concentration was > 60% saturation.

Test results are therefore valid.

Effects:

Figure 1.1 shows the concentration effect curve. There is no adverse effect on the mobility of daphnia in the test range (up to 1000 mg/l).

Fig.1.2: concentration effect curve for Provifrost KA ECO (DAC 12023)



Conclusion:

PROVIFROST KA ECO has no adverse effect on the daphnia mobility at test concentrations up to 1000 mg/l.

Test report: ALGAL GROWTH INHIBITION TEST

Test substance: PROVIFROST KA ECO (50% solution of potassium-acetate in water with additives)

Test substance Code: 12J001

Test protocol

Principles: The test protocol is based on the OECD guideline for testing chemicals N°201: Growth inhibition test on unicellular green algae :

Unicellular algae *Pseudokirchneriella subcapitata* are exposed to a dilution series of the test substance. The number of cells is assessed after 24, 48 and 72 hours of exposure.

Conditions:

- Erlenmeyers (500 ml) with cotton stoppers
- Nominal Test concentrations: a stock solution was made of 500 mg/l in algal mineral medium (annex 1) and further 1/2 dilutions in medium were prepared. 500-250-125-62.5-31.25 mg/l.
- Recipients: 100 ml control (algal mineral medium) or test dilutions, 100 µl algal stock (batch 1203 *Pseudokirchneriella subcapitata* – $6.4 \cdot 10^6$ cells per ml) except in the abiotic controls. As the algal stock is diluted 1000 times in the test the final algal concentration is $6.4 \cdot 10^3$ cells per ml.
- 3 replicates for test conditions and abiotic controls, 6 replicates for controls
- Tests were performed in incubator BIO 0658: 23°C, continuous illumination (3620 lux), continuous shaker at 100 rpm
- Test duration: 72h
- Assessments: Daily measurements of the number of cells (using coulter counter BIO 1012) Calculations: increase in biomass as a function of time (growth curves), specific growth, growth rate. The inhibition of these parameters when compared to controls is assessed.

The test was performed from 5-8/11/2012

Testcode: ALGE12018

Physical chemical assessments:

pH (BIO 1334) at start and end of the test in all test vessels

No chemical measurements were performed to confirm the stability of the test substance.

Test Facility: VITO BIO – Industriezone Vlasmeer 7 – B2400 MOL

Test results

Algae growth inhibition test

Raw data in table 2.1.

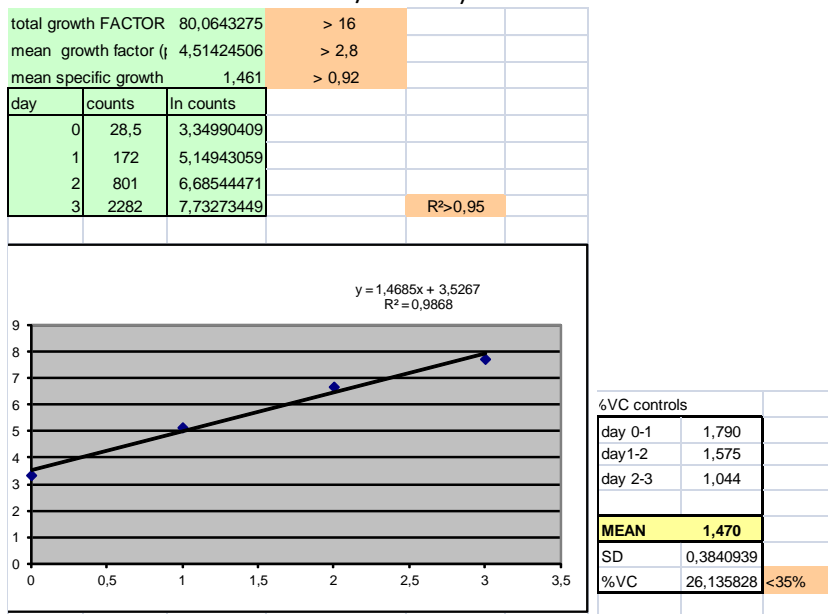
Table 2.1: raw data of the algal growth inhibition test.

ALGE12018		erlenmeyer		OECD protocol							
12J001 proviron		5/11/12-8/11/12									
	name	time (h)	cell concentration				pH start	pH end	mean		
			0	24	48	72			pH start	pH end	
blanks	abiotic control	0	2	7	9	10	8,47	8,02			
blanks	abiotic control	0	6	8	5	3	8,46	7,98			
blanks	abiotic control	0	5	5	6	5	8,45	7,98	8,46	7,99	
controls	biotic control	0	35	161	752	2449	8,41	8,01			
	controle	0	26	161	850	2331	8,4	8,19			
	controle	0	27	192	877	2250	8,42	8,07			
	controle	0	21	187	741	2214	8,42	8,07			
	controle	0	35	172	773	2260	8,4	8,05			
	controle	0	27	161	811	2187	8,42	8,05	8,41	8,07	
replica 1	test concentration 1	31,25		146	670	1754	8,37	7,99			
replica 2	test concentration 1	31,25		167	813	1865	8,37	8,04			
replica 3	test concentration 1	31,25		188	653	1791	8,34	7,98	8,36	8,00	
replica 1	test concentration 2	62,5		165	695	1744	8,33	8,04			
replica 2	test concentration 2	62,5		159	634	1705	8,33	8			
replica 3	test concentration 2	62,5		160	747	1999	8,34	8,03	8,33	8,02	
replica 1	test concentration 3	125		181	784	2487	8,32	8,15			
replica 2	test concentration 3	125		192	798	2194	8,33	8,07			
replica 3	test concentration 3	125		177	664	1999	8,32	8,09	8,32	8,10	
replica 1	test concentration 4	250		188	841	2267	8,33	8,04			
replica 2	test concentration 4	250		161	773	2295	8,34	8,01			
replica 3	test concentration 4	250		199	740	2243	8,34	8,03	8,34	8,03	
replica 1	test concentration 5	500		194	689	1257	8,35	8,01			
replica 2	test concentration 5	500		174	403	534	8,37	7,97			
replica 3	test concentration 5	500		150	669	1918	8,34	8,04	8,35	8,01	

Validity of the test:

Assessment of the growth parameters in the control recipients (figure 2.1) show that all criteria were fulfilled and test results are accepted.

Fig.2.1: Growth parameters in controls (measured values in green, limit values in orange)(VC = coefficient of variance: mean/SD*100)



pH was within the boundary conditions for algae (7-9: see raw data). The conductivities of the different dilutions (compare to values in table 1.1) were all within the acceptable range for algae (i.e. < 4 mS/cm).

Effects:

Figure 2.2 shows the concentration effect range for growth rate. Figure 2.3 shows the growth curves.

Figures 2.2 and 2.3 show there was no inhibiting effect on the algal growth or growth rate within the test concentration range (up to 500 mg/l). There is a slight inhibition at 500 mg/l but this is not statistically significant.

Fig. 2.2: Concentration-effect curve: ALGE12018 Effect on the growth rate of unicellular algae.

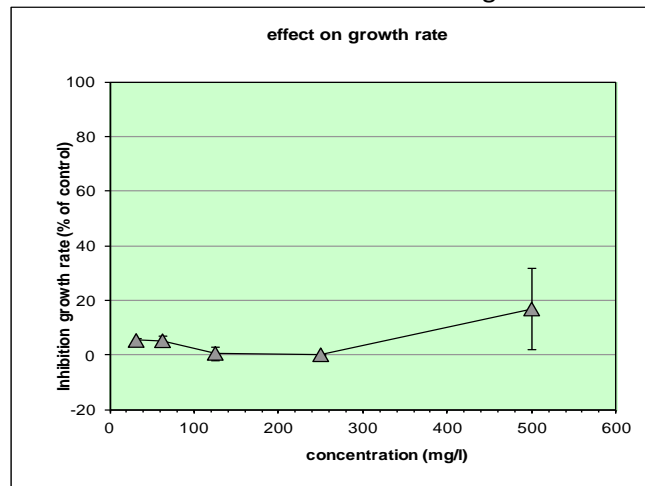
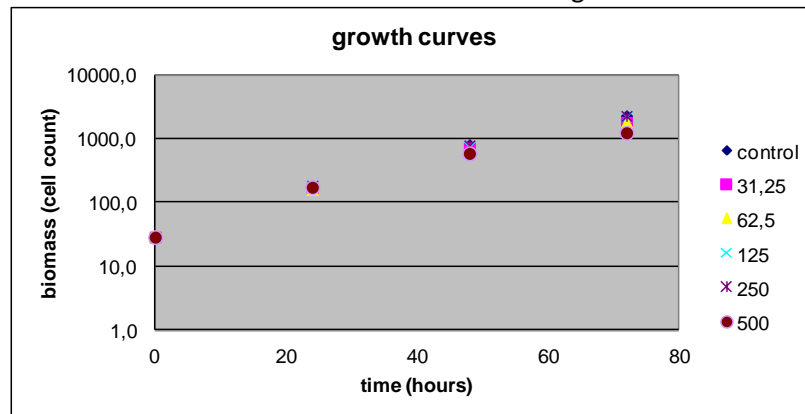


Fig. 2.3: Concentration-effect curve: ALGE12018. Effect on the growth of unicellular algae.



Conclusion:

Provifrost KA ECO has no statistical significant adverse effect on the growth and growth rate of the unicellular green algae *Pseudokirchneriella subcapitata* up to 500 mg/l (nominal concentration).

Test report: ACUTE FISH MORTALITY TEST – LIMIT TEST

Test substance: PROVIFROST KA ECO (50% solution of potassium-acetate in water with additives)

Test substance Code: 12J001

Test protocol

Principles: The test protocol is based on the OECD guideline for testing chemicals N°203: Fish Acute Mortality Test (Limit test) using *Danio rerio* (zebra fish) as test species

Fish are exposed to one test concentration of the test substance and mortality is assessed after 2, 24, 48, 72 and 96 hours of exposure.

Conditions:

- Aquaria 10 l
- 7 fish/aquarium (*Danio rerio*)
- 23.3-25.3°C, continuous pressed air supply
- Nominal Test concentration: 1000 mg/l (in aerated tap water)
- control = aerated tap water*
- Test duration: 96h
- Static test
- Assessments: at daily intervals pH, oxygen, behaviour and mortality of the organisms are assessed
- Reported values: % mortality in the different test conditions.

* Reference PIDPA (delivery of tap water): Tap water quality at VITO (Geel, retieseweg): 5,6 °F or 0.56 ppm (very soft water).

According to OECD 203 fish medium preferably has a total hardness of 10-250 ppm. The tap water in our institution has a lower total hardness, but as survival of fish in stock and controls is normal this is not adapted.

The test was performed from 5-9/11/2012

Testcode: VAC12008

Physical chemical assessments:

- pH (BIO 1581) daily in all dilutions
- Conductivity (BIO 01359) in freshly prepared solution
- O₂ (BIO 0870) daily in all dilutions
- Temperature (BIO 1581) daily in all dilutions

No chemical measurements were performed to confirm the stability of the test substance.

Test Facility: VITO BIO – Industriezone Vlasmeer 7 – B2400 MOL

Test results

Physical chemical measurements

Table 3.1: physical chemical measurements in freshly prepared dilutions of Provifrost NF

Dilutions (g/l)	pH	Oxygen (%)	Conductivity (mS/cm)
0	8.46	92	0.303
0.5	8.45	91	0.583
Boundary conditions for fish	6-9	>60	<22

Values are within the normal range. No effects are expected due to these confounding factors.

Table 3.2 shows the daily measurements of pH, Oxygen and temperature in the dilutions.

Table 3.2: daily measurements of pH, Oxygen and temperature in the aquaria.

Dilutions (g/l)	pH	Oxygen (%)	Temperature (°C)
0	8.49-8.53-8.53-8.55	91-97-90-94	25.3-24.8-24.6-24.7
1	8.49-8.53-8.55-8.58	96-96-91-93	23.5-23.5-23.3-23.3

Validity of the test:

- No mortality was seen in controls
- Oxygen was >60 % during the experiment
- pH values were within the normal range.

Test results are therefore valid.

Effects:

No mortality was seen in any of the two test conditions, and fish behaved normal in both conditions.

Conclusion:

1000 mg/l test substance PROVIFROST NF (nominal concentration) has no adverse effect on the survival of *Danio rerio* at nominal concentrations up to 500 mg/l. No adverse effects on behaviour of the fish were seen in this test condition.

Test report: ZAHN-WELLENS biodegradation test

Test substance: PROVIFROST KA ECO (50% solution of potassium-acetate in water with additives)

Test substance Code: 12J001

Test protocol

Principles: The test protocol is based on the OECD guideline for testing chemicals N°302: Zahn Wellens/EMPA Test (Inherent biodegradability test).

A mixture containing the test substance, mineral nutrients and a relatively large amount of activated sludge is agitated and aerated at room temperature in diffuse light for up to 28 days. Blank controls are run in parallel. The biodegradation process is monitored by determination of DOC in filtered samples taken at regular intervals.

Conditions:

- duplicate set up
- 4 Brown bottles 10 l , with an aeration tube and a catheter for sampling (2 controls, 2 test vessels)
- 3 liter medium/test dilution per vessel
- Magnetic stirrer for continuous stirring of the vessels
- 21-22°C
- Diffuse light
- Blank and dilution medium: BOD medium according to OECD (annex 3)
- Test concentration:
 - Provifrost KA ECO contains 50% CH₃CO₂K (24.47 % carbon) and 50% water. Density is 13g/l. The intended concentration of carbon in the test vessel is 200 mg/l or 600 mg/ vessel. Therefore 3 ml of the test substance per vessel was added to the medium.
- Inoculum: 2 liter active sludge was collected at the municipal waste water treatment plant of Geel (Aquafin). After washing and concentrating the sludge was aerated overnight. DW of the prepared sludge was 1.48 g/100 ml. The concentration added to the test vessels was 0.5 g Dry Matter/ l test substance.
- Test duration: 28d or until all DOC has been removed
- Assessments: at regular intervals 10 ml samples were taken from each recipient. The samples were filtered (0.22µ) and immediately acidified (pH 2) with HNO₃ and were kept refrigerated until the measurement.
- Reported values: time to reduce DOC to values not different from control value.

The test was performed from 24/10-5/11/2012

Testcode: BIO12002

Physical chemical assessments:

- DOC was measured in the filtered samples using Hach Lange Kits for TOC (KCK 385/386: DL 3 – 300 mg/l)

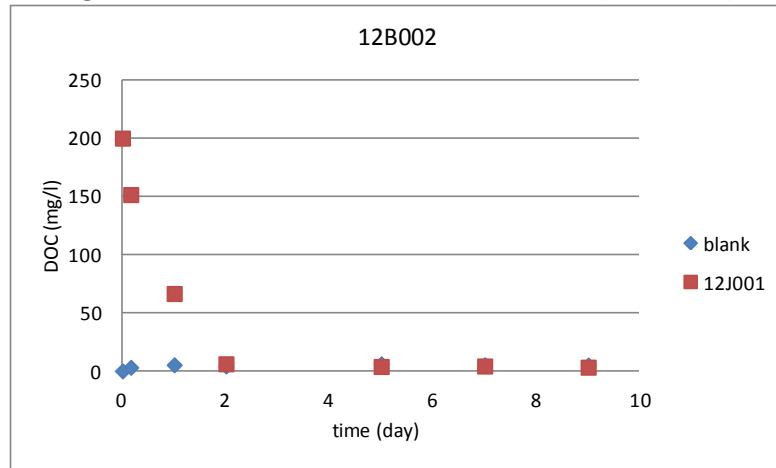
Test Facility: VITO BIO – Industriezone Vlasmeer 7 – B2400 MOL

Test results

Table 4.1: DOC measurements in samples taken on the different days during BIO12002

	day					
	3h	1	2	5	7	9
blank	3,49	7,1	3,93	6,7	4,7	4,71
blank	2,69	3,4	4,47	5,5	5,91	5,3
12J001	129	56	3,16	4,42	4,6	3,53
12J001	174	77	9,1	3,21	3,8	2,91

Fig. 4.1: DOC values in blank and test vessels (BIO12002)



The data show that the test substance has disappeared from the test solutions within 2 days. There is an initial decrease of 50 mg/3 hours due to adsorption to the particles. After that there is a steady decrease in DOC concentration (slope: 78 mg DOC/day) and total depletion after 2 days. The test was stopped at day 9.

Conclusion:

The test substance PROVIFROST KA ECO (bio)degrades very fast in aerobic conditions with an inoculum collected from a municipal waste water treatment plant.

ANNEX 1: COMPOSITION OF THE DAPHNIA JP4 MEDIUM

Concentrations of the different ingredients in the reconstituted water (JP4) used in Daphnia toxicity tests and in the breeding compartments.

Substance	Final concentration in diluting water	
CaCl ₂ .2H ₂ O	294.0	mg/l
MgSO ₄ .7H ₂ O	123.3	mg/l
KCl	58	mg/l
NaHCO ₃	64.8	mg/l
K ₂ -EDTA.2H ₂ O	1086	µg/l
FeCl ₃ .6H ₂ O	1500	µg/l
MnSO ₄ .7H ₂ O	31	µg/l
Na ₂ MoO ₄ .2H ₂ O	12.6	µg/l
ZnSO ₄ .7H ₂ O	4.4	µg/l
SeO ₂	1.4	µg/l
Vitamine B ₁₂	1.0	µg/l
Water: MilliRO; conductivity ≤ 20 µS/cm	upto 1 l	

Physical chemical parameters:

pH = 7.9 +/- 0.3

Hardness = 250 +/- 25 mg/l CaCO₃, Ca/Mg ~ 4

Na/K ~ 10

ANNEX 2: COMPOSITION OF THE ALGAL OECD MINERAL MEDIUM

Concentrations of the different ingredients in the reconstituted water (OECD algal medium) used in algal toxicity tests.

Substance	mg/l
H ₃ BO ₃	0,185
MnCl ₂ .4H ₂ O	0,415
ZnCl ₂	0,003
FeCl ₃ .6H ₂ O	0,08
Na ₂ EDTA.2H ₂ O	0,1
CoCl ₂ .6H ₂ O	0,0015
Na ₂ MoO ₄ .2H ₂ O	0,007
CuCl ₂ .2H ₂ O	0,00001
NH ₄ Cl	15
MgCl ₂ .6H ₂ O	12
CaCl ₂ .2H ₂ O	18
MgSO ₄ .7H ₂ O	7,4
KH ₂ PO ₄	1,6
NaHCO ₃	50

ANNEX 3: COMPOSITION OF THE BOD MINERAL MEDIUM

Concentrations of the different ingredients in the BOD medium (OECD) used in biodegradation tests.

Substance	Concentration in the medium
NH ₄ Cl	500 µg/L
CaCl ₂	27.5 mg/L
MgSO ₄ .7H ₂ O	22.5 mg/L
KH ₂ PO ₄	8.5 mg/L
K ₂ HPO ₄	21.7 mg/L
Na ₂ HPO ₄ .2 H ₂ O	33.4 mg/L
FeCl ₃ .6H ₂ O	250 µg/L
EDTA	400 µg/L
Demineralised water*	Up to 1 L

*Produced by a water demineralising system (Millipore) based on reverse osmosis.