Hydrolysed Proteins

DOCUMENT M-CA, Section 8

ECOTOXICOLOGICAL STUDIES ON THE ACTIVE SUBSTANCE

Version history[1]

Date	Data points containing amendments or additions and brief description	Document identifier and version number
21/02/2020	Additional data and information on aquatic organisms and potential for endocrine disruption in CA 8.1.5, CA 8.2, CA 8.2.1, CA 8.2.3, CA 8.2.4.1 and CA 8.2.6.1 highlighted in yellow	Hydrolysed Proteins document M-CA 8

[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

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CA 8 ECOTOXICOLOGICAL STUDIES ON THE ACTIVE SUBSTANCE

Introduction

Hydrolysed proteins are naturally occurring compounds of degradation from the hydrolysis of living organisms' tissues that can have vegetable or animal origin. The degradation of the hydrolysed proteins results in more simple metabolites called amino acids. Proteins and amino acids are abundant organic molecules in living cells. They can be found in every single cell, since they are fundamental in all aspects of the cell structure and function, and intervene in the most essential biochemical processes.
Thus, the hydrolysed proteins are biodegradable, so their persistence in the environment is very short without any tendency for bioaccumulation.
Due to the nature of the hydrolysed proteins and their characteristics regarding the fate and behaviour in the environment, it is deemed very unlikely the existence of relevant residues resulting from applications as plant protection product in the soil, surface water or sediment.
Furthermore, the proteins are one of the three basic principal nourishment of living beings. The proteins that are found in food and eaten by human beings and mammals are normally degraded metabolically by means of enzymatic processes and results in amino acids, that are then used by the living cells for the biosynthesis of new specific proteins.
Therefore, the hydrolysed proteins and resulting metabolites are not expected to cause any danger to human beings and mammals in general because as explained, these compounds take place in every living cells and are therefore essential for life.

Moreover, it should be noted that the review of the scientific literature within the last 10 years did not give any results indicating a hazardous effect or a potential risk for the terrestrial and aquatic wildlife and ecosystems in general. Please refer to Document M-CA 9.

For all these reasons, the use of Hydrolysed proteins is considered to pose a low risk to non-target organisms (birds, aquatic organisms, wild mammals, bees, non-target arthropods, earthworms and other soil-macroorganisms, soil microorganisms and non-target plants) and no testing toxicity data are required.

CA 8.1 Effects on Birds and Other Terrestrial Vertebrates

No EU data/endpoints available.

No new data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.1.1 Effect on birds

CA 8.1.1.1 Acute oral toxicity to birds

No data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.1.1.2 Short-term dietary toxicity to birds

No data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.1.1.3 Sub-chronic and reproductive toxicity to birds

No data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.1.2 Effects on terrestrial vertebrates other than birds

CA 8.1.2.1 Acute oral toxicity to mammals

No data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.1.2.2 Long-term and reproductive toxicity to mammals

No data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.1.3 Effects of active substance bioconcentration in prey of birds and mammals

No EU data/endpoints available.

No new data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.1.4 Effects on terrestrial vertebrate wildlife (birds, mammals, reptiles and amphibians)

No data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.1.5 Endocrine disrupting properties

No data submitted, not required. Please refer to the argumentation presented under Point B 8.
Due to the nature of the Hydrolysed proteins and their essential role in living cells, it is deemed acceptable to consider that the active substance Hydrolysed proteins have no endocrine disrupting properties.
Therefore, Hydrolysed Proteins meet the criteria for the approval of low-risk active substance because not considered to be an endocrine disruptor.

In response to the RMS request, the applicant would like to highlight that Hydrolysed proteins are naturally occurring compounds whose degradation leads to simple metabolites called amino acids that are abundant organic molecules in living cells and used for the biosynthesis of new specific proteins and therefore essential for life.
Furthermore, in addition to the use as fertilisers as explained for environmental fate and behaviour section, hydrolysed proteins are also used as raw material for feed in aquaculture or animal feeding market.
On request, the applicant can provide several articles or examples of final products containing hydrolysed proteins in the market linked to the aquaculture and animal feeding uses, to reinforce this argumentation.
Moreover, the active substance hydrolysed proteins is not classified according to the ECHA.
The applicant is of the opinion that these information provide an argumentation strong enough to justify the exemption of further studies, considered as unnecessary from a scientific and rational point of view.

However, for completeness purposes, the applicant provides a study performed with Hydrolysed proteins ([redacted], *"Bioefficacy of parentally-administered Norlan L 25 in piglets" (2004)* [redacted], and supplementary report) to prove that the administration of such compounds to mammals has no endocrine disruption effects.
The conclusion of this study is that the use of Hydrolysed proteins resulted in significant increases in the concentration of monocytes and platelets and in mean platelet volume, but without having any significant effect on the growth and feed conversion index. Moreover, it was clearly highlighted that the increased concentration of monocytes observed after administration reveals an immune efficacy, inducing increased defences against various pathogenic microorganisms without any endocrine disruption properties.

CA 8.2 Effects on Aquatic Organisms

No EU data/endpoints available. According to the EFSA Journal 2012;10(2):2545, studies on aquatic organisms were considered necessary to fulfil the Annex II requirements directly related to classification and labelling. A data gap was identified.

Therefore, confirmatory data were presented and the designated RMS (Greece) in the Addendum IV to the Draft Assessment Report (Volume 3, Annex B-9: Ecotoxicology, September 2014), has concluded the following:
"Although no specific testing toxicity data on either of the hydrolysed proteins notified have been submitted, by taking into account:

(i) the lack of any information or evidence in the scientific literature related to the aquatic toxicity potential of hydrolysed proteins,

(ii) the indication of low hazard and risk entailed by the use of hydrolysed proteins (e.g. beet molasses-urea hydrolysate) in insect attractants for bait spray applications compared to other nitrogen compounds and

(iii)the nature of the active substance and its characteristics regarding the fate and behavior in the environment (biodegradable, non-persistent, non bioaccumulative),

it can be concluded that the use of hydrolysed proteins is of low danger for the aquatic ecosystems in general and for the aquatic organisms in particular. In consequence, from the RMS's point of view, hydrolysed proteins should not be assigned any classification for aquatic hazards and should be deemed as non-dangerous for the environment substances."

Furthermore, it should be noted that according to the notifications provided to ECHA in REACH registrations and CLP notifications, no hazards have been classified for the active substance Hydrolysed proteins, referred as "*Protein hydrolyzates, animal*" by ECHA. Please refer to the summary from ECHA provided in Document M-CA 10.

According to the EFSA Conclusion (2012) a data gap was identified concerning toxicity studies for aquatic organisms: "*Studies on aquatic organisms that are necessary to fulfil the Annex II requirements directly related to classification and labelling*".
Therefore, and in agreement with the current data requirements related to Regulation (EU) 283/2013 the following toxicity studies have been submitted for aquatic organisms:

- Acute toxicity to fish (*Danio rerio*).
- Acute toxicity for aquatic invertebrates (*Daphnia magna*).
- Growth inhibition test for freshwater alga (*Pseudokirchneriella subcapitata*)

The endpoints drawn from these studies are summarized in the table below and will be used for risk assessment.

Table 8.2-1: Endpoints and effect values relevant for the risk assessment for aquatic organisms – Hydrolysed proteins

Species	Substance	Exposure System	Results	Reference
Danio rerio	SVMA14-004	96h, s	EC_{50} > 100 mg/L	[redacted], 2019, Report No [redacted]
Daphnia magna	SVMA14-004	48h	EC_{50} > 100 mg/L_{nom}	C. Giarei, 2019, Report No STULV19AA4423-1 GLP
Pseudokirchneriella subcapitata	SVMA14-004	72h	EC_{50} = 313.493 mg/L	C. Giarei, 2019, Report No STULV19AA4421-1 GLP
Higher-tier studies (micro- or mesocosm studies)				
No data				

s: static; ss: semi-static; f: flow-through; nom: based on nominal concentrations; mm: based on mean measured concentration

Please note that endpoints for the formulation SVMA14-004 are the same than those for the active substance Hydrolysed proteins due to the composition of the formulation SVMA14-004 (please refer to Part C).

CA 8.2.1 Acute toxicity to fish

A summary of the new submitted study is available below.

Reference:	KCA 8.2.1
Report	ACUTE TOXICITY TEST ON AQUATIC ORGANISMS (*Danio rerio*) ON "NORLAN AMPL 201901001007", [redacted], 2020, report No. [redacted]
Guideline(s):	Yes, OECD 203
Deviations:	No
GLP:	Yes
Acceptability:	Yes/No/Supplementary
Duplication (if vertebrate study)	-

SVMA14-004 (called "NORLAN AMPL 201901001007" in the study) is a brown liquid formulation containing 300 g/L of animal proteins hydrolysate. The aim of the study is to determine the ecotoxicological effects of the test item on biotic systems: in particular aquatic organism such as Danio rerio fish in order to perform a test to define a limit not toxic threshold.
The toxicity of the test item, "NORLAN AMPL 201901001007", on the fish, *Danio rerio*, has been evaluated according to OECD guideline N. 203 concerning Acute toxicity test – LC_{50}.

In particular, the assay of acute toxicity with fish *Danio rerio* has been performed by means of a static assay in which the solution has not been changed for the whole test period. After a range finding test, a limit test has been performed using 14 fish, 7 of them treated with the test item at 100 mg/L and 7 fish used as control, kept in the same assay conditions without adding the test item.
Fish have been observed for a period of 96 hours and mortality and/or possible anomalous behaviours have been observed both in control and in treated.
Temperature, pH and percentage of saturation of oxygen in the water of each tank have been measured at the beginning of the treatment and with intervals of 24 hours.
Stability of the test item in the definitive test has been performed measuring organic carbon concentration at the beginning and at the end of the test in the tanks.

Material and methods

- **Assay conditions**

Water parameters at the beginning of the assay

To reduce the eventual presence of chlorine, the tap water used for the assay has been allowed to stabilize for 48 hours.
Tanks have been kept under control for temperature and oxygen for approximately 1 hour before introducing fish.
pH, temperature and oxygen have been measured at the beginning of the test in the water used for the experimentation.

Exposition

Animals have been added to the assay tanks with a biomass density lower than 0.8 g/L.
The treated group and the control group have been observed for 96 hours with a photoperiod of 12 hours of light and 12 hours of darkness.
Fish have been observed after 2 h ± 0.5h and 5 h ±1 h from the beginning of the test and twice per day until the end of the test.

- **Experimental design (Sample preparation)**

Range finding test

A solution at 1000 mg/L of the test item has been prepared in tap water. Then 5 different concentrations have been prepared with ten-fold dilutions from 100 mg/L to 0.01 mg/L.
The test has been performed on the diluted solutions.

N° of groups: 5 treated + 1 control
N° of animals : 3/tank

Definitive test (LC_{50} test)

After the range finding test a solution at 1000 mg/L has been prepared. Then the solution has been diluted to 100 mg/L in culture medium. The test has been performed on the diluted solution.
14 fish have been used for the definitive test subdivided as follows:

Group	Animals (N°)	Administered solution Nominal concentration	Observation times – Check point chemical-physical parameters (hours)
Control	7	Tap water	0-24-48-72-96
Treated	7	100 mg/L	0-24-48-72-96

N° of groups: 5 treated + 1 control
N° of animals : 7/tank

- **Determination of analytical concentration of total organic carbon (TOC)**

Determination of total organic carbon has been done using an automatic Shimadzu TOC analyser. It is a high sensitivity instrument based on the combustion catalytic oxidation method (680°C). The carbon dioxide generated by oxidation has been detected using an infrared gas analyser (NDIR). Samples, properly

diluted with deionised water with low TOC contents, have been analysed by means of an integrated sampling system directly from vials containing 40 ml volume.
For each sample under test the corresponding control is analysed.

Results and discussion
Number of dead fish and symptomatology of fish in control and treated groups during the definitive test

Time / Group	2h	5h	1 day	1 day	2 day	2 day	3 day	3 day	4 day	4 day
Check N°	-	-	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Control	0/0/7	0/0/7	0/0/7	0/0/7	0/0/7	0/0/7	0/0/7	0/0/7	0/0/7	0/0/7
Treated 100 mg/L	0/0/7	0/0/7	0/0/7	0/0/7	0/0/7	0/0/7	0/0/7	0/0/7	0/0/7	0/0/7

x/x/x (number of death/number of abnormal changes or symptoms/number of fishes used)

❖ **Validity criteria**

The assay is considered valid if the following criteria are satisfied:

- the mortality in the control animals should not exceed one fish at the end of the test;
- the dissolved oxygen concentration must have been at least 60% of the air saturation value throughout the test.
- Chemical conditions should be maintained during the test;

The validity criteria of the test are satisfied.
There are no mortality detected in control and treated animals.
There are no toxic symptoms detected in control and treated animals.
The temperature (24°C ± 1°C), % of oxygen saturation (above 60% both in control and in treated), pH (6.0-8.5) parameters as well of the stability of the test item (80-120%) are all satisfying the validity criteria.

Conclusion
The obtained results, in compliance with assay validity criteria, interpreted in accordance to OECD 203, showed that the *Danio rerio* LC_{50} of the test item "NORLAN AMPL 201901001007" after 96 hours is >100 mg/L.

CA 8.2.2 Long-term and chronic toxicity to fish

No data submitted, not required. Please refer to point 8.2.

CA 8.2.2.1 Fish early life stage toxicity test

No data submitted, not required. Please refer to point 8.2.

CA 8.2.2.2 Fish full life cycle test

No data submitted, not required. Please refer to point 8.2.

CA 8.2.2.3 Bioconcentration in fish

No data submitted, not required. Please refer to point 8.2.

CA 8.2.3 Endocrine disrupting properties

No data submitted, not required. Please refer to point 8.2.
Due to the nature of the Hydrolysed proteins and their essential role in living cells, it is deemed acceptable to consider that the active substance Hydrolysed proteins have no endocrine disrupting properties.
Therefore, Hydrolysed Proteins meet the criteria for the approval of low-risk active substance because not considered to be an endocrine disruptor.

In response to the RMS request, the applicant would like to highlight that Hydrolysed proteins are naturally occurring compounds whose degradation leads to simple metabolites called amino acids that are abundant organic molecules in living cells and used for the biosynthesis of new specific proteins and therefore essential for life.
Furthermore, in addition to the use as fertilisers as explained for environmental fate and behaviour section, hydrolysed proteins are also used as raw material for feed in aquaculture or animal feeding market.
On request, the applicant can provide several articles or examples of final products containing hydrolysed proteins in the market linked to the aquaculture and animal feeding uses, to reinforce this argumentation.
Moreover, the active substance hydrolysed proteins is not classified according to the ECHA.
The applicant is of the opinion that these information provide an argumentation strong enough to justify the exemption of further studies, considered as unnecessary from a scientific and rational point of view.

However, for completeness purposes, the applicant provides a study performed with Hydrolysed proteins ([redacted], *"Bioefficacy of parentally-administered Norlan L 25 in piglets" (2004)* [redacted], and supplementary report) to prove that the administration of such compounds to mammals has no endocrine disruption effects.
The conclusion of this study is that the use of Hydrolysed proteins resulted in significant increases in the concentration of monocytes and platelets and in mean platelet volume, but without having any significant effect on the growth and feed conversion index. Moreover, it was clearly highlighted that the increased concentration of monocytes observed after administration reveals an immune efficacy, inducing increased defences against various pathogenic microorganisms without any endocrine disruption properties.

CA 8.2.4 Acute toxicity to aquatic invertebrates

CA 8.2.4.1 Acute toxicity to *Daphnia magna*

A summary of the new submitted study is available below.

Reference:	KCA 8.2.4.1
Report	ACUTE TOXICITY ON AQUATIC ORGANISMS (*Daphnia sp.*) ON "NORLAN AMPL 201901001007", C. Giarei, 2020, report No. STULV19AA4423-1 GLP
Guideline(s):	Yes, OECD 202
Deviations:	No
GLP:	Yes
Acceptability:	Yes/No/Supplementary
Duplication (if vertebrate study)	-

SVMA14-004 (called "NORLAN AMPL 201901001007" in the study) is a brown liquid formulation containing 300 g/L of animal proteins hydrolysate. The aim of the study is to determine the ecotoxicological effects of the test item "NORLAN AMPL 201901001007" on biotic systems: in particular on aquatic organism such as *Daphnia magna* in order to define a limit not toxic threshold according to the guideline OECD 202.
After a range finding test, the organisms have been exposed to the test item at a concentration of 100 mg/L for a total period of 48 hours.
Daphnie have been kept under observations for a period of 48 hours and the number of immobilised organisms and/or possible abnormal behaviours both in control and in treatment group vessel have been observed.
At the beginning and at the end of the test (48 hours) dissolved oxygen and pH have been measured in the highest concentration and in control group.
Temperature of the assay environment have been recorded at the beginning and at the end of the test.
Organic carbon concentration (TOC) has been measured at the beginning and at the end of the test in a pool derived from control and treated vessels.

Material and methods

- **Experimental design**

Medium preparation

As dilution water, reconstituted water has been used; it has been prepared dissolving the following salts in 1 litre of deionised water:

N. Solution	Reagents	Formula	Quantity (g/L)
A	Bihydrated calcium chloride	$CaCl_2$ $2H_2O$	11.76
B	Eptahydrated magnesium sulphate	$MgSO_4$ $7H_2O$	4.93
C	Acid sodium bicarbonate	$NaHCO_3$	2.59
D	Potassium chloride	KCl	0.23

25 mL of solution A, B, C and D have been taken to 1000 mL volume with deionised water.
Medium has been prepared according to the same proportion and reported on internal logbook.

Assay sample preparation

Range finding test

A solution at 1000 mg/L of the test item has been prepared in culture medium. Then 5 different concentrations have been prepared with ten-fold dilutions from 1000 mg/L to 0.10 mg/L. The test has been performed on the diluted solutions.
120 Daphnie have been used, 100 of them treated with the test item at different concentration and 20 used as control, kept in the same assay conditions without adding the test item.
At 24 e 48 hours immobilisation of the organisms has been evaluated.

Definitive test

After the range finding test, the test item has been prepared at 100 mg/L in culture medium. 40 Daphnie have been used, 20 of them treated with the test item at 100 mg/L and 20 used as control, kept in the same assay conditions without adding the test item, according to the design described in the following table.
At 24 e 48 hours immobilisation of the organisms has been evaluated.

Group	Replica N°	N° of individuals/replica	Administered Solution Nominal concentration	Observation times – Daphnia immobilization (hours)
Treated	4	5	100.00 mg/L	24-48
Control	4	5	Medium	24-48

Assay conditions
Lightning: None during the course of the test.
Feeding: None during the course of the test.
Temperature: 20°C ± 2°C
Water: Reconstituted dilution water
Dissolved oxygen: > 3 mg/L
Hardness: between 140 and 250 mg $CaCO_3$/L

❖ **Determination of analytical concentration of total organic carbon (TOC)**
Determination of total organic carbon has been done using an automatic SHIMADZU TOC analyser. It is a high sensitivity instrument based on the combustion catalytic oxidation method (680°C). The carbon dioxide generated by oxidation has been detected using an infrared gas analyser (NDIR). Samples, properly diluted with deionised water with low TOC contents, have been analysed by means of an integrated sampling system directly from vials containing 40 ml volume.
For each sample under test the corresponding blank has been analysed.

Results and discussion
Number of immobilised Daphnie at 24 and 48 hours in treated and control groups in the definitive control

Control	**24 hours**	**48 hours**
Control replication N.1	0/5	0/5
Control replication N.2	0/5	0/5
Control replication N.3	0/5	0/5
Control replication N.4	0/5	0/5
Concentration : 100.00 mg/L		
Treated -replication N.1	0/5	0/5
Treated -replication N.2	0/5	0/5
Treated -replication N.3	0/5	0/5
Treated -replication N.4	0/5	0/5

❖ **Validity criteria**
The assay is considered valid if the following criteria are satisfied:
- The immobilisation of control animals must not be higher than 10% at the end of the test.
- The concentration of dissolved oxygen in the vessels ought to be higher than 3 mg/L during the assay.
- However, in any case, the concentration of dissolved oxygen must not be lower than 2 mg/L.
- pH must not change more than 1.5 units.

The validity criteria of the test are satisfied.
There are no immobilisation detected in control and treated animals.
The temperature, oxygen concentration, pH parameters as well of the stability of the test item are all satisfying the validity criteria.

Conclusion
The obtained results, in compliance with assay validity criteria, showed that *Daphnia magna* EC_{50} after 48 hours of the nominal concentration of the test item "NORLAN AMPL 201901001007" is >100 mg/L.

CA 8.2.4.2 Acute toxicity to an additional aquatic invertebrate species

No data submitted, not required. Please refer to point 8.2.

CA 8.2.5 Long-term and chronic toxicity to aquatic invertebrates

No data submitted, not required. Please refer to point 8.2.

CA 8.2.5.1 Reproductive and development toxicity to *Daphnia magna*

No data submitted, not required. Please refer to point 8.2.

CA 8.2.5.2 Reproductive and development toxicity to an additional aquatic invertebrate species

No data submitted, not required. Please refer to point 8.2.

CA 8.2.5.3 Development and emergence in *Chironomus riparius*

No data submitted, not required. Please refer to point 8.2.

CA 8.2.5.4 Sediment dwelling organisms

No data submitted, not required. Please refer to point 8.2.

CA 8.2.6 Effects on algal growth

CA 8.2.6.1 Effects on growth of green algae

A summary of the new submitted study is available below.

Reference:	KCA 8.2.6.1
Report	FRESHWATER ALGA, GROWTH INHIBITION TEST ON "NORLAN AMPL 201901001007", C. Giarei, 2020, report No. STULV19AA4421-1 GLP
Guideline(s):	Yes, OECD 201
Deviations:	No
GLP:	Yes
Acceptability:	Yes/No/Supplementary
Duplication (if vertebrate study)	-

SVMA14-004 (called "NORLAN AMPL 201901001007" in the study) is a brown liquid formulation containing 300 g/L of animal proteins hydrolysate.
On the test item "NORLAN AMPL 201901001007" the growth inhibition test on a culture of algae Pseudokirchneriella subcapitata has been performed on a culture in exponential growth phase.
This approach, according to OECD 201, allowed to calculate the inhibition concentration for 50% of the organisms (IC_{50} or EC_{50}).
During the range finding test the algae have been exposed to 5 different dilutions of the test item for 72 hours. After the results of the range finding test an EC_{50} test has been performed. The algae have been exposed to 5 dilutions of the test item for 72 hours. Algae have been measured using a Burker chamber.
At the end of the test, microscopic observation was performed to verify a normal and healthy appearance of the inoculum culture and to observe any abnormal appearance of the algae.
At the beginning and at the end of the test (72 hours) pH has been measured in all the vessels.
Temperature of the ambient has been recorded every day.
Stability of the test item in the definitive test has been performed using TOC analyser. Organic carbon concentration has been measured at the beginning and at the end of the test in a pool derived from vessels at the higher, intermediate and lower tested concentration.

Materials and methods

❖ **Experimental design**

Culture medium
The culture medium has been prepared by mixing the 4 stock solutions in order to obtain final concentrations specified in the following table:

Solution N.	**Nutrient**	**Quantity dissolved in 500 ml of deionized water**
1	NH_4Cl	0,75 g
	$CaCl_2$ $2H_2O$	0,9 g
	$MgCl_2$ $6H_2O$	0,6 g
	$MgSO_4$ $7H_2O$	0,75 g
	KH_2PO_4	0,08 g
2	$FeCl_3$ $6H_2O$	0,032 g
	Na_2EDTA $2H_2O$	0,05 g
3	H_3BO_3	0,0925 g
	$MnCl_2$ $4H_2O$	0,207 g
	$ZnCl_2$	1 mL of the following solution: 150 mg in 100 mL of deionized water
	$CoCl_2$ $6H_2O$	1 mL of the following solution: 75 mg in 100 mL of deionized water
	$CuCl_2$ $2H_2O$	1 mL of the following solution: 25 mg in 500 mL of deionized water, then diluting 1 mL of it in 10 mL
	Na_2MoO_4 $2H_2O$	1 mL of the following solution: 35 mg in 10 mL of deionized water
4	$NaHCO_3$	25 g

Solutions 1, 2, 3 have been sterilised by autoclaving (120°C, 15 min). Solution 4 has been sterilised via membrane filtration (0.22 mm). 10 mL of solution 1 and 1 mL of solutions 2, 3, 4 have been made up to 1000 mL with deionised water.
For higher volumes the medium has been prepared according to the same proportion.

❖ **Sample preparation**

Range finding test

A solution at 1000 mg/L of the test item has been prepared in culture medium. Then 5 different concentrations have been prepared, with ten-fold dilutions from 1000 mg/L to 0.1 mg/L. The test has been performed on the diluted solutions.

Definitive test
After the range finding test a solution at 2000 mg/L of the test item has been prepared in culture medium. The test has been performed on the diluted solutions. (see Table below). The total volume of the culture medium and the concentration of the algae inoculum added were the same in all vessels.

Group	Number	Cellular density (cells/ml)	Administered solution Nominal concentration	Cell observation times (hours)
Treated 1	3	10^4	1700.00 mg/L	0-24-48-72
Treated 2	3	10^4	531.25 mg/L	0-24-48-72
Treated 3	3	10^4	166.02 mg/L	0-24-48-72
Treated 4	3	10^4	51.88 mg/L	0-24-48-72
Treated 5	3	10^4	16.21 mg/L	0-24-48-72
Control	6	10^4	Culture Medium	0-24-48-72

❖ **Determination of organic carbon for analytical verification**
Determination of Total Organic Carbon (TOC) has been done using an automatic SHIMADZU TOC analyser. It is a high sensitivity instrument based on the combustion catalytic oxidation method (680°C). The carbon dioxide generated by oxidation has been detected using an infrared gas analyser (NDIR). Samples, properly diluted with deionised water with low TOC contents, have been analysed by means of an integrated sampling system directly from vials containing 40 mL volume. For each sample, the corresponding control has been analysed.

Results and discussion
% inhibition of growth rate

Parameter	Treated sample				
	1700.00 mg/L	531.25 mg/L	166.02 mg/L	51.88 mg/L	16.21 mg/L
%I_r	68.80	47.65	45.99	39.83	19.14

❖ **Validity criteria**
The assay is considered valid if the following criteria are satisfied:
- the cell concentration in the control cultures should have increased by a factor of at least 16 within three days corresponds to a specific growth rate of 0.92 day -1.
- the mean coefficient of variation for section by section (CVss) specific growth rates in the control cultures must not exceed 35%.
- the coefficient of variation of average specific growth rates during the whole period (CVwp) in replicate control cultures must not exceed 7%.

The validity criteria of the test are satisfied.
A normal and healthy appearance of the algae at the end of the test was observed at microscopic for control and treated group.
The temperature and pH parameters as well of the stability of the test item are all satisfying the validity criteria.

Conclusion
The obtained results, in compliance with assay validity criteria, showed that Algae EC_{50} of the concentration of the test item “NORLAN AMPL 201901001007” after 72 hours is 313.493 mg/L.

CA 8.2.6.2 Effects on growth of an additional algal species

No data submitted, not required. Please refer to point 8.2.

CA 8.2.7 Effects on aquatic macrophytes

No data submitted, not required. Please refer to point 8.2.
Furthermore, Hydrolysed proteins are not intended to be used as an herbicide or a plant growth regulator, and are not known to have any herbicidal activities.

CA 8.2.8 Further testing on aquatic organisms

No data submitted, not required. Please refer to point 8.2

CA 8.3 Effects on Arthropods

CA 8.3.1 Effects on bees

No EU data/endpoints available.

No new data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.3.1.1 Acute toxicity to bees

CA 8.3.1.1.1 Acute oral toxicity

No data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.3.1.1.2 Acute contact toxicity

No data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.3.1.2 Chronic toxicity to bees

No data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.3.1.3 Effects on honeybee development and other honeybee life stages

No data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.3.1.4 Sub-lethal effects

No data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.3.2 Effects on non-target arthropods other than bees

No EU data/endpoints available.

No new data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.3.2.1 Effects on *Aphidius rhopalosiphi*

No data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.3.2.2 Effects on *Typhlodromus pyri*

No data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.4 Effects on Non-Target Soil Meso- and Macrofauna

No EU data/endpoints available.

No new data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.4.1 Earthworms – sub-lethal effects

No data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.4.2 Effects on non-target soil meso- and macrofauna (other than earthworms)

CA 8.4.2.1 Species level testing

No EU data/endpoints available.

No new data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.5 Effects on Nitrogen Transformation

No EU data/endpoints available.

No new data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.6 Effects on Terrestrial Non-Target Higher Plants

No EU data/endpoints available.

No new data submitted, not required. Please refer to the argumentation presented under Point B 8.

Furthermore, Hydrolysed proteins are not intended to be used as an herbicide or a plant growth regulator, and are not known to have any herbicidal activities.

CA 8.6.1 Summary of screening data

No data submitted, not required. Please refer to point 8.6.

CA 8.6.2 Testing on non-target plants

No data submitted, not required. Please refer to point 8.6.

CA 8.7 Effects on Other Terrestrial Organisms (Flora and Fauna)

No EU data/endpoints available.

No new data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.8 Effects on Biological Methods for Sewage Treatment

No EU data/endpoints available.

No new data submitted, not required. Please refer to the argumentation presented under Point B 8.

CA 8.9 Monitoring Data

No data submitted, not required.
It should be noted that since proteins are naturally occurring in the environment, it would not be possible to distinguish the naturally compounds from those resulting from the use of plant protection products. Thus, the concept of environmental monitoring is not applicable to Hydrolysed proteins.