SPONSOR
APME
Avenue E. van Niewenhuyse 4
BOX 3
B-1160 Brussels
Belgium

TEST ITEM
AMMONIUM PERFLUOROOCTANOATE (APFO)

STUDY TITLE
ALGAL INHIBITION TEST

DATA REQUIREMENT
Directive 92/69/EEC C.3, 31st July 1992
OECD Guideline No. 201, 7th June 1984
US EPA/OPPTS 850.5400 Guidelines, April 1996

STUDY DIRECTOR
Jacques L'Haridon

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TEST FACILITY
CIT
BP 563 - 27005 Evreux - France

LABORATORY STUDY NUMBER
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Safety & Health Research Laboratories

IFM Recherche
S.N.C. au Capital de 846.092 €
788 060 465 R.C.S. Evreux
VAT : FR19 788 060 465
Agrément Crédit Impôt Recherche

B.P. 563 27005 Evreux Cedex France
Tél. : +33 (0)2 32 29 26 26
Fax : +33 (0)2 32 67 87 05
E-mail : CIT@citox.com
Web site : www.citox.com

STATEMENT OF CONFIDENTIALITY OR NO CONFIDENTIALITY CLAIMS

(To be completed by the Sponsor)

STATEMENT OF THE STUDY DIRECTOR AND CIT SCIENTIFIC MANAGEMENT

The study was performed in compliance with the principles of Good Laboratory Practice as described in:

- OECD Principles on Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM (98) 17.
- Décret N° 98-1312 du 31 décembre 1998 concernant les Bonnes Pratiques de Laboratoire, (Journal Officiel du 1er janvier 1999) Ministère de l'Economie, des Finances et de l'Industrie.
- Commission Directive 1999/11/EC of 8 March 1999 adapting to technical progress the Principles of Good Laboratory Practice as specified in Council Directive 87/18/EEC on the harmonisation of laws, regulations and administrative provisions relating to the application of the Principles of Good Laboratory Practice and the verification of their applications for tests on chemical substances (OJ No. L 77 of 23.3.1999).
- US Environmental Protection Agency, Federal Register, 40 CFR Part 792; Toxic Substances Control Act; Good Laboratory Practice Standards, August 17, 1989 (and subsequent amendments).
- Japanese Ministry of International Trade and Industry, Good Laboratory Practice Standards, Basic Industries Bureau, KanHogyo No. 39, March 31, 1984.
- Japanese Ministry of Health and Welfare, Good Laboratory Practice Standards, Pharmaceutical Affairs Bureau, YakuHatsu No. 229 and Environmental Agency, 59 KiKyoku No. 85, March 31, 1984.

I declare that this report constitutes a true and faithful record of the procedures undertaken and the results obtained in the performance of the study.

This study was performed at CIT, BP 563, 27005 Evreux, France.

Ecotoxicology

J. L'Haridon Study completion date: 16 January 2004
Doctor of Ecotoxicology
Study Director

R. Groult Date: 16 Jan 2004
Doctor of Chemistry
Scientific Management

OTHER SCIENTIST INVOLVED IN THIS STUDY

For Pharmacy: X. Manciaux
Doctor of Pharmacy

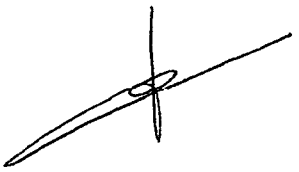
STATEMENT OF QUALITY ASSURANCE UNIT

Type of inspections	Dates		
	Inspections	Reported to Study Director (*)	Reported to Management (*)
Study plan	24 July 2002	26 July 2002	29 July 2002
Study	14 October 2002	25 October 2002	28 October 2002
Report	30 July 2003	22 August 2003	8 December 2003

In addition to the above-mentioned inspections, at about the same time as the study described in this report, "process-based" and routine facility inspections of critical procedures relevant to this study type were also made by the Quality Assurance Unit.
The findings of these inspections were reported to the Study Director and to CIT Management.

The inspections were performed in compliance with CIT Quality Assurance Unit procedures and Principles of Good Laboratory Practice.

The reported methods and procedures were found to describe those used and the results to constitute an accurate and complete reflection of the study raw data.



C. Galli-Kar Date: 16 Jan. 2004
Ing. Biol.
Head of Quality Assurance Unit

(*) The dates indicated correspond to the dates of signature of audit reports by Study Director and Management.

PAGE RESERVED FOR 40 CFR 158.34 (c) (1) CERTIFICATION

(To be completed by the Sponsor)

CONTENTS

SUMMARY

At the request of APME, Brussels, Belgium, the acute toxicity of the test item AMMONIUM PERFLUOROOCTANOATE (APFO) was evaluated in the algal strain *Pseudokirchneriella subcapitata* using a 96-hour static test based on:

- Directive 92/69/EEC C.3, 31st July 1992,
- OECD Guideline No. 201, 7th June 1984,
- US EPA/OPPTS 850.5400 Guidelines, April 1996,
- Requirements of the Japanese Government under the revised Chemical Substance Law according to the Notification No. 700 of the Environmental Agency, No. 1039 of the Ministry of Health and Welfare and No. 1014 of Ministry of International Trade and Industry, 9th December 1986.

The main criterion measured is the EC50 (Median Effective Concentration), a statistically derived concentration which can be expected to cause a reduction in algal growth of 50% in comparison with the control. This criterion is evaluated as:

- The ErC50 - the concentration of test item resulting in 50% reduction of the specific growth rate with respect to the control. Specific growth rate can be defined as the rate of increase in the natural log of the cell number (ln no. of cells/mL) over specific intervals (e.g. 0 to 24, 0 to 48, 0 to 72 and 0 to 96 hours).
- The EbC50 - the concentration of test item resulting in 50% reduction of the biomass with respect to the control. Biomass, in this case, may be defined as the increase in the cell number per mL of solution x time in hours and therefore represents an area ($cell.mL^{-1}.h$).

The No Observed Effect Concentration (NOEC), the highest concentration tested which does not induce a statistically significant reduction of the algal growth (based on specific growth rate or biomass), is also determined.

Methods

The test item was dissolved in reconstituted water (LC) with a hardness of approximately 34 mg/L as $CaCO_3$.

The study included three tests.

Range-finding test

Three replicate solutions of algae with an initial dilution of 1 x 10^4 cells/mL were exposed to a nominal concentration of 100 mg/L expressed as the APFO (solids) content of the test item, while four other groups of two replicate flasks were exposed to nominal concentrations of 0.01, 0.1, 1 and 10 mg/L expressed as the APFO (solids) content of the test item.
A further group of six replicate algal solutions without test item was used as the control.
The number of replicates of the control and the 100 mg/L group was higher compared to other groups to provide a limit test if there was no effect at this concentration.

The growth of the cell culture in each flask was monitored by counting the number of cells at 24, 48, 72 and 96 hours.

First test
Seven concentrations of AMMONIUM PERFLUOROOCTANOATE (APFO) were used together with a control at: 0, 6.25, 12.5, 25.0, 50.0, 100, 200 and 400 mg/L expressed as the APFO (solids) content of the test item.
Three replicates were exposed to each concentration and six replicates to dilution water only (to act as the control) for 96 hours. Observations of cell growth were recorded at 24, 48, 72 and 96 hours.

Second test
A second test was undertaken since the results of the range-finding test were not in accordance with those of the first test. This second test was considered as the reference test for determination of all toxicity parameters.

Seven concentrations of AMMONIUM PERFLUOROOCTANOATE (APFO) were used together with a control at: 0, 6.25, 12.5, 25.0, 50.0, 100, 200 and 400 mg/L expressed as the APFO (solids) content of the test item.
Three replicates were exposed to each concentration and six replicates to dilution water only (to act as the control) for 96 hours. Observations of cell growth were recorded at 24, 48, 72 and 96 hours.

Environmental parameters were:
- pH: 6.61 to 10.74,
- temperature: 22.6°C to 23.8°C,
- lighting: 6710 lux to 7620 lux.

Chemical analysis
Chemical analysis was undertaken to measure the concentration of the test item in each test solution of the second test, except for the control, at the beginning (T0 hour) and the end (T96 hours) of the test.

Results

Range-finding test
The following results are based on nominal concentrations.

According to the results obtained in this test, the 72-hour and 96-hour EC50s were > 100 mg/L. After 72 and 96 hours, the growth rate was equivalent to that of the control at 100 mg/L (highest concentration tested) while there was evidence of effect on biomass at this concentration. A further test was therefore performed (limit test not applicable).

First test
The following results are based on nominal concentrations.

According to the results obtained in this test, the 72-hour and 96-hour EC50s were > 400 mg/L.

The growth rate NOEC was 200 mg/L after 72 hours (using the two replicates giving valid results at this concentration) and only 6.25 mg/L after 96 hours. However, after 96 hours, inhibition of the growth rate at 400 mg/L was low (14%).
The biomass NOEC was 400 mg/L after 72 hours and 100 mg/L after 96 hours.

The large differences between the growth rate NOECs (72 and 96 hours) and between the growth rate and biomass NOECs at 96 hours, were considered unusual, and it was considered possible that the low slope of the concentration-effect curve may have prevented determination of precise NOECs. The results were not considered conclusive and, consequently, a second test was performed to determine whether the results of the first test were reproducible, using the same range of concentrations.

Second test
The following results are based on nominal concentrations.

The second test confirmed that EC50 values after 72 and 96 hours were > 400 mg/L.

The growth rate NOEC was 200 mg/L after 72 hours and only 12.5 mg/L after 96 hours. However, inhibition of growth rate at 400 mg/L was once again low after 96 hours (15%).
The biomass NOEC was 200 mg/L after 72 hours and 12.5 mg/L after 96 hours. The biomass NOEC at 96 hours was therefore equivalent to the growth rate NOEC at 96 hours in this second test.

Then, the second test generally confirmed that the low slope of the concentration-effect curve made it difficult to determine precisely NOECs after 72 and 96 hours using statistical analysis, as observed in the first test. However, the second test was considered to provide more reliable data because there was not the large difference in the growth rate and biomass NOEC values that had been observed in the first test.
Therefore, the second test was considered as the reference test for determination of all toxicity parameters and, consequently, all samples taken during this test were analyzed to measure actual concentrations of AMMONIUM PERFLUOROOCTANOATE (APFO) in test solutions.

Measured concentrations in the solutions of the second test with and without algae were within ± 20% of the corresponding nominal values at the beginning (T0 hour) and the end (T96 hours) of the test. Hence, the test item was stable in solution throughout the test and was not significantly bioaccumulated or adsorbed at the surface of the cells. Furthermore, the study results could be based on nominal concentrations.

These study results were as follows:

	Growth rate (mg/L) (1)		Biomass (mg/L) (1)	
Time (h)	ErC50	NOEC	EbC50	NOEC
72	> 400	200	> 400	200
96	> 400	12.5	> 400	12.5

(1) concentrations expressed as the APFO (solids) content of the test item

Conclusion

Under our experimental conditions, the 72-hour ErC50 and the 72-hour EbC50 of AMMONIUM PERFLUOROOCTANOATE (APFO) in a static test system were > 400 mg/L for *Pseudokirchneriella subcapitata.*

The NOEC at 72 hours was 200 mg/L based on the specific growth rate and biomass data.
The NOEC at 96 hours was 12.5 mg/L based on the specific growth rate and biomass data.

These results are based on the APFO (solids) content of the test item.

1. INTRODUCTION

The objective of this study was to assess the acute toxicity of AMMONIUM PERFLUOROOCTANOATE (APFO), to *Pseudokirchneriella subcapitata*, in a 96-hour static test based on:

- Directive 92/69/EEC C.3, 31st July 1992,
- OECD Guideline No. 201, 7th June 1984,
- US EPA/OPPTS 850.5400 Guidelines, April 1996,
- Requirements of the Japanese Government under the revised Chemical Substance Law according to the Notification No. 700 of the Environmental Agency, No. 1039 of the Ministry of Health and Welfare and No. 1014 of Ministry of International Trade and Industry, 9th December 1986.

The criterion measured is the EC50 (Median Effective Concentration), a statistically derived concentration of the test item in water which can be expected to cause a reduction of algal growth of 50% in comparison with the control. This criterion is evaluated as:

- The ErC50 - the concentration of test item resulting in 50% reduction of the specific growth rate with respect to the control.
- The EbC50 - the concentration of test item resulting in 50% reduction of the biomass with respect to the control.

The No Observed Effect Concentration (NOEC), the highest concentration tested which does not induce a statistically significant reduction of the algal growth (based on specific growth rate or biomass), is also determined.

2. MATERIALS AND METHODS

2.1 TEST ITEM

2.1.1 Identification

- supplier: 3M
- name:
  - Study plan: AMMONIUM PERFLUOROOCTANOATE (APFO)
  - labeling [redacted]

Both names correspond to the same test item.

- batch number [redacted]
- description at receipt: colorless liquid
- description on the analytical certificate: [redacted]
- containers: three drums
- date of receipt: 28 January 2002
- storage conditions: at room temperature
- purity [redacted]

Data relating to the characterisation of the test item are documented in an analytical certificate and in the composition of the test item (presented in appendix 1) provided by the Sponsor.

The test concentrations were expressed as concentrations of APFO, based on the stated APFO content of the test item [redacted]

2.1.2 Preparation of the test solutions

The stock solutions, for the three tests carried out, were prepared by dissolving the test item directly in LC reconstituted water (see § 2.2.2).

The conditions of preparation of the stock solutions are reported in the following table:

	Quantity of test item (as received) (mg)	Quantity of APFO (solids) (mg)	Concentration of the test item (mg/L) (1)	Duration of the agitation (minutes)
Range-finding test	510.2	100	100	10
First test	2041	400	400	15
Second test	2041	400	400	15

(1) expressed as the APFO (solids) content of the test item

The agitation was continued until the stock solutions were used to prepare the test solutions.

Test solutions were prepared by further dilution of the stock solution with LC reconstituted water to provide a geometric series of concentrations (expressed as the APFO (solids) content of the test item):

- 0, 0.01, 0.1, 1, 10 and 100 mg/L for the range-finding test,
- 0, 6.25, 12.5, 25.0, 50.0, 100, 200 and 400 mg/L for the first and second tests.

Glass test vessels (250 mL Erlenmeyer flasks) containing algae and test solutions (or dilution water in the case of the controls) were filled directly from the test solution containers immediately after preparation and test solutions remained unchanged throughout the study.
The pH of the test solutions remained within acceptable limits (between 6 and 9 and in the range ± 0.5 unit of the test water) after preparation and there was no adjustment of pH before addition of the algae.

For the test solution at 100 mg/L in the range-finding test and all test solutions of the first and second tests (except for the controls), a further group of vessels was prepared for possible chemical analysis by adding test solutions but no algal pre-culture.

2.2 TEST SYSTEM

2.2.1 Algae

Species:	*Pseudokirchneriella subcapitata.*
Strain No.:	CCAP 278/4.
Reason for this choice:	species commonly used in Europe for aquatic toxicity testing and recommended in OECD, EEC and EPA guidelines.
Cultured at:	CIT.
Origin:	Culture Collection of Algae and Protozoa, Institute of Freshwater Ecology, Far Sawrey, Ambleside, Cumbria, LA22 OLP, UK.
Culture method:	the algae are cultured under sterile conditions and maintained at exponential growth rate.

2.2.2 Environmental conditions during culture

During the culture period the conditions were as follows:

Water:	reconstituted water (LC oligo medium, see appendix 2), recommended in the French guideline AFNOR T 90-304. A primary stock solution is made up every month from which a final culture medium is prepared. This solution is autoclaved at which point it has a stock life of one week. Before use, the pH is verified at 7.5 ± 0.3 or adjusted until this pH is obtained. The hardness of this solution is approximately 34 mg/L as $CaCO_3$. This medium does not fully comply with the requirement of OECD guideline concerning P, N and chelators concentrations (in excess). However, it is considered as suitable for this type of study, according to the above-mentioned French guideline and experience of our laboratory.
Culture period:	algae are cultivated for 7 days before harvesting for use in a new culture.
Temperature:	between 21°C and 25°C in water. Algae are maintained at ± 2°C during the acclimation period. Temperature is checked daily.
Illumination:	24 hours per day of constant illumination maintained at approximately 2000 lux throughout the culture period. For lighting details see § 2.2.3.
Aeration:	the cultures are constantly aerated with air filtered using a 0.22 µm porosity filter to maximize the CO_2 availability (and thereby the cell growth) while maintaining the sterility.
Preculture loading:	new cultures are loaded at a concentration of 1 x 10^4 cells/mL. Each weekly culture is prepared under sterile conditions using autoclaved medium while week old cell cultures are checked for contamination before being transferred to fresh medium.

2.2.3 Environmental conditions during the test

Test water:	reconstituted-LC (see § 2.2.2).
Temperature:	controlled daily in a flask containing test water but no algae, run alongside the test. Between 21°C and 25°C (regulated to be as close as possible to 23°C at the beginning of the test).
Illumination:	continuous. A set of 15 and 30 W Osram Fluora® fluorescent tubes between "white" and "daylight" type (spectral range 400 to 700 nm) were set approximately 40 cm above the cultures. These emit light measured using a lux meter equipped with a spherical collector at a level corresponding to half the height of the cultures in their conical flasks (corresponding to an irradiance of 252 mW/m^2 for 15 W and 476 mW/m^2 for 30 W tubes x no. of bulbs = 3724 mW/m^2). The light intensity at each position to be occupied by a culture flask is measured regularly. The color temperature of the fluorescent lamps is between 4400 K and 5000 K.
Duration of test:	96 hours.
Transfer:	the number of cells in the week-old culture was counted and the quantity of algal pre-culture to be added to the fresh test medium calculated to give a test solution concentration of 1 x 10^4 cells/mL. The algal pre-cultures were then added where appropriate to the 100 mL test solutions and all the 250 mL conical flasks stoppered with sterile cotton wool wrapped with lint and transferred to the agitator.
Culture homogeneity:	the solutions were agitated throughout the test.
pH:	the pH values of the control and of all the test concentrations, except for the range-finding concentrations at 0.01, 0.1, 1 and 10 mg/L, were measured at the beginning of the tests and after 72 and 96 hours.
Hardness:	water hardness was measured once in LC reconstituted water before the start of the test.
Forced aeration:	was not used during the test but the cultures were constantly agitated.

2.3 TREATMENT

2.3.1 Study design

The study included three tests.
The duration of each test was 96 hours.

Range-finding test

	Nominal concentration (mg/L) (1)	Number of replicates with algae	without algae
Control	0	6	0
Treated	0.01	2	0
Treated	0.1	2	0
Treated	1	2	0
Treated	10	2	0
Treated	100	3	1

(1) expressed as the APFO (solids) content of the test item

The number of replicates of the control and the 100 mg/L group was higher compared to other groups to provide a limit test if there was no effect at this concentration.

First and second tests

	Nominal concentration (mg/L) (1)	Number of replicates with algae	without algae
Group 1 (control)	0	6	0
Group 2	6.25	3	1
Group 3	12.5	3	1
Group 4	25.0	3	1
Group 5	50.0	3	1
Group 6	100	3	1
Group 7	200	3	1
Group 8	400	3	1

(1) expressed as the APFO (solids) content of the test item.

2.3.2 Time schedule

Experimental starting date (first day of treatment): 14 October 2002,
Experimental completion date: 7 March 2003.

2.4 OBSERVATIONS

The number of cells in solutions was calculated at T0 as 1 x 10^4 cells/mL. For the remaining observation times (T24, T48, T72 and T96 hours), cell numbers were determined using a Malassez cell counter.

An observation of less than 1 x 10^4 cells/mL is below the sensitivity of the Malassez cell counter. Possible observations of this type were considered as 1 x 10^4 cells/mL - i.e. the test item was taken to be algaestatic at this test concentration rather than algaecidal, as no further experiment was undertaken to determine whether algae exposed to algaestatic concentrations were capable of growth post-exposure.

2.5 CHEMICAL ANALYSIS

Each sample contained 5 mL.

2.5.1 Range-finding test

At T0 hour, a sample was taken from the test solution container at 100 mg/L nominal and stored at -20°C.
At T24, T48, T72 and T96 hours, samples were taken from each test solution replicate at 100 mg/L nominal, pooled (5 mL in total) and stored at -20°C.
Further samples were also taken at T24, T48, T72 and T96 hours from the test solution at 100 mg/L nominal which contained no algae and had been run alongside the test to determine the influence of adsorption (at the surface of algae cells) and/or bioaccumulation on the possible decrease in test item concentration throughout the test.

Although samples were taken during the range-finding test, no chemical analysis was performed on these samples because the test item was found to be toxic at 100 mg/L and consequently, at least one further test was necessary.

2.5.2 First and second tests

At T0 hour, samples were taken from each test solution container, except for the control, and stored at -20°C.
At T24, T48, T72 and T96 hours, samples were taken from all test replicate groups at each concentration (except for the control), pooled by concentration (5 mL in total) and stored at -20°C.
Further samples were also taken at T24, T48, T72 and T96 hours from the solutions which contained no algae and had been run alongside the test to determine the influence of adsorption (at the surface of algae cells) and/or bioaccumulation on the possible decrease in test item concentration throughout the test.

Chemical analysis was only performed on samples taken at the beginning (T0 hour) and the end (T96 hours) of the second test (see § 3.3.2).

The analytical procedure is presented in appendix 3.

2.6 DATA EVALUATION

2.6.1 Determination of the ErC50 and the EbC50

Specific growth rate can be defined as the rate of increase in the natural log of the cell number (ln no. of cells/mL) over specific intervals (e.g. 0 to 24, 0 to 48, 0 to 72, 0 to 96 hours) (see appendix 4 for details). The concentration causing a reduction of growth rate to 50% that of the control (ErC50) is obtained for each observation time (T24, T48, T72 and T96 hours) by calculation from the average specific growth rate at each concentration.
Biomass, in this case may be defined as the number of cells per mL of solution x time in hours and therefore represents an area (cells.mL^{-1}.h). The biomass at 72 or 96 hours was determined using the trapezoïdal method from the number of cells observed at T0, T24, T48, T72 and T96 hours. The concentration causing a reduction of biomass at 72 or 96 hours to 50% that of the control (EbC50) is then calculated.
When a partial inhibition (0% < inhibition < 100%) is observed at at least two concentrations, the EC50 is calculated according to Probit analysis (i.e. Finney's method, published by E. Weber, combined with Bliss's method). The confidence interval limits are calculated statistically according to Fieller's method.

When a partial inhibition (0% < inhibition < 100%) is observed at only one concentration, the EC50 is estimated using Probit analysis or an interpolation procedure (depending on the data obtained). In this case, the highest concentration causing no inhibition and the lowest concentration producing 100% inhibition are used as confidence limits.
If at all concentrations, inhibition is 0% or 100%, an EC50 can not be calculated. It is estimated using the geometric mean of the highest concentration causing no inhibition and the lowest concentration producing 100% inhibition.

2.6.2 Determination of the No Observed Effect Concentration (NOEC)

After checking of the normality of the data with Chi-square and Shapiro-Wilks tests (normal at $p = 0.01$) as well as the variance homogeneity (Bartlett test; $p = 0.01$), the NOEC is determined by ANOVA (the Bonferroni T-test or the Dunnett test; $p = 0.05$) using the individual replicates of the area under the curve and the specific growth rate.

2.7 ARCHIVING

The following study materials are archived by CIT (BP 563, 27005 Evreux, France) for 5 years after the end of the *in vivo* phase of the study:
- Study plan and possible amendments,
- raw data,
- correspondence,
- final report and possible amendments.

On completion of this period, the archived study materials will be returned to the Sponsor, or may be archived at CIT for a further period (at additional cost).
The total duration of archiving (depending on regulations) will be the responsibility of the Sponsor.
In addition, raw data not specific to the study including, but not limited to, records of environmental data and equipment calibration, will also be archived by CIT and retained for at least 30 years.

2.8 STUDY PLAN ADHERENCE

The study was performed in accordance with the Study plan No. 23685 EAA and subsequent amendments, with the following deviations from the agreed Study plan:
- after 72 hours, the pH varied by more than 1.5 units in the first and second tests, based on individual treatment values. The maximum variation was observed in the control for both tests (1.67 and 2.94 units between T0 hour and T72 hours in the controls of the first and second tests, respectively). These pH variations were due to the high algal growth rate under our experimental conditions.
- the temperature varied by more than ± 1°C during the first test (± 1.35°C).

These minor deviations were not considered to have compromised the validity or integrity of the study.

3. RESULTS

3.1 RANGE-FINDING TEST

3.1.1 Water quality

The test solution was a translucent colorless solution at all test item concentrations.

The minimum and maximum parameters measured during the range-finding test were:
- pH: 6.98 and 9.67 between T0 and T72 hours; 6.98 and 10.35 between T0 and T96 hours,
- temperature: 23.6°C and 24.3°C,
- lighting: 6930 lux and 7410 lux.

The temperature and pH data are presented in table 1.

3.1.2 Chemical analysis

No chemical analysis was performed since the test item was found to be toxic at 100 mg/L and consequently, at least one further test was necessary.

3.1.3 Growth inhibition

The results after 72 and 96 hours are presented in tables 2, 3, 4 and 5.

The following results are based on nominal concentrations.

Inhibitions of the growth rate at 0.01, 0.1, 1, 10 and 100 mg/L were respectively 0%, 0%, 0%, 0% and 4%, relative to the control, at T72 hours.
Inhibitions of the growth rate at 0.01, 0.1, 1, 10 and 100 mg/L were respectively 0%, 2%, 1%, 1% and 1%, relative to the control, at T96 hours.

Inhibitions of the biomass at 0.01, 0.1, 1, 10 and 100 mg/L were respectively 0%, 1%, 10%, 8% and 31%, relative to the control, at T72 hours.
Inhibitions of the biomass at 0.01, 0.1, 1, 10 and 100 mg/L were respectively 0%, 4%, 5%, 4% and 17%, relative to the control, at T96 hours.

Acccording to the results obtained in this test, the 72-hour and 96-hour EC50s were > 100 mg/L. After 72 and 96 hours, the growth rate was equivalent to that of the control at 100 mg/L (highest concentration tested) while there was evidence of effect on biomass at this concentration. A further test was therefore performed (limit test not applicable).

3.2 FIRST TEST

3.2.1 Water quality

The test solution was a translucent colorless solution at all test item concentrations.

The minimum and maximum parameters measured during the first test were:
- pH: 6.99 and 9.37 between T0 and T72 hours; 6.44 and 9.63 between T0 and T96 hours,
- temperature: 21.9°C and 24.6°C,
- lighting: 6600 lux and 7580 lux.

The temperature and pH data are presented in table 6.

3.2.2 Chemical analysis

No chemical analysis was performed since this first test was not retained for determination of all toxicity parameters.

3.2.3 Growth inhibition

The results after 72 and 96 hours are presented in tables 7, 8, 9 and 10.

The following results are based on nominal concentrations.

According to the results obtained in this test, the 72-hour and 96-hour EC50s were > 400 mg/L.

After 72 hours, the growth rate was significantly lower than that of the control at 400 mg/L. NOEC at 72 hours was 200 mg/L using the two replicates giving valid results at this concentration.
After 96 hours, the growth rate was significantly lower than that of the control at and above a concentration of 12.5 mg/L. The NOEC at 96 hours was 6.25 mg/L.
However, after 96 hours, inhibition of the growth rate at 400 mg/L was only 14%.

After 72 hours, the biomass was not significantly lower than that of the control at 400 mg/L. Therefore, the NOEC at 72 hours was 400 mg/L.
After 96 hours, the biomass was significantly lower than that of the control at and above 200 mg/L. The NOEC at 96 hours was 100 mg/L.

The large differences between the growth rate NOECs at 72 and 96 hours and between the NOECs at 96 hours for growth rate and biomass, were considered unusual, and it was considered possible that the low slope of the concentration-effect curve may have prevented determination of precise NOECs. The results were not considered conclusive and, consequently, a second test was performed to determine whether the results of the first test were reproducible, using the same range of concentrations.

3.3 SECOND TEST

3.3.1 Water quality

The test solution was a translucent colorless solution at all test item concentrations.

The minimum and maximum parameters measured during the second test were:
- pH: 6.66 and 10.24 between T0 and T72 hours; 6.61 and 10.74 between T0 and T96 hours,
- temperature: 22.6°C and 23.8°C,
- lighting: 6710 lux and 7620 lux.

The temperature and pH data are presented in table 11.

3.3.2 Growth inhibition and chemical analysis

The results after 72 and 96 hours are presented in tables 12, 13, 14 and 15.

The following results are based on nominal concentrations.

The second test confirmed that EC50 values after 72 and 96 hours were > 400 mg/L.

After 72 hours, the growth rate was significantly lower than that of the control at 400 mg/L. The NOEC at 72 hours, i.e. 200 mg/L, was the same as that obtained in the first test.
After 96 hours, the growth rate was significantly lower than that of the control at and above 25.0 mg/L. The NOEC at 96 hours, i.e. 12.5 mg/L, was only twice that obtained in the first test (6.25 mg/L), confirming a significant reduction of the growth rate at a much lower concentration after 96 hours compared to 72 hours. However, inhibition of growth rate at 400 mg/L was once again low after 96 hours (15%).

After 72 hours, the biomass was significantly lower than that of the control at 400 mg/L. The NOEC at 72 hours corresponded to 200 mg/L while it was 400 mg/L in the first test.
After 96 hours, the biomass was significantly lower than that of the control at and above 25.0 mg/L. At the maximum concentration tested (400 mg/L), the biomass after 96 hours was 43% lower than that of the control. The NOEC at 96 hours, i.e. 12.5 mg/L, was therefore eight times lower than in the first test but equivalent to the NOEC at 96 hours based on growth rate obtained in this second test.

Then, the second test generally confirmed that the low slope of the concentration-effect curve made it difficult to determine precisely NOECs after 72 and 96 hours using statistical analysis, as observed in the first test. However, the second test was considered to provide more reliable data because there was not the large difference in the growth rate and biomass NOEC values that had been observed in the first test.
Therefore, the second test was considered as the reference test for determination of all toxicity parameters and, consequently, all samples taken during this test were analyzed to measure actual concentrations of AMMONIUM PERFLUOROOCTANOATE (APFO) in test solutions.

Results of chemical analysis are presented in table A, appendix 3.

Measured concentrations in the solutions of the second test with and without algae were within ± 20% of the corresponding nominal values at the beginning (T0 hour) and the end (T96 hours) of the test.
Hence, the test item was stable in solution throughout the test and was not significantly bioaccumulated or adsorbed at the surface of the cells.
Furthermore, the study results could be based on nominal concentrations.

All EC50s (24, 48, 72, and 96-hour ErC50s and 72 and 96-hour EbC50s) were not calculated since the inhibition percentage of the growth rate or biomass at the highest concentration (400 mg/L) was systematically lower than 50% at the corresponding time.

Consequently, the ErC50 at each of the measured growth intervals was as follows:

Time (h)	ErC50 (mg/L) (1)	95% confidence limits (mg/L)
24	> 400	
48	> 400	
72	> 400	
96	> 400	

(1) expressed as the APFO (solids) content of the test item

The 72-hour EbC50 and 96-hour EbC50 were as follows:

Time (h)	EbC50 (mg/L) (1)	95% confidence limits (mg/L)
72	> 400	
96	> 400	

(1) expressed as the APFO (solids) content of the test item

The NOEC at 72 hours was calculated as 200 mg/L (p = 0.05) based on the specific growth rate and biomass data.
The NOEC at 96 hours was calculated as 12.5 mg/L (p = 0.05) based on the specific growth rate and biomass data.

4. CONCLUSION

Under our experimental conditions, the 72-hour ErC50 and the 72-hour EbC50 of AMMONIUM PERFLUOROOCTANOATE (APFO) in a static test system were > 400 mg/L for *Pseudokirchneriella subcapitata.*

The NOEC at 72 hours was 200 mg/L based on the specific growth rate and biomass data.
The NOEC at 96 hours was 12.5 mg/L based on the specific growth rate and biomass data.

These results are based on the APFO (solids) content of the test item.

5. REFERENCES

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Table 1: Range-finding test - temperature and pH

Nominal concentration (mg/L)	pH at T 0 hour	pH at T 72 hours	pH at T 96 hours
0	7.80	9.67	10.35
100	7.76	6.98	7.93

Temperature measured between 0 and 2 hours: 23.6°C
Temperature at 24 hours (measured in the same test vessel): 23.9°C
Temperature at 48 hours (measured in the same test vessel): 23.8°C
Temperature at 72 hours (measured in the same test vessel): 23.9°C
Temperature at 96 hours (measured in the same test vessel): 24.3°C

Table 2: Range-finding test - T72 hours - number of cells and calculation of the specific growth rate at each concentration

Nominal concentration (mg/L)	Rep.	Total No. of cells at 72 hours (10^4/mL)	Ln No. of cells	Mean Ln No. of cells	Specific growth rate	% Inhibition of growth rate
0	1	133.0	14.10	14.21	0.07	
	2	154.0	14.25			
	3	157.0	14.27			
	4	150.0	14.22			
	5#	-				
	6#	-				
0.01	1	163.0	14.30	14.35	0.07	-2.84
	2	179.0	14.40			
0.1	1	168.0	14.33	14.27	0.07	-1.24
	2	148.0	14.21			
1	1	135.0	14.12	14.20	0.07	0.23
	2	159.0	14.28			
10	1	170.0	14.35	14.22	0.07	-0.14
	2	131.0	14.09			
100	1	121.0	14.01	14.01	0.07	3.97
	2	122.0	14.01			
	3#	-				

Ln number of cells at T0 (10 000 cells) = 9.21

Rep. : replicate

Values obtained in two control replicates (Nos. 5 and 6) and one replicate at 100 mg/L (No. 3) were not retained for data interpretation since the value(s) obtained after 72 and/or 96 hours was/were at least 3 times lower than the mean of other replicate values.

Variation coefficient of the control cell density at 72 hours: 7.22%

Table 3: Range-finding test - T96 hours - number of cells and calculation of the specific growth rate at each concentration

Nominal concentration (mg/L)	Rep.	Total No. of cells at 96 hours (10^4/mL)	Ln No. of cells	Mean Ln No. of cells	Specific growth rate	% Inhibition of growth rate
0	1	338.0	15.03	15.11	0.06	
	2	396.0	15.19			
	3	336.0	15.03			
	4	388.0	15.17			
	5#	-				
	6#	-				
0.01	1	364.0	15.11	15.12	0.06	-0.16
	2	370.0	15.12			
0.1	1	334.0	15.02	15.00	0.06	1.85
	2	318.0	14.97			
1	1	338.0	15.03	15.06	0.06	0.70
	2	360.0	15.10			
10	1	368.0	15.12	15.08	0.06	0.51
	2	338.0	15.03			
100	1	346.0	15.06	15.02	0.06	1.39
	2	324.0	14.99			
	3#	-				

Ln number of cells at T0 (10 000 cells) = 9.21

Rep. : replicate

Values obtained in two control replicates (Nos. 5 and 6) and one replicate at 100 mg/L (No. 3) were not retained for data interpretation since the value(s) obtained after 72 and/or 96 hours was/were at least 3 times lower than the mean of other replicate values.

Variation coefficient of the control cell density at 96 hours: 8.76%

Table 4: Range-finding test - Number of cells (10^4 cells x mL^{-1}) at each observation time and calculation of the area under the growth curve (biomass) at each concentration after 72 hours.
Area = cell x mL^{-1} x hour

		Total number of cells			Biomass					Inhibition (%)
Nominal concentration (mg/L)	Rep.	at 24 hours	at 48 hours	at 72 hours	0 to 24 hours	24 to 48 hours	48 to 72 hours	0 to 72 hours	Average (0-72 hours)	
0	1	6.30	33.50	133.00	63.60	453.60	1974.00	2491.20	2754.60	
	2	5.70	45.70	154.00	56.40	592.80	2372.40	3021.60		
	3	4.60	29.00	157.00	43.20	379.20	2208.00	2630.40		
	4	5.30	42.00	150.00	51.60	543.60	2280.00	2875.20		
	5#	-	-	-						
	6#	-	-	-						
0.01	1	5.60	44.70	163.00	55.20	579.60	2468.40	3103.20	3105.60	-12.74
	2	4.50	38.00	179.00	42.00	486.00	2580.00	3108.00		
0.1	1	3.80	34.30	168.00	33.60	433.20	2403.60	2870.40	2730.00	0.89
	2	5.90	30.50	148.00	58.80	412.80	2118.00	2589.60		
1	1	4.50	28.00	135.00	42.00	366.00	1932.00	2340.00	2472.00	10.26
	2	3.50	28.00	159.00	30.00	354.00	2220.00	2604.00		
10	1	4.20	30.30	170.00	38.40	390.00	2379.60	2808.00	2536.80	7.91
	2	5.40	26.00	131.00	52.80	352.80	1860.00	2265.60		
100	1	1.90	20.80	121.00	10.80	248.40	1677.60	1936.80	1911.60	30.60
	2	1.50	18.60	122.00	6.00	217.20	1663.20	1886.40		
	3#	-	-	-						

Rep. : replicate
Values obtained in two control replicates (Nos. 5 and 6) and one replicate at 100 mg/L (No. 3) were not retained for data interpretation since the value(s) obtained after 72 and/or 96 hours was/were at least 3 times lower than the mean of other replicate values.

Variation coefficient of the control cell density at 72 hours: 7.22%

Table 5: Range-finding test - Number of cells (10^4 cells x mL^{-1}) at each observation time and calculation of the area under the growth curve (biomass) at each concentration after 96 hours.
Area = cell x mL^{-1} x hour

		Total number of cells				Biomass						Inhibition (%)
Nominal concentration (mg/L)	Rep.	at 24 hours	at 48 hours	at 72 hours	at 96 hours	0 to 24 hours	24 to 48 hours	48 to 72 hours	72 to 96 hours	0 to 96 hours	Average (0-96 hours)	
0	1	6.30	33.50	133.00	338.0	63.60	453.60	1974.00	5628.00	8119.20	8886.60	
	2	5.70	45.70	154.00	396.0	56.40	592.80	2372.40	6576.00	9597.60		
	3	4.60	29.00	157.00	336.0	43.20	379.20	2208.00	5892.00	8522.40		
	4	5.30	42.00	150.00	388.0	51.60	543.60	2280.00	6432.00	9307.20		
	5#	-	-	-	-							
	6#	-	-	-	-							
0.01	1	5.60	44.70	163.00	364.0	55.20	579.60	2468.40	6300.00	9403.20	9537.60	-7.33
	2	4.50	38.00	179.00	370.0	42.00	486.00	2580.00	6564.00	9672.00		
0.1	1	3.80	34.30	168.00	334.0	33.60	433.20	2403.60	6000.00	8870.40	8514.00	4.19
	2	5.90	30.50	148.00	318.0	58.80	412.80	2118.00	5568.00	8157.60		
1	1	4.50	28.00	135.00	338.0	42.00	366.00	1932.00	5652.00	7992.00	8400.00	5.48
	2	3.50	28.00	159.00	360.0	30.00	354.00	2220.00	6204.00	8808.00		
10	1	4.20	30.30	170.00	368.0	38.40	390.00	2379.60	6432.00	9240.00	8554.80	3.73
	2	5.40	26.00	131.00	338.0	52.80	352.80	1860.00	5604.00	7869.60		
100	1	1.90	20.80	121.00	346.0	10.80	248.40	1677.60	5580.00	7516.80	7365.60	17.12
	2	1.50	18.60	122.00	324.0	6.00	217.20	1663.20	5328.00	7214.40		
	3#	-	-	-	-							

Rep.: replicate
Values obtained in two control replicates (Nos. 5 and 6) and one replicate at 100 mg/L (No. 3) were not retained for data interpretation since the value(s) obtained after 72 and/or 96 hours was/were at least 3 times lower than the mean of other replicate values.

Variation coefficient of the control cell density at 96 hours: 8.76%

Table 6: First test - temperature and pH

Nominal concentration (mg/L)	pH at T 0 hour	pH at T 72 hours	pH at T 96 hours
0	7.70	9.37	9.63
6.25	7.77	9.22	9.27
12.5	7.66	8.89	9.10
25.0	7.63	7.82	8.94
50.0	7.57	7.63	8.37
100	7.60	7.05	7.64
200	7.59	6.99	6.73
400	7.67	7.05	6.44

Temperature measured between 0 and 2 hours: 21.9°C
Temperature at 24 hours (measured in the same test vessel): 24.2°C
Temperature at 48 hours (measured in the same test vessel): 24.2°C
Temperature at 72 hours (measured in the same test vessel): 24.3°C
Temperature at 96 hours (measured in the same test vessel): 24.6°C

Table 7: First test - T72 hours - number of cells and calculation of the specific growth rate at each concentration

Nominal concentration (mg/L)	Rep.	Total No. of cells at 72 hours (10^4/mL)	Ln No. of cells	Mean Ln No. of cells	Specific growth rate	% Inhibition of growth rate
0	1	189.0	14.45	14.58	0.07	
	2	235.0	14.67			
	3	205.0	14.53			
	4	230.0	14.65			
	5	242.0	14.70			
	6	197.0	14.49			
6.25	1	223.0	14.62	14.66	0.08	-1.42
	2	220.0	14.60			
	3	256.0	14.76			
12.5	1	233.0	14.66	14.65	0.08	-1.32
	2	208.0	14.55			
	3	255.0	14.75			
25.0	1	241.0	14.69	14.62	0.08	-0.69
	2	204.0	14.53			
	3	227.0	14.63			
50.0	1	240.0	14.69	14.72	0.08	-2.51
	2	289.0	14.88			
	3	216.0	14.59			
100	1	239.0	14.69	14.65	0.08	-1.21
	2	234.0	14.67			
	3	217.0	14.59			
200	1	222.0	14.61	14.58	0.07	0.04
	2#	-				
	3	208.0	14.55			
400	1	183.0	14.42	14.29	0.07	5.42*
	2	139.0	14.14			
	3	164.0	14.31			

Ln number of cells at T0 (10 000 cells) = 9.21

Rep. : replicate

Values obtained in this replicate (No. 2) at 200 mg/L were not retained for data interpretation since the value(s) obtained after 72 and/or 96 hours was/were at least 3 times lower than the mean of other replicate values.

* Significant inhibition (relative to the control)

Variation coefficient of the control cell density at 72 hours: 10.2%

Table 8: First test - T96 hours - number of cells and calculation of the specific growth rate at each concentration

Nominal concentration (mg/L)	Rep.	Total No. of cells at 96 hours (10^4/mL)	Ln No. of cells	Mean Ln No. of cells	Specific growth rate	% Inhibition of growth rate
0	1	379.0	15.15	15.28	0.06	
	2	470.0	15.36			
	3	432.0	15.28			
	4	418.0	15.25			
	5	466.0	15.35			
	6	436.0	15.29			
6.25	1	408.0	15.22	15.21	0.06	1.19
	2	422.0	15.26			
	3	378.0	15.14			
12.5	1	396.0	15.19	15.12	0.06	2.70*
	2	322.0	14.98			
	3	388.0	15.17			
25.0	1	404.0	15.21	15.06	0.06	3.56*
	2	308.0	14.94			
	3	340.0	15.04			
50.0	1	364.0	15.11	15.01	0.06	4.37*
	2	288.0	14.87			
	3	348.0	15.06			
100	1	334.0	15.02	15.04	0.06	3.92*
	2	324.0	14.99			
	3	366.0	15.11			
200	1	270.0	14.81	14.82	0.06	7.64*
	2#	-				
	3	274.0	14.82			
400	1	183.0	14.42	14.42	0.05	14.20*
	2	185.0	14.43			
	3	180.0	14.40			

Ln number of cells at T0 (10 000 cells) = 9.21

Rep. : replicate

Values obtained in this replicate (No. 2) at 200 mg/L were not retained for data interpretation since the value(s) obtained after 72 and/or 96 hours was/were at least 3 times lower than the mean of other replicate values.

* Significant inhibition (relative to the control)

Variation coefficient of the control cell density at 72 hours: 7.73%

Table 9: First test - Number of cells (10^4 cells x mL^{-1}) at each observation time and calculation of the area under the growth curve (biomass) at each concentration after 72 hours. Area = cell x mL^{-1} x hour

		Total number of cells			Biomass					Inhibition (%)
Nominal concentration (mg/L)	Rep.	at 24 hours	at 48 hours	at 72 hours	0 to 24 hours	24 to 48 hours	48 to 72 hours	0 to 72 hours	Average (0-72 hours)	
0	1	2.10	26.00	189.00	13.20	313.20	2556.00	2882.40	3478.00	
	2	3.90	34.30	235.00	34.80	434.40	3207.60	3676.80		
	3	5.90	29.80	205.00	58.80	404.40	2793.60	3256.80		
	4	4.90	34.70	230.00	46.80	451.20	3152.40	3650.40		
	5	4.90	39.70	242.00	46.80	511.20	3356.40	3914.40		
	6	3.60	45.70	197.00	31.20	567.60	2888.40	3487.20		
6.25	1	3.60	39.30	223.00	31.20	490.80	3123.60	3645.60	3747.20	-7.74
	2	4.30	33.70	220.00	39.60	432.00	3020.40	3492.00		
	3	4.80	40.70	256.00	45.60	522.00	3536.40	4104.00		
12.5	1	3.90	38.70	233.00	34.80	487.20	3236.40	3758.40	3751.20	-7.86
	2	4.70	43.70	208.00	44.40	556.80	2996.40	3597.60		
	3	4.10	33.30	255.00	37.20	424.80	3435.60	3897.60		
25.0	1	3.80	50.50	241.00	33.60	627.60	3474.00	4135.20	3711.20	-6.71
	2	4.00	38.70	204.00	36.00	488.40	2888.40	3412.80		
	3	4.10	34.30	227.00	37.20	436.80	3111.60	3585.60		
50.0	1	4.40	38.00	240.00	40.80	484.80	3312.00	3837.60	3893.60	-11.95
	2	2.50	35.30	289.00	18.00	429.60	3867.60	4315.20		
	3	3.50	38.00	216.00	30.00	474.00	3024.00	3528.00		
100	1	3.70	42.30	239.00	32.40	528.00	3351.60	3912.00	3750.40	-7.83
	2	4.30	37.70	234.00	39.60	480.00	3236.40	3756.00		
	3	3.30	40.00	217.00	27.60	495.60	3060.00	3583.20		
200	1	3.40	41.70	222.00	28.80	517.20	3140.40	3686.40	3561.60	-2.40
	2#	-	-	-						
	3	3.70	38.00	208.00	32.40	476.40	2928.00	3436.80		
400	1	2.70	46.30	183.00	20.40	564.00	2727.60	3312.00	2864.00	17.65
	2	2.10	35.70	139.00	13.20	429.60	2072.40	2515.20		
	3	2.40	33.30	164.00	16.80	404.40	2343.60	2764.80		

Rep. : replicate
Values obtained in this replicate (No. 2) at 200 mg/L were not retained for data interpretation since the value(s) obtained after 72 and/or 96 hours was/were at least 3 times lower than the mean of other replicate values.
* Significant inhibition (relative to the control)

Variation coefficient of the control cell density at 72 hours: 10.2%

Table 10: First test - Number of cells (10^4 cells x mL^{-1}) at each observation time and calculation of the area under the growth curve (biomass) at each concentration after 96 hours. Area = cell x mL^{-1} x hour

		Total number of cells				Biomass						Inhibition (%)
Nominal concentration (mg/L)	Rep.	at 24 hours	at 48 hours	at 72 hours	at 96 hours	0 to 24 hours	24 to 48 hours	48 to 72 hours	72 to 96 hours	0 to 96 hours	Average (0-96 hours)	
0	1	2.10	26.00	189.00	379.00	13.20	313.20	2556.00	6792.00	9674.40	11252.00	
	2	3.90	34.30	235.00	470.00	34.80	434.40	3207.60	8436.00	12112.80		
	3	5.90	29.80	205.00	432.00	58.80	404.40	2793.60	7620.00	10876.80		
	4	4.90	34.70	230.00	418.00	46.80	451.20	3152.40	7752.00	11402.40		
	5	4.90	39.70	242.00	466.00	46.80	511.20	3356.40	8472.00	12386.40		
	6	3.60	45.70	197.00	436.00	31.20	567.60	2888.40	7572.00	11059.20		
6.25	1	3.60	39.30	223.00	408.00	31.20	490.80	3123.60	7548.00	11193.60	11351.20	-0.88
	2	4.30	33.70	220.00	422.00	39.60	432.00	3020.40	7680.00	11172.00		
	3	4.80	40.70	256.00	378.00	45.60	522.00	3536.40	7584.00	11688.00		
12.5	1	3.90	38.70	233.00	396.00	34.80	487.20	3236.40	7524.00	11282.40	10935.20	2.82
	2	4.70	43.70	208.00	322.00	44.40	556.80	2996.40	6336.00	9933.60		
	3	4.10	33.30	255.00	388.00	37.20	424.80	3435.60	7692.00	11589.60		
25.0	1	3.80	50.50	241.00	404.00	33.60	627.60	3474.00	7716.00	11851.20	10583.20	5.94
	2	4.00	38.70	204.00	308.00	36.00	488.40	2888.40	6120.00	9532.80		
	3	4.10	34.30	227.00	340.00	37.20	436.80	3111.60	6780.00	10365.60		
50.0	1	4.40	38.00	240.00	364.00	40.80	484.80	3312.00	7224.00	11061.60	10849.60	3.58
	2	2.50	35.30	289.00	288.00	18.00	429.60	3867.60	6900.00	11215.20		
	3	3.50	38.00	216.00	348.00	30.00	474.00	3024.00	6744.00	10272.00		
100	1	3.70	42.30	239.00	334.00	32.40	528.00	3351.60	6852.00	10764.00	10582.40	5.95
	2	4.30	37.70	234.00	324.00	39.60	480.00	3236.40	6672.00	10428.00		
	3	3.30	40.00	217.00	366.00	27.60	495.60	3060.00	6972.00	10555.20		
200	1	3.40	41.70	222.00	270.00	28.80	517.20	3140.40	5880.00	9566.40	9381.60	16.62*
	2#	-	-	-	-							
	3	3.70	38.00	208.00	274.00	32.40	476.40	2928.00	5760.00	9196.80		
400	1	2.70	46.30	183.00	183.00	20.40	564.00	2727.60	4368.00	7680.00	6976.00	38.00*
	2	2.10	35.70	139.00	185.00	13.20	429.60	2072.40	3864.00	6379.20		
	3	2.40	33.30	164.00	180.00	16.80	404.40	2343.60	4104.00	6868.80		

Rep. : replicate
Values obtained in this replicate (No. 2) at 200 mg/L were not retained for data interpretation since the value(s) obtained after 72 and/or 96 hours was/were at least 3 times lower than the mean of other replicate values.
* Significant inhibition (relative to the control)

Variation coefficient of the control cell density at 96 hours: 7.73%

Table 11: Second test - temperature and pH

Nominal concentration (mg/L)	pH at T 0 hour	pH at T 72 hours	pH at T 96 hours
0	7.30	10.24	10.74
6.25	7.47	9.90	10.03
12.5	7.45	9.66	10.11
25.0	7.44	9.52	9.94
50.0	7.44	9.38	9.87
100	7.42	8.58	9.50
200	7.41	6.73	7.13
400	7.40	6.66	6.61

Temperature measured between 0 and 2 hours: 22.6°C
Temperature at 24 hours (measured in the same test vessel): 23.6°C
Temperature at 48 hours (measured in the same test vessel): 23.3°C
Temperature at 72 hours (measured in the same test vessel): 23.4°C
Temperature at 96 hours (measured in the same test vessel): 23.8°C

Table 12: Second test - T72 hours - number of cells and calculation of the specific growth rate at each concentration

Nominal concentration (mg/L)	Rep.	Total No. of cells at 72 hours (10^4/mL)	Ln No. of cells	Mean Ln No. of cells	Specific growth rate	% Inhibition of growth rate
0	1	115.0	13.95	14.06	0.07	
	2	114.0	13.95			
	3	154.0	14.25			
	4	127.0	14.05			
	5	129.0	14.07			
	6	133.0	14.10			
6.25	1	121.0	14.01	14.08	0.07	-0.33
	2	129.0	14.07			
	3	141.0	14.16			
12.5	1	130.0	14.08	14.17	0.07	-2.30
	2	179.0	14.40			
	3	126.0	14.05			
25.0	1	145.0	14.19	14.17	0.07	-2.17
	2	134.0	14.11			
	3	148.0	14.21			
50.0	1	133.0	14.10	14.10	0.07	-0.79
	2	131.0	14.09			
	3	135.0	14.12			
100	1	129.0	14.07	14.13	0.07	-1.33
	2	138.0	14.14			
	3	143.0	14.17			
200	1	117.0	13.97	14.00	0.07	1.34
	2	120.0	14.00			
	3	123.0	14.02			
400	1	104.0	13.85	13.88	0.06	3.78*
	2	113.0	13.94			
	3	103.0	13.84			

Ln number of cells at T0 (10 000 cells) = 9.21

Rep. : replicate

* Significant inhibition (relative to the control)

Variation coefficient of the control cell density at 72 hours: 11.3%

Table 13: Second test - T96 hours - number of cells and calculation of the specific growth rate at each concentration

Nominal concentration (mg/L)	Rep.	Total No. of cells at 96 hours (10^4/mL)	Ln No. of cells	Mean Ln No. of cells	Specific growth rate	% Inhibition of growth rate
0	1	476.0	15.38	15.37	0.06	
	2	420.0	15.25			
	3	474.0	15.37			
	4	510.0	15.44			
	5	442.0	15.30			
	6	518.0	15.46			
6.25	1	394.0	15.19	15.33	0.06	0.59
	2	460.0	15.34			
	3	520.0	15.46			
12.5	1	368.0	15.12	15.26	0.06	1.80
	2	460.0	15.34			
	3	446.0	15.31			
25.0	1	328.0	15.00	15.01	0.06	5.78*
	2	298.0	14.91			
	3	370.0	15.12			
50.0	1	316.0	14.97	15.03	0.06	5.44*
	2	348.0	15.06			
	3	350.0	15.07			
100	1	316.0	14.97	14.96	0.06	6.68*
	2	332.0	15.02			
	3	292.0	14.89			
200	1	208.0	14.55	14.65	0.06	11.63*
	2	248.0	14.72			
	3	238.0	14.68			
400	1	184.0	14.42	14.46	0.05	14.79*
	2	200.0	14.51			
	3	186.0	14.44			

Ln number of cells at T0 (10 000 cells) = 9.21
Rep. : replicate
* Significant inhibition (relative to the control)

Variation coefficient of the control cell density at 96 hours: 8.00%

Table 14: Second test - Number of cells (10^4 cells x mL^{-1}) at each observation time and calculation of the area under the growth curve (biomass) at each concentration after 72 hours.
Area = cell x mL^{-1} x hour

		Total number of cells			Biomass					Inhibition (%)
Nominal concentration (mg/L)	Rep.	at 24 hours	at 48 hours	at 72 hours	0 to 24 hours	24 to 48 hours	48 to 72 hours	0 to 72 hours	Average (0-72 hours)	
0	1	6.50	31.00	115.00	66.00	426.00	1728.00	2220.00	2530.00	
	2	8.60	42.30	114.00	91.20	586.80	1851.60	2529.60		
	3	6.40	33.70	154.00	64.80	457.20	2228.40	2750.40		
	4	6.30	37.00	127.00	63.60	495.60	1944.00	2503.20		
	5	8.10	39.00	129.00	85.20	541.20	1992.00	2618.40		
	6	5.60	37.00	133.00	55.20	487.20	2016.00	2558.40		
6.25	1	6.70	33.30	121.00	68.40	456.00	1827.60	2352.00	2540.00	-0.40
	2	7.20	39.00	129.00	74.40	530.40	1992.00	2596.80		
	3	6.60	36.70	141.00	67.20	495.60	2108.40	2671.20		
12.5	1	6.30	32.00	130.00	63.60	435.60	1920.00	2419.20	2640.80	-4.38
	2	6.50	38.00	179.00	66.00	510.00	2580.00	3156.00		
	3	6.80	30.50	126.00	69.60	423.60	1854.00	2347.20		
25.0	1	5.90	31.50	145.00	58.80	424.80	2094.00	2577.60	2556.80	-1.06
	2	6.80	32.00	134.00	69.60	441.60	1968.00	2479.20		
	3	6.40	31.00	148.00	64.80	424.80	2124.00	2613.60		
50.0	1	5.50	30.50	133.00	54.00	408.00	1938.00	2400.00	2569.60	-1.57
	2	7.00	40.30	131.00	72.00	543.60	2031.60	2647.20		
	3	6.60	39.30	135.00	67.20	526.80	2067.60	2661.60		
100	1	5.50	33.30	129.00	54.00	441.60	1923.60	2419.20	2447.20	3.27
	2	5.20	30.80	138.00	50.40	408.00	2001.60	2460.00		
	3	5.60	28.00	143.00	55.20	379.20	2028.00	2462.40		
200	1	5.70	21.00	117.00	56.40	296.40	1632.00	1984.80	2263.20	10.55
	2	7.10	39.70	120.00	73.20	537.60	1892.40	2503.20		
	3	5.90	31.00	123.00	58.80	418.80	1824.00	2301.60		
400	1	4.60	27.50	104.00	43.20	361.20	1554.00	1958.40	1987.20	21.45*
	2	4.50	27.00	113.00	42.00	354.00	1656.00	2052.00		
	3	4.00	28.30	103.00	36.00	363.60	1551.60	1951.20		

Rep. : replicate
* Significant inhibition (relative to the control)

Variation coefficient of the control cell density at 72 hours: 11.3%

Table 15: Second test - Number of cells (10^4 cells x mL^{-1}) at each observation time and calculation of the area under the growth curve (biomass) at each concentration after 96 hours.
Area = cell x mL^{-1} x hour

		Total number of cells				Biomass						Inhibition (%)
Nominal concentration (mg/L)	Rep.	at 24 hours	at 48 hours	at 72 hours	at 96 hours	0 to 24 hours	24 to 48 hours	48 to 72 hours	72 to 96 hours	0 to 96 hours	Average (0-96 hours)	
0	1	6.50	31.00	115.00	476.00	66.00	426.00	1728.00	7068.00	9288.00	9730.00	
	2	8.60	42.30	114.00	420.00	91.20	586.80	1851.60	6384.00	8913.60		
	3	6.40	33.70	154.00	474.00	64.80	457.20	2228.40	7512.00	10262.40		
	4	6.30	37.00	127.00	510.00	63.60	495.60	1944.00	7620.00	10123.20		
	5	8.10	39.00	129.00	442.00	85.20	541.20	1992.00	6828.00	9446.40		
	6	5.60	37.00	133.00	518.00	55.20	487.20	2016.00	7788.00	10346.40		
6.25	1	6.70	33.30	121.00	394.00	68.40	456.00	1827.60	6156.00	8508.00	9576.00	1.58
	2	7.20	39.00	129.00	460.00	74.40	530.40	1992.00	7044.00	9640.80		
	3	6.60	36.70	141.00	520.00	67.20	495.60	2108.40	7908.00	10579.20		
12.5	1	6.30	32.00	130.00	368.00	63.60	435.60	1920.00	5952.00	8371.20	9452.80	2.85
	2	6.50	38.00	179.00	460.00	66.00	510.00	2580.00	7644.00	10800.00		
	3	6.80	30.50	126.00	446.00	69.60	423.60	1854.00	6840.00	9187.20		
25.0	1	5.90	31.50	145.00	328.00	58.80	424.80	2094.00	5652.00	8229.60	8224.80	15.47*
	2	6.80	32.00	134.00	298.00	69.60	441.60	1968.00	5160.00	7639.20		
	3	6.40	31.00	148.00	370.00	64.80	424.80	2124.00	6192.00	8805.60		
50.0	1	5.50	30.50	133.00	316.00	54.00	408.00	1938.00	5364.00	7764.00	8197.60	15.75*
	2	7.00	40.30	131.00	348.00	72.00	543.60	2031.60	5724.00	8371.20		
	3	6.60	39.30	135.00	350.00	67.20	526.80	2067.60	5796.00	8457.60		
100	1	5.50	33.30	129.00	316.00	54.00	441.60	1923.60	5316.00	7735.20	7823.20	19.60*
	2	5.20	30.80	138.00	332.00	50.40	408.00	2001.60	5616.00	8076.00		
	3	5.60	28.00	143.00	292.00	55.20	379.20	2028.00	5196.00	7658.40		
200	1	5.70	21.00	117.00	208.00	56.40	296.40	1632.00	3876.00	5860.80	6455.20	33.66*
	2	7.10	39.70	120.00	248.00	73.20	537.60	1892.40	4392.00	6895.20		
	3	5.90	31.00	123.00	238.00	58.80	418.80	1824.00	4308.00	6609.60		
400	1	4.60	27.50	104.00	184.00	43.20	361.20	1554.00	3432.00	5390.40	5523.20	43.24*
	2	4.50	27.00	113.00	200.00	42.00	354.00	1656.00	3732.00	5784.00		
	3	4.00	28.30	103.00	186.00	36.00	363.60	1551.60	3444.00	5395.20		

Rep. : replicate
* Significant inhibition (relative to the control)

Variation coefficient of the control cell density at 96 hours: 8.00%

APPENDICES

1. Analytical certificate and composition of the test item

Certificate of Compliance/Analysis

This is to certify that the material shipped on this order is in conformance with 3M specifications dated April 25, 2001 with the following analysis:

3M Invoice Nbr: [redacted]
3M Product ID: [redacted]
3M Product Code: [redacted]
Description: [redacted]

Customer P.O.Nbr: SAMPLE/ SIERAKOWS

Lot Nbr: 10004 Order Qty: 40 LBS Lot Qty: 40 LBS 1 DRUM @ 40 LBS

Customer: 3M/ PERF MATLS LAB
MIKE SIERAKOWSKI
BLDG236-2A-01
ST PAUL, MN 55144

Ship Date: Jan 24, 2002
Date Of Mfg: Mar 30, 2001

Product Test	Unit	Specifications Lower Limit	Target	Upper Limit	Result/ Range	Test Method
pH 2 % Aqueous Solution		5.0	-	6.5	6.0	30.2.C
Solids	%	19.5	-	20.5	19.6	14.20.C
APHA Color		-	-	50	15	96.1.C
Iron Based on 100% Solids	ppm	-	-	5.0	0.2	58.47.C
Turbidity	NTU	-	-	3	3	47.5.C
[redacted]	%	-	-	0.08	0.02	71.5.1.C
UV Extinction	abs/cm	-	-	0.60	0.04	71.5.1.C
[redacted]	%	-	-	1.4	0.1	300.114
	%	96.5	-	-	99.7	300.114

Shelf Life: The shelf life of this material is two years from the date of manufacture if left in the original, unopened container under recommended storage conditions.

These are introductory specifications based upon limited data and are subject to change.

3M Specialty Materials
3M Center Bldg. 223-6S-04
St. Paul, MN 55144-1000

3M Corporate Toxicology and
Regulatory Services

3M Center, Building 0220-02-E-02
St. Paul, MN 55144-1000
651 733 1773 Fax

May 2, 2003

3M

Dr. Jacques L'Haridon
CIT
BP 563
27005 Evreux, France

Dear Dr. L'Haridon:

This is to confirm that the sample of ammonium perfluorooctanoate [redacted] supplied to to CIT, BP 563, 27005 Evreux, France (3M Product Code [redacted] would be expected to contain approximately [redacted] material (mole percentages). [redacted] is a [redacted] solution of ammonium perfluorooctanoate in water. An example analysis on a batch of solid ammonium perfluorooctanoate (3M Product Code [redacted] gave the following isomer distribution, determined using ^{19}F-NMR techniques:

Isomer	Mole percentage
[redacted]	77.6
	12.6
	9.0
	0.2
	0.3

Sincerely,

John L. Butenhoff

John L. Butenhoff, Ph.D., CIH, DABT
Staff Scientist

JLB/rm

2. Substances required for the preparation of LC reconstituted water

LC RECONSTITUTED WATER
(as described in the French guideline T 90-304)

	Final Concentrations
Solution No. 1:	
- calcium nitrate ($Ca(NO_3)_2.4H_2O$)	40 mg/L
Solution No. 2:	
- potassium nitrate (KNO_3)	100 mg/L
Solution No. 3:	
- magnesium sulphate ($MgSO_4.7H_2O$)	30 mg/L
Solution No. 4:	
- monohydrogen potassium phosphate (K_2HPO_4)	40 mg/L
Trace element solutions:	
Solution No. 5:	
- copper sulphate ($CuSO_4.5H_2O$)	15 µg/L
- ammonium heptamolybdate [$(NH_4)_6Mo_7O_{24}.4H_2O$]	30 µg/L
- zinc sulphate ($ZnSO_4.7H_2O$)	30 µg/L
- cobalt chloride ($CoCl_2.6H_2O$)	30 µg/L
- manganese nitrate ($Mn(NO_3)_2.4H_2O$)	30 µg/L
- citric acid ($C_6H_8O_2.H_2O$)	30 µg/L
- boric acid (H_3BO_3)	30 µg/L
Solution No. 6:	
- iron citrate (III) ($C_6H_5FeO_7$)	0.616 mg/L
- iron sulphate (II) ($FeSO_4.7H_2O$)	0.3125 mg/L
- iron chloride (III) ($FeCl_3.6H_2O$)	0.3125 mg/L

All solutions are made up with injectable grade deionized water (conductivity < 10 μScm^{-1}).
Prepared solutions are kept for no more than 1 week after autoclaving.
Final pH = 7.5 ± 0.3; hardness = approximately 34 mg/L as $CaCO_3$.

3. Chemical analysis of test solutions

CHEMICAL ANALYSIS

Principle

An aliquot of each sample was diluted and analyzed by Ion Chromatography with Electrochemical Detection (Conductimetry). The concentrations of AMMONIUM PERFLUOROOCTANOATE (APFO) were determined from a calibration curve of peak area against concentration of AMMONIUM PERFLUOROOCTANOATE (APFO) in standard solutions.

Sample preparation

All samples were mixed and those with algae were centrifuged (4000 rpm, 15 min, +4°C) to discard algae for analysis. If necessary, samples were then diluted with Milli-Q water to achieve concentrations in the range 1-100 mg/L of AMMONIUM PERFLUOROOCTANOATE (APFO).

Chromatographic conditions

Pump : GP 50 Gradient Pump (Dionex)

Mobile phase : phase A: aqueous sodium hydroxide solution 50 mM*
phase B: Milli-Q water
phase C: acetonitrile

* Aqueous sodium hydroxide solution 50 mM: 2.6 mL of sodium hydroxide solution at 46/48% (Fisher Chemicals, Ref.: S/4930/05) was added to 1 L of Milli-Q Water.

Time (min)	phase A (%)	phase B (%)	phase C (%)	curve
0	10	85	5	6
5	40	0	60	6
12	40	0	60	-
13	10	85	5	-
25	10	85	5	-

Flow rate : 1 mL/min

Precolumn : Ionpac ATC-3, 4 mm (Dionex)

Column : Ionpac AS16 (Dionex)
length = 250 mm,
inner diameter = 4 mm

Temperature : 30°C

Detector : ED 50 Electrochemical Detector (Dionex)
Conductimetry after eluent neutralization

Neutralization : AMMS III (Dionex)

Injector : AS50 (Dionex), at 10°C

Injected volume : 25 µL

Data acquisition software : Multichrom 2 (Fisons Instruments)

Retention time : APFO,
approx. 9 min

Analysis time : 30 min

Calibration curve

Peak areas were determined for standard solutions ranging from 1 to 100 mg/L of AMMONIUM PERFLUOROOCTANOATE (APFO) (six levels). A calibration curve was obtained by plotting test item peak areas against concentrations.

The regression analysis of the calibration data gave an equation of the following form:

$$Y = aX + b$$

where Y = test item peak area (µVs)
X = concentration of test item (mg/L)
a = slope value
b = intercept

Assay

Samples of AMMONIUM PERFLUOROOCTANOATE (APFO) were analyzed by Ion Chromatography with Electrochemical Detection (Conductimetry).

One dilution was prepared for each sample and one injection (of 25-µL aliquots) was performed for each final dilution.

The peak area was determined for each sample. The concentration of AMMONIUM PERFLUOROOCTANOATE (APFO) in each sample was calculated using the equation obtained from the calibration data.

All the results are expressed as mg/L of APFO.

Table A: Concentration of AMMONIUM PERFLUOROOCTANOATE (APFO) in the test solutions of the second test (mg/L)

Nominal	Measured (1)					
	0 hour		96 hours			
	without algae		with algae		without algae	
6.25	5.73	(92)	6.03	(96)	5.53	(88)
12.5	10.8	(86)	12.7	(102)	10.6	(85)
25.0	22.4	(90)	24.3	(97)	21.4	(86)
50.0	45.8	(92)	47.3	(95)	45.9	(92)
100	96.7	(97)	95.9	(96)	95.0	(95)
200	179	(90)	180	(90)	183	(92)
400	364	(91)	362	(91)	383	(96)

(1): numbers in brackets represent percentages of the nominal concentration

VALIDATION OF THE ANALYTICAL METHOD

The validation of the analytical method was performed according to CIT Standard Operating Procedures.
The specificity, limit of quantification, linearity, repeatability of injections, accuracy and precision (Coefficient of Variation: CV %) of the analytical method were determined.

Specificity

The specificity of the analytical method was demonstrated as follows:
- analysis of a standard solution of AMMONIUM PERFLUOROOCTANOATE (APFO) in Milli-Q water,
- analysis of Milli-Q water or test water (LC, M4 or dechlorinated: desionised water) without dilution,

No relevant interference between the test item peak and Milli-Q water or test water was observed on chromatograms.

Limit of quantification

The limit of quantification of the analytical method was established as 1 mg/L for a standard solution of APFO.
This limit corresponds to a limit of quantification of 1 mg/L for the test item in aqueous phase.

Linearity

Linearity was checked by analysis of three different sets of six standard solutions containing 1, 5, 10, 20, 50 and 100 mg/L of AMMONIUM PERFLUOROOCTANOATE (APFO) in Milli-Q water.
Satisfactory linearity was demonstrated in the range 1 to 100 mg/L since the coefficients of determination obtained were higher than 0.999.

Repeatability of injections

Replicate analysis (n = 10) of a solution containing 50 mg/L of the test item gave satisfactory results since the coefficients of variation values obtained were as follows:
- 1% based on peak height,
- 1% based on peak area.

Based on these results, the repeatability of injections of the analytical method was validated taking into account peak area.

Accuracy and precision

Six analyses of solutions containing 2.03 and 50.7 mg/L of the test item in Milli-Q water were carried out.

The accuracy and the precision (CV%) obtained were as follows:

Concentration (mg/L)		Dilution	CV	Accuracy
Nominal	Mean measured*	factor	(%)	(%)
2.03	2.02	1	6	99
50.7	53.7	1	2	106

*: mean values of six determinations

Conclusion

The analytical method was validated and considered to be suitable for the analysis of the samples of the study.

4. Comparison of areas under the growth curves

COMPARISON OF AREAS UNDER THE GROWTH CURVES (Biomass)

The areas between the growth curves and the horizontal line $N = N_0$ were calculated according to the formula:

$$A = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_2 - 2N_0}{2} \times (t_2 - t_1) + \ldots + \frac{N_n + N_{n-1} - 2N_0}{2} \times (t_n - t_{n-1})$$

where,

A = area
N_0 = number of cells/mL at time t_0 (beginning of the test),
N_1 = measured number of cells/mL at time t_1
N_n = measured number of cells/mL at time t_n
t_1 = time in hours of first measurement after beginning of test
t_n = time in hours of n^{th} measurement after beginning of test
n = number of measurements taken after the beginning of the test.

The percentage inhibition of the cell growth at each test item concentration (I_A) was calculated according to the formula:

$$I_A = \frac{A_c - A_t}{A_c} \times 100$$

where,
A_c = area between the control growth curve and the horizontal line $N = N_0$
A_t = area between the growth curve at the concentration t and the horizontal line $N = N_0$

I_A values are plotted on semilogarithmic paper or on semilogarithmic probit paper against the corresponding concentrations. If plotted on probit paper, the points are fitted by a straight line, either by eye or by a computed regression.

The EC50 is estimated from the regression line by reading off the concentration that is equivalent to a 50% inhibition ($I_A = 50\%$). To denote this value unambiguously in relation to this method of calculation, it is proposed to use the symbol E_bC_{50} is quoted with the appropriate exposure period, e.g. E_bC_{50}(0-96h).

COMPARISON OF GROWTH RATES

The average specific growth rate (μ) for exponentially growing cultures was calculated as:

$$\mu = \frac{\text{Ln } N_n - \text{Ln } N_0}{t_n - t_0}$$

where t_0 is the time at the beginning of the test and t_n the time in hours of n^{th} measurement after beginning of test.

The percentage inhibition of specific growth rate at each test item concentration ($I_\mu t$) was calculated according to the formula:

$$I_{\mu t} = \frac{\mu_c - \mu_t}{\mu_c} \times 100$$

where,
μ_c = mean control specific growth rate
μ_t = mean specific growth rate for the test concentration t

The percentage reduction of average specific growth rate at each test item concentration compared with the control value is plotted against the logarithm of the concentration. The EC50 may be red from the resulting graph. To denote unambiguously the EC50 derived by this method it is proposed to use the symbol E_rC_{50}. The times of measurement must be indicated, e.g. if the value relates to times 0 and 96 hours, the symbol becomes E_rC_{50}(0-96h).

Note: specific growth rate is a logarithmic term and small changes in growth rate may lead to great changes in biomass. E_bC and E_rC values are therefore not numerically comparable.

CALCULATION OF THE NOEC

The No Observed Effect Concentration was determined by first checking for normality (i.e. Chi-square test or Shapiro-Wilks test) as well as variance homogeneity (i.e. Bartlett test) and then by analysis of variance (i.e. Bonferroni T-test), using the individual replicate values of the areas under the growth curves or the specific growth rates.