Λ_o of the acid can be calculated using literature values for H^+ and Na^+. The pKa can be calculated from $\alpha = \Lambda_i/\Lambda_o$ and $Ka = \alpha^2 C/1 - \alpha$ for each concentration. Better values for Ka can be obtained by making corrections for mobility and activity. The mean and standard deviations of the pKa values should be calculated.

• **T e s t   r e p o r t**

All raw data and calculated pK$_a$ values should be submitted together with method of calculation (preferably in a tabulated format, such as suggested in ref. 1) as should the statistical parameters described above. For titration methods, details of the standardisation of titrants should be given.

For spectrophotometric methods, all spectra should be submitted. For the conductometric method, details of the cell constant determination should be reported. Information on technique used, analytical methods and the nature of any buffers used should be given.

The test temperature(s) should be reported.

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OECD GUIDELINE FOR TESTING OF CHEMICALS

Adopted by the Council on 30th March 1989

Partition Coefficient (n-octanol/water), High Performance Liquid Chromatography (HPLC) Method

Introduction

1.   The partition coefficient (P) is defined as the ratio of the equilibrium concentrations of a dissolved substance in a two-phase system consisting of two largely immiscible solvents. In the case of n-octanol and water,

$$P_{ow} = \frac{c_{n\text{-}octanol}}{c_{water}}$$

The partition coefficient being the quotient of two concentrations, is dimensionless and is usually given in the form of its logarithm to base ten.

2. P_{ow} is a key parameter in studies of the environmental fate of chemical substances. A highly-significant relationship between the P_{ow} of substances and their bioaccumulation in fish has been shown. It has also been shown that P_{ow} is a useful parameter in the prediction of adsorption on soil and sediments andfor establishing quantitative structure-activity relationships for a wide range of biological effects.

3. P_{ow} values in the range log P_{ow} between -2 and 4 can be experimentally determined by the Shake-Flask method (OECD Test Guideline 107). P_{ow} values in the range log P_{ow} between 0 and 6 can be estimated using high performance liquid chromatography (HPLC) (1) (2) (3) (4). The HPLC method requires a preliminary estimation of P_{ow}, generally done through calculation. Calculation methods are briefly discussed in the Annex to this guideline.

4.   The original proposal for this guideline was based on an article by C.V. Eadsforth and P. Moser (1). The development of the guideline and an OECD inter-laboratory comparison test were coordinated by the Umweltbundesamt of the Federal Republic of Germany during 1986 (5).

Principle of the method

5.   HPLC is performed on analytical columns packed with a commercially available solid phase containing long hydrocarbon chains (e.g. C_8, C_{18}) chemically bound onto silica.

Chemicals injected onto such a column move along it by partitioning between the mobile solvent phase and the hydrocarbon stationary phase. The chemicals are retained in proportion to their hydrocarbon-water partition coefficient, with water-soluble chemicals eluted first and oil-soluble chemicals last. This enables the relationship between the retention time on a reverse-phase column and the n-octanol/water partition coefficient to be established. The partition coefficient is deduced from the capacity factor k, given by the expression

$$k = \frac{tR - t_o}{t_o}$$

where, tR is the retention time of the test substance, and t_o is the dead-time, i.e. the average time a solvent molecule needs to pass the column. Quantitative analytical methods are not required and only the determination of retention times is necessary. If standard reference compounds are available and standard experimental conditions are used, the HLPC method can be performed faster than the Shake-Flask method.

6.   The HPLC method enables partition coefficients to be estimated in the log P_{ow} range between 0 and 6. The method is not applicable to strong acids and bases, metal complexes, substances which react with the eluent, or surface-active agents. Measurements should be made on ionisable substances in their non-ionised from (free acid or free base) only by using an appropriate buffer with a pH below the pK for a free acid or above the pK for a free base (e.g. phosphoric acid for pH = 2 and 0.01 - 0.02 M phosphate buffer for pH = 7.5).

7.   The HPLC method is less sensitive to the presence of impurities in the test substance than the Shake-Flask method. Nevertheless, in some cases impurities can make the interpretation of the results difficult due to uncertainly in peak assignments. For mixtures which result in an unresolved band, upper and lower limits of log P should be stated (3).

Information on the test substance

8.   The structural formula and the dissociation constant should be known before using the method. Information on solubility and hydrolysis characteristics is useful.

Repeatability and accuracy

9.   In order to increase the confidence in the measurement, duplicate determinations must be made. The values of log P_{ow} derived from the different measurements should fall within a range of +/- 0.1 log units.

10.   The inter-laboratory comparison test has shown that with the HPLC method log P_{ow} values can be obtained to within +/- 0.5 units of the Shake-Flask values (5). Other comparisons can be found in the literature (3) (4) (6) (7) (8). Correlation graphs based on structurally related reference compounds give the most accurate results (9).

Reference compounds

11. In order to correlate the measured capacity factor k of a compound with its P_{ow}, a calibration graph using at least 6 points has to be established. It is up to the user to select the appropriate reference compounds. It is preferable that these should be structurally related to the test substance. Whenever possible, at least one reference compound should have a P_{ow} above that of the test substance, and another a P_{ow} below that of the test substance. For log P_{ow} values below 4, the calibration can be based on data obtained by the Shake Flask method. For log P_{ow} values above 4, the calibration can be based on literature values if they correspond to calculated values.

12. Extensive lists of log P_{ow} values for many groups of chemicals are available (10) (11). If data on the partition coefficients of structurally related compounds are not available, a more general calibration, established with other reference compounds, may be used. Recommended reference compounds and their P_{ow} values are listed in Table 1. For ionisable substances the values given apply to the non-ionised form. The values were checked for plausibility and quality during the inter-laboratory comparison test.

Description of the method

Preliminary estimate of the partition coefficient

13. The partition coefficient of the test substance is estimated preferably by using a calculation method (see Annex), or where appropriate, by using the ratio of the solubilities of the test substance in the pure solvents (12).

Table 1: Recommended Reference Compounds

Reference substance	log P_{ow}	pKa
2-Butanone	0,3	
4-Acetylpyridine	0,5	
Aniline	0,9	
Acetanilide	1,0	
Benzil alcohol	1,1	
4-Methoxyphenol	1,3	pKa = 10,26
Phenoxyacetic acid	1,4	pKa = 3,12
Phenol	1,5	pKa = 9,92
2,4-Dinitrophenol	1,5	pKa = 3,96
Benzonitrile	1,6	
Phenylacetonitrile	1,6	
4-Methylbenzyl alcohol	1,6	
Acetophenone	1,7	
2-Nitrophenol	1,8	pKa = 7,17
3-Nitrobenzoic acid	1,8	pKa = 3,47
4-Chloraniline	1,8	pKa = 4,15
Nitrobenzene	1,9	
Cinnamic alcohol	1,9	
Benzoic acid	1,9	pKa = 4,19
p-Cresol	1,9	pKa = 10,17

Table 1: Recommended Reference Compounds
(Continued)

Reference substance	log P_{ow}	pKa
Cinnamic acid	2,1	pKa = 3,89 cis
		4,44 trans
Anisole	2,1	
Methyl benzoate	2,1	
Benzene	2,1	
3-Methylbenzoic acid	2,4	pKa = 4,27
4-Chlorophenol	2,4	pKa = 9,1
Trichloroethene	2,4	
Atrazine	2,6	
Ethyl benzoate	2,6	
2,6-Dichlorobenzonitrile	2,6	
3-Chlorobenzoic acid	2,7	pKa = 3,82
Toluene	2,7	
1-Naphthol	2,7	pKa = 9,34
2,3-Dichloroaniline	2,8	
Chlorobenzene	2,8	
Allyl phenyl ether	2,9	
Bromobenzene	3,0	
Ethylbenzene	3,2	
Benzophenone	3,2	
4-Phenyl phenol	3,2	pKa = 9,54
Thymol	3,3	
1,4-Dichlorobenzene	3,4	
Diphenylamine	3,4	pKa = 0,79
Naphthalene	3,6	
Phenyl benzoate	3,6	
Isopropylbenzene	3,7	
2,4,6-Trichlorophenol	3,7	pKa = 6
Biphenyl	4,0	
Benzyl benzoate	4,0·	
2,4-Dinitro-6 sec. butyl phenol	4,1	
1,2,4-Trichlorobenzene	4,2	
Dodecanoic acid	4,2	
Diphenyl ether	4,2	
Phenanthrene	4,5	
n-Butylbenzene	4,6	
Fluoranthene	4,7	
Dibenzyl	4,8	
2,6-Diphenylpyridine	4,9	
Triphenylamine	5,7	
DDT	6,2	

Apparatus

14. A liquid-phase chromatograph, fitted with a pulse-free pump and a suitable detection device is required. The use of an injection valve with injection loops is recommended. The

presence of polar groups in the stationary phase may seriously impair the performance of the HPLC column. Therefore, stationary phases should have a minimal percentage of polar groups (13). Commercial microparticulate reverse-phase packings or ready-packed columns can be used. A guard column may be positioned between the injection system and the analytical column.

Mobile phase

15. HPLC-grade methanol and distilled water are used to prepare the eluting solvent, which is degassed before use. Isocratic elution should be employed. Methanol/water ratios with a minimum water content of 25% should be used. Typically a 3:1 (v/v) methanol-water mixture is satisfactory for eluting compounds with a log P of 6 within an hour, at a flow rate of 1 ml/min. For compounds with a log P above 6 it may be necessary to shorten the elution time (and those of the reference compounds) by decreasing the polarity of the mobile phase or the column length.

16. The test substance and the reference compounds should be soluble in the mobile phase in sufficient concentration to allow their detection. Additives may be used with the methanol-water mixture in exceptional cases only, since they will change the properties of the column. In these cases a separate column of the same type should be used. If methanol-water is not appropriate, other organic solvent-water mixtures can be used, e.g. ethanol-water, and acetonitrile-water.

17. The pH of the eluent is critical for ionisable compounds. It should be within the operating pH range of the column, usually between 2 and 8. Buffering is recommended. Care must be taken to avoid salt precipitation and column deterioration which occur with some organic phase/buffer mixtures. HPLC measurements with silica-based stationary phases above pH 8 are not advisable since the use of an alkaline mobile phase may cause rapid deterioration in the performance of the column.

Solutes

18. The test and reference compounds should be the purest available. Compounds to be used for test or calibration purposes are dissolved in the mobile phase if possible.

Test conditions

19. The temperature during the measurements should not vary by more than +/- 2 K.

Determination of dead time t_0

20. The dead time t_0 can be measured by using unretained organic compounds (e.g. thiourea or formamide). It can also be derived from the retention times measured for a set of approximately seven members of a homologous series (e.g. n-alkyl methyl ketones) (14). The retention times t_R (n_c+1) are plotted against t_R (n_c), where n_c is the number of carbon atoms. A straight line, t_R (n_c+1) = $At_R(n_c)$ + ($1-A)t_0$, is obtained, where A, representing $k(n_c+1)/k(n_c)$, is constant. The dead time t_0 is obtained from the intercept ($1-A)t_0$ and the slope A.

Calibration graph

21. The next step is to plot a correlation graph of log k versus log P for appropriate reference compounds with log P values near the value expected for the test substance. In practice, from 5 to 10 reference compounds are injected simultaneously. The retention times are determined, preferably on a recording integrator linked to the detection system. The corresponding logarithms of the capacity factors, log k, are calculated and plotted as a function of log P. The calibration is performed at regular intervals, at least once daily, so that account can be taken of possible changes in column performance.

Determination of the P_{ow} of the test substance

22. The test substance is injected in the smallest quantity possible. The retention time is determined in duplicate. The partition coefficient of the test substance is obtained by interpolation of the calculated capacity factor on the calibration graph. For very low and very high partition coefficients extrapolation is necessary. Especially in these cases attention must be given to the confidence limits of the regression line.

Report

23. The following should be included in the report:

- test and reference substances, and their purity;
- description of equipment and operating conditions: analytical column, guard column,
- mobile phase, means of detection, temperature range, pH;
- elution profiles;
- deadtime and how it was measured;
- quantities of test and references substances introduced in the column;
- retention data and literature log P values for reference compounds used in calibration;
- details on fitted regression line (log k versus log P);
- preliminary estimate of the partition coefficient and the method used; and if a calculation method was used, its full description including identification of the data base and detailed information on the choice of fragments;
- average retention data and interpolated log P value for the test substance.

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A N N E X

P_{ow} Calculation Methods

Introduction

This annex provides a short introduction to the calculation of P_{ow}. For further information the reader is referred to textbooks (1) (2).

Calculated values of P_{ow} are used for:
- deciding which experimental method to use: Shake Flask method for log P_{ow} between -2 and 4 and HPLC method for log P_{ow} between 0 and 6;
- selecting conditions to be used in HPLC (reference compounds, methanol/water ratio);
- checking the plausibility of values obtained through experimental methods;
- providing an estimate when experimental methods cannot be applied.

Principle of calculation methods

Calculation methods are based on the theoretical fragmentation of the molecule into suitable substructures for which reliable log P_{ow} increments are known. The log P_{ow} is obtained by summing the fragment values and the correction terms for intramolecular interactions. Lists of fragment constants and correction terms are available, [(1) to (6)]. Some are regularly updated (3).

Reliability of calculated values

In general, the reliability of calculation methods decreases as the complexity of the compound under study increases. In the case of simple molecules of low molecular weight and with one or two functional groups, a deviation of 0.1 to 0.3 log P_{ow} units between the results of the different fragmentation methods and the measured vaslue can be expected. The margin of error will depend on the reliability of the fragment constants used, the ability to recognise intramolecular interactions (e.g. hydrogen bonds) and the correct use of correction terms. In the case of ionising compounds the charge and degree of ionisation must be taken into consideration (10).

Fujita-Hansch π - method

The hydrophobic substituent constant, π, originally introduced by Fujita et al. (7) is defined as:

$$\pi X = \log P_{ow} (PhX) - \log P_{ow} (PhH),$$

where PhX is an aromatic derivative and PhH the parent compound

[e.g. $^\pi$Cl = log P_{ow} (C$_6$H$_5$Cl) - log P_{ow} (C$_6$H$_6$) = 2.84 - 2.13 = 0.71].

The π - method is primarily of interest for aromatic compounds. π - Values for a large number of substituents are available (4) (5).

Rekker method

Using the Rekker method (8) the log P_{ow} value is calculated as:

$$\log P_{ow} = \sum_i a_i \, f_i + \sum (interaction \ terms)$$

where a_i is the number at which a given fragment is present in the molecule and f_i is the log P_{ow} increment of the fragment. The interaction terms can be expressed as an integral multiple of one single constant C_m (so-called "magic constant"). The fragment constants f_i and C_m have been determined from a list of 1054 experimental P_{ow} values of 825 compounds using multiple regression analysis (6) (8). The determination of the interaction terms is carried out according to set rules (6) (8) (9).

Hansch-Leo method

Using the Hansch and Leo method (4), the log P_{ow} value is calculated as:

$$\log P_{ow} = \sum_i a_i \, f_i + \sum_j b_j \, F_j$$

where f_i is a fragment constant, F_j a correction term (factor), a_i and b_j the corresponding frequency of occurence. Lists of atomic and group fragmental values and of correction terms F_j were derived by trial and error from experimental P_{ow} values. The correction terms have been divided into several different classes (1) (4). Sofware packages have been developed to take into account all the rules and correction terms (3).

Combined method

The calculation of log P_{ow} of complex molecules can be considerably improved, if the molecule is dissected into larger substructures for which reliable log P_{ow} values are available, either from tables (3) (4) or by existing measurements. Such fragments (e.g. heterocycles, anthraquinone, azobenzene) can then be combined with the Hansch- π values or with Rekker or Leo fragment constants.

Remarks

i) The calculation methods are only applicable to partly or fully ionised compounds when the necessary correction factors are taken into account.

ii) If the existence of intramolecular hydrogen bonds can be assumed, the corresponding correction terms (approx. +0.6 to +1.0 log P_{ow} units) must be added (1). Indications on the presence of such bonds can be obtained from stereo models or spectroscopic data.

iii) If several tautomeric forms are possible, the most likely form should be used as the basis of the calculation.

iv) The revisions of lists of fragment constants should be followed carefully.

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OECD GUIDELINE FOR TESTING OF CHEMICALS

Adopted:
7 June 1984

"Alga, Growth Inhibition Test"

1. INTRODUCTORY INFORMATION

- **Prerequisites**

- Water solubility
- Vapour pressure

- **Guidance information**

- Structural formula
- Purity of the substance
- Chemical stability in water and light
- Methods of analysis for quantification of the substance in water
- pK_a value
- n-Octanol/water partition coefficient
- Results of a ready biodegradability test (see Test Guideline 301)

- **Qualifying statements**

- This guideline is suitable for a number of fresh-water green algae.

- This guideline is most easily applied to water-soluble substances which, under the conditions of the test, are likely to remain in the water.

- For substances with limited solubility in the test medium, it may not be possible to quantitatively determine the EC 50 (see Definitions, below).

- This guideline can be used for substances that do not interfere directly with the measurement of algal growth.

- **Standard documents**

See Section 4, Literature.

"Alga, Growth Inhibition Test"

2. M E T H O D

A. INTRODUCTION, PURPOSE, SCOPE, RELEVANCE, APPLICATION AND LIMITS OF TEST

The purpose of this test is to determine the effects of a substance on the growth of a unicellular green algal species. Relatively brief tests can assess effects over several generations. This guideline can be adapted for use with several unicellular algal species, in which case a description of the method used must be provided with the test report.

- **D e f i n i t i o n s**

Cell concentration is the number of cells per ml.

Growth is the increase in cell concentration over the test period.

Growth rate is the increase in cell concentration per unit of time.

EC 50 in this guideline is that concentration of test substance which results in a 50 per cent reduction in either growth or growth rate relative to the control.

NOEC (no observed effect concentration) in this guideline is the highest concentration tested at which the measured parameter(s) show(s) no significant inhibition of growth relative to control values.

- **R e f e r e n c e   s u b s t a n c e s**

A reference substance may be tested as a means of detecting unsatisfactory test conditions. If a reference substance is used, the results should be given in the test report. Potassium dichromate can be used as a reference substance.

- **Principle of the test method**

    Exponentially-growing cultures of selected green algae are exposed to various concentrations of the test substance over several generations under defined conditions. The inhibition of growth in relation to a control culture is determined over a fixed period.

- **Conditions for the validity of the test**

- The cell concentration in the control cultures should have increased by a factor of at least 16 within three days.

- Disappearance of the test substance from the water into the biomass does not necessarily invalidate the test.

B. DESCRIPTION OF THE TEST PROCEDURE

- **Preparations**

    Equipment

- Normal laboratory equipment.

- Test flasks of suitable volume (e.g. 250 ml conical flasks are suitable when the volume of the test solution is 100 ml).

- Culturing apparatus: a cabinet or chamber is recommended in which a temperature in the range 21 to 25°C can be maintained at ± 2°C and continuous uniform illumination provided with a quantum flux of 0.72×10^{20} photons/m²s ± 20 per cent* in the spectral range 400-700 nm.

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* A quantum flux of 0.72×10^{20} photons/m²s equals 120 µE/m²s. This quantum flux can be obtained with universal white-type fluorescent lamps [light-temperature of approximately 4200K] yielding approximately 8000 Lux measured with a spherical collector.

— Apparatus to determine cell concentrations, e.g. electronic particle counter, microscope with counting chamber, fluorimeter, spectrophotometer, colorimeter [Note: in order to provide useful measurements at low cell concentrations when using a spectrophotometer, it may be necessary to use cuvettes with a light path of at least 4 cm].

Algal medium

The following medium is recommended:

NH_4Cl	15	mg/l
$MgCl_2.6H_2O$	12	mg/l
$CaCl_2.2H_2O$	18	mg/l
$MgSO_4.7H_2O$	15	mg/l
KH_2PO_4	1.6	mg/l
$FeCl_3.6H_2O$	0.08	mg/l
$Na_2EDTA.2H_2O$	0.1	mg/l
H_3BO_3	0.185	mg/l
$MnCl_2.4H_2O$	0.415	mg/l
$ZnCl_2$	3×10^{-3}	mg/l
$CoCl_2.6H_2O$	1.5×10^{-3}	mg/l
$CuCl_2.2H_2O$	10^{-5}	mg/l
$Na_2MoO_4.2H_2O$	7×10^{-3}	mg/l
$NaHCO_3$	50	mg/l

The pH of this medium after equilibration with air is approximately 8.

The use of other media is not precluded by the above recommendation, provided, however, that following limits of essential constituents are respected:

P	≤ 0.7	mg/l
N	≤ 10	mg/l
chelators	$\leq 10^{-3}$	mmol/l
hardness (Ca + Mg)	≤ 0.6	mmol/l

The recommended medium and the medium given in reference (6) meet this requirement.

- ## Experimental organisms

Selection of species

It is suggested that the species of green algae used be a fast-growing species that is convenient for culturing and testing. The following species are considered suitable:

- *Selenastrum capricornutum* ATCC 22662
- *Scenedesmus subspicatus* 86.81 SAG
- *Chlorella vulgaris* CCAP 211/11b

If other species are used, the strain should be reported.

- ## Test design

Initial cell concentration

It is recommended that the initial cell concentration in the test cultures be approximately 10^4 cells/ml for *Selenastrum capricornutum* and *Scenedesmus subspicatus*. When other species are used the biomass should be comparable.

Concentrations of test substance

The concentration range in which effects are likely to occur is determined on the basis of results from range-finding tests. For the test, at least five concentrations arranged in a geometric series, should be selected. The lowest concentration tested should have no observed effect on the growth of the algae.

The highest concentration tested should inhibit growth by at least 50 per cent relative to the control and, preferably, stop growth completely.

Replicates and controls

The test design should include preferably three replicates at each test concentration and ideally twice that number of controls. If justified the test design may be altered to increase the number of concentrations and reduce the number of replicates per concentration.

When a vehicle is used to solubilise the test substance additional controls containing the vehicle at the highest concentration used in the test cultures should be included in the test design.

- Performance of the test

This section contains guidance for the testing of readily soluble and poorly soluble substances and of volatile substances.

(I) Testing readily water-soluble substances

Test cultures containing the desired concentrations of test substance and the desired quantity of algal inoculum are prepared by diluting with filtered algal medium aliquots of stock solutions of the test substance and of algal suspension.

The culture flasks are shaken and placed in the culturing apparatus. During the test it is necessary to keep the algae in suspension and to facilitate transfer of CO_2. To this end shaking, stirring or aeration may be used. The cultures should be maintained at a temperature in the range of 21 to 25°C, controlled at ±2°C.

The cell concentration in each flask is determined at least at 24, 48 and 72 hours after the start of the test. Filtered algal medium is used to determine the background when using particle counters or as a blank when using spectrophotometers.

The pH is measured at the beginning of the test and at 72 hours. The pH of the solutions should not normally deviate by more than one unit during the test.

(2)   Testing substances with limited water solubility

When the solubility of the test substance is of the order of the highest concentration used in the test, only slight deviations from the above procedure are necessary to make up the test solutions. A saturated solution may serve as the stock solution of the test substance. Another approach can be to dissolve the test substance at the desired concentration in the algal medium prior to the introduction of algal suspension.

Stock solutions of substances of low water-solubility may be prepared by mechanical dispersion or by the use of vehicles of low toxicity to algae, such as organic solvents, emulsifiers or dispersants. When a vehicle is used the concentration should not exceed 100 mg/l, and additional controls, in which the vehicle is incorporated at the highest concentration present in the test solutions, must be included in the test design.

(3)   Testing volatile substances

There is to date no generally accepted way to test volatile substances. When a substance is known to have a tendency to vaporise, closed test flasks with increased head-space may be used. Variations to this method have been proposed (see reference 11). Attempts should be made to determine the amount of the substance which remains in solution, and extreme caution is advised when interpreting results of tests with volatile chemicals using closed systems.

3. DATA AND REPORTING

• Treatment of results

The measured cell concentrations in the test cultures and controls are tabulated together with the concentrations of the test substance and the times of measurements.

The mean value of the cell concentration for each test substance concentration and for the controls is plotted against time to produce growth curves.

To determine the concentration effect relationship one of the following recommended approaches can be used.

(1) Comparison of areas under the growth curves

The area below the growth curves may be calculated according to the formula:

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

where

A = area

N_0 = nominal number of cells/ml at time t_0

N_i = measured number of cells/ml at t_i

N_n = measured number of cells/ml at time t_n

t_1 = time of first measurement after beginning of test

t_n = time of nth measurement after beginning of test