INTERNATIONAL STANDARD

ISO 9888

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Water quality — Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium — Static test (Zahn-Wellens method)

Qualité de l'eau — Évaluation, en milieu aqueux, de la biodégradabilité aérobie ultime des composés organiques — Essai statique (méthode Zahn-Wellens)

Reference number
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Contents

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

International Standard ISO 9888 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods.*

This second edition cancels and replaces the first edition (ISO 9888:1991), which has been technically revised.

Annex A of this International Standard is for information only.

Water quality — Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium — Static test (Zahn-Wellens method)

WARNING — Activated sludge and sewage may contain potentially pathogenic organisms. Take appropriate precautions when handling them. Handle with care toxic test compounds and those with unknown properties.

1 Scope

This International Standard specifies a method for the evaluation in aqueous medium of the ultimate biodegradability and, as additional information, the primary biodegradability and the total elimination from water, of organic compounds at a given concentration by aerobic microorganisms.

The conditions described in this International Standard normally correspond to optimal conditions for allowing the maximum value of biodegradation to occur with the chosen inoculum in the test time. These conditions may even be more favourable than in full-scale wastewater treatment plants, especially if their hydraulic retention time, sludge age or the adaptation of the activated sludge is not optimal.

The method applies to organic compounds which are

a) water-soluble at the concentration used under the test conditions and not expected to be transformed to insoluble metabolites if biodegradation and not elimination only shall be determined;

b) nonvolatile, or which have a negligible vapour pressure under the test conditions;

c) not lost by foaming from the test solution;

d) not inhibitory to the test microorganisms at the concentration chosen for the test. Inhibitory effects can be determined using a suitable test method (e.g. see ISO 8192). If the test compound is toxic, the test concentration must be lowered, or a pre-exposed inoculum can be used.

This International Standard is also applicable to the measurement of biodegradation and elimination of dissolved organic compounds in wastewater (also called "test compound" in the method).

NOTE If more information is required to predict the behaviour of test compounds or wastewater in a treatment plant, a simulation test (e.g. the activated sludge simulation test ISO 11733) should be performed. For appropriate use of this method and for alternative biodegradation methods, see ISO 15462.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 6060:1989, *Water quality — Determination of the chemical oxygen demand*.

ISO 8245, *Water quality — Guidelines for the determination of total organic carbon (TOC) and dissolved organic carbon (DOC).*

3 Terms and definitions

For the purposes of this International Standard, the following terms and definitions apply.

3.1
ultimate aerobic biodegradation
breakdown of a chemical compound or organic matter by microorganisms in the presence of oxygen to carbon dioxide, water and mineral salts of any other elements present (mineralization) and the production of new biomass

3.2
primary biodegradation
structural change (transformation) of a chemical compound by microorganisms resulting in the loss of a specific property

3.3
activated sludge
biomass produced in the aerobic treatment of wastewater by the growth of bacteria and other microorganisms in the presence of dissolved oxygen

3.4
concentration of suspended solids of an activated sludge
amount of solids obtained by filtration or centrifugation of a known volume of activated sludge and drying at about 105 °C to constant mass

3.5
total organic carbon
TOC
all that carbon present in organic matter which is dissolved and suspended in the water

3.6
dissolved organic carbon
DOC
that part of the organic carbon in the water which cannot be removed by specified phase separation

NOTE Phase separation may be specified for example by centrifugation at 40 000 $m \cdot s^{-2}$ for 15 min or by membrane filtration using membranes with pores of 0,2 µm to 0,45 µm diameter.

3.7
chemical oxygen demand
COD
mass concentration of oxygen equivalent to the amount of a specified oxidant consumed by a chemical compound or organic matter when a water sample is treated with that oxidant under defined conditions

NOTE COD is expressed in this case as milligrams oxygen consumed per milligram or gram of test compound.

3.8
lag phase
time from the start of a test until adaptation and/or selection of the degrading microorganisms are achieved and the biodegradation degree of a chemical compound or organic matter has increased to about 10 % of the maximum level of biodegradation

NOTE Lag phase is recorded in days.

3.9
maximum level of biodegradation
maximum degree of biodegradation of a chemical compound or organic matter in a test, above which no further biodegradation takes place during the test

NOTE Maximum level of biodegradation is recorded in percent.

3.10
biodegradation phase
time from the end of the lag phase of a test until about 90 % of the maximum level of biodegradation has been reached

NOTE Biodegradation phase is recorded in days.

3.11
plateau phase
time from the end of the biodegradation phase until the end of the test

NOTE Plateau phase is recorded in days.

3.12
pre-exposure
pre-incubation of an inoculum in the presence of the test chemical compound or organic matter, with the aim of enhancing the ability of this inoculum to biodegrade the test material by adaptation and/or selection of the microorganisms

3.13
preconditioning
pre-incubation of an inoculum under the conditions of the subsequent test in the absence of the test chemical compound or organic matter, with the aim of improving the performance of the test by acclimatization of the microorganisms to the test conditions

4 Principle

The biodegradation or elimination of water-soluble organic compounds or wastewater ingredients by aerobic microorganisms is determined using a static aqueous test system. The test mixture contains an inorganic medium, activated sludge as a mixed inoculum and an organic test compound as the sole source of carbon and energy other than the sludge. The amount of test compound added is chosen to result in an initial concentration of dissolved organic carbon (DOC) between 50 mg/l and 400 mg/l, or of chemical oxygen demand (COD) between 100 mg/l and 1000 mg/l, depending on its water solubility and on its toxicity to the bacteria in the inoculum.

Measurement of the concentration of DOC (or COD) is made at the beginning and end of the test (normally 28 d) and at intermediate time intervals, as required. To allow for any significant adsorption of the test compound onto the sludge, samples are also taken 3 h after the beginning of the test. Values obtained at this time are used as the basis for calculating the percentage of ultimate biodegradability at each sampling time. As additional information, the total elimination of DOC or COD from the aqueous phase may be obtained by calculating the removal based on a measured value before addition of the salts of the test medium and the inoculum. Possible other abiotic elimination processes, such as stripping into the air, may be determined by an abiotic elimination control without inoculum.

Use of specific analysis may give additional information on primary biodegradability of test compounds.

5 Test environment

Incubation shall take place in the dark or in diffuse light, at a temperature within the range 20 °C to 25 °C which shall not vary by more than 2 °C during the test and in an enclosure which shall be free from gases and vapours which are toxic to microorganisms.

6 Reagents

Use only reagents of recognized analytical grade, including the following.

6.1 Water, distilled or deionized, containing less than 1 mg/l DOC.

6.2 Test medium

6.2.1 Composition

6.2.1.1 Solution a)

Anhydrous potassium dihydrogenphosphate (KH_2PO_4)	8,5 g
Anhydrous dipotassium hydrogenphosphate (K_2HPO_4)	21,75 g
Disodium hydrogenphosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$)	33,4 g
Ammonium chloride (NH_4Cl)	0,5 g
Dissolve in water (6.1) to make up to	1000 ml

NOTE In order to check this buffer solution, it is recommended that the pH is measured. If it is not at about 7,4 a fresh solution should be prepared.

6.2.1.2 Solution b)

Dissolve 22,5 g magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) in water (6.1) to make up to 1000 ml.

6.2.1.3 Solution c)

Dissolve 36,4 g calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) in water (6.1) to make up to 1000 ml.

6.2.1.4 Solution d)

Dissolve 0,25 g iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) in water (6.1) to make up to 1000 ml. Prepare this solution freshly before use or add a drop of concentrated hydrochloric acid (HCl) to avoid precipitation.

6.2.2 Preparation of the test medium

1000 ml test medium shall contain 10 ml of solution a) and 1 ml of each of solutions b) to d). Add the correct amounts of the stock solutions as described in 8.3. Prepare the test medium freshly before use. The solutions a) to c) may be stored up to 6 months in the dark at room temperature.

NOTE If a test compound influences the pH value of the mixture at the chosen test concentration, an increase in the buffer capacity of the test medium may be required. At high test concentrations, a lack of nitrogen may also occur. In these cases it is recommended that the nutrient composition of the test medium be improved by adding, for example, 100 ml of solution a) instead of 10 ml or just the phosphate amount of solution a). The C:N:P ratio for a test concentration of 400 mg/l DOC is then changed from 100:0,3:30 to 100:3:300. As at higher nitrogen concentrations nitrification may occur, higher phosphate buffer concentrations are required to keep the pH stable.

6.3 Sodium hydroxide solution

Dissolve sodium hydroxide (NaOH) in the water (6.1) to obtain a solution of concentration 0,1 mol/l to 0,5 mol/l.

6.4 Hydrochloric acid solution

Dissolve hydrochloric acid (HCl) in the water (6.1) to obtain a solution of concentration 0,1 mol/l to 0,5 mol/l.

6.5 Mercury chloride solution

Dissolve 1 g of mercury(II) chloride ($HgCl_2$) in 100 ml of the water (6.1).

7 Apparatus

Ordinary laboratory equipment and the following shall be used

7.1 Glass vessels, of capacity 1 litre to 5 litres equipped with agitators with glass or metal stirrers; rotate to ensure adequate mixing.

Each vessel shall be fitted with 2 mm to 4 mm inner diameter glass tubes or glass frits to introduce air. The air shall be free from organic carbon and toxic vapours and shall be presaturated with water vapour to reduce losses by evaporation.

The glassware shall be carefully cleaned and, in particular, be free from traces of organic or toxic matter.

7.2 Measuring equipment, of sufficient sensitivity for the measurement of dissolved organic carbon (see ISO 8245) or the measurement of chemical oxygen demand (see ISO 6060) and, if required, for substance-specific analysis.

7.3 Centrifuge or device for filtration, with paper filters or membrane filters (of pore size/nominal aperture diameter 0,2 to 0,45 μm) which adsorb or release organic carbon to a minimum degree.

7.4 pH-meter (usual laboratory equipment).

8 Procedure

8.1 Preparation of the test solutions

8.1.1 Solution of the test compound in the water (6.1) or in the test medium (6.2.2), at a suitable concentration (e.g. 3 000 mg/l).

In the case of wastewater, determine the TOC, DOC and pH. The test is acceptable to the wastewater if the DOC is >90 % of the TOC, which indicates sufficiently water-soluble compounds for this method based on DOC measurement. Add the wastewater directly or dilute it with water (6.1) to obtain the desired test concentration. If the wastewater has an extreme pH value (<3 or >10), neutralize to pH 7 ± 1 with sodium hydroxide (6.3) or hydrochloric acid (6.4) solution.

8.1.2 Solution of the reference compound in the water (6.1) or in the test medium (6.2.2), at a suitable concentration (e.g. 3 000 mg/l). Use a known biodegradable water-soluble organic compound (e.g. diethylene glycol, ethylene glycol, sodium benzoate or aniline) which have degradation degrees >90 %).

8.2 Preparation of the inoculum

Take a sample of activated sludge from the aeration tank of a biological wastewater treatment plant. Mix the sample well and wash the activated sludge by repeatedly (e.g. two to three times) adding tap water or the test medium (6.2), centrifuging or settling, and decanting of the supernatant. Before use, determine the concentration of suspended solids (use e.g. ISO 11923). If necessary, concentrate the sludge by settling so that the volume of sludge added to obtain the desired concentration of suspended solids is minimal (about 10 % of the total volume). Keep the inoculum aerated at room temperature until just before use.

NOTE Depending on the purpose of the test, the wastewater treatment plant should receive wastewater which is predominantly municipal. Activated sludge may also be taken from a laboratory treatment plant. To get as many different species or strains of bacteria as possible, it may be preferable in special cases to make a mixture from various sources. Pre-exposed inocula may be used in certain circumstances. When such inocula are used, this should be clearly stated in the test results (e.g. percentage biodegradation using pre-exposed inocula) and the method of pre-exposure detailed in the test report. Pre-exposed inocula can be obtained from laboratory biodegradation tests conducted under a variety of conditions as appropriate. Suitable tests are e.g. a Zahn-Wellens test (this test, ISO 9888) already performed with the test compound (see 8.3) or the SCAS test (ISO 9887). Inocula may also be collected from locations where relevant environmental conditions exist (e.g. wastewater treatment plants dealing with similar compounds).

8.3 Test

Provide a sufficient number of glass vessels (7.1) in order to have:

— at least 1 vessel (symbol F_T) for the test compound (8.1.1);

— at least 1 blank vessel (symbol F_B) containing test medium and inoculum;

— at least 1 vessel, for checking the procedure (symbol F_C) containing the reference compound (8.1.2);

— if needed, 1 vessel for checking a possible abiotic elimination (symbol F_S) containing the test compound (8.1.1) but no inoculum, sterilized e.g. by addition of a suitable inorganic toxic compound to prevent microbial activity.

NOTE Use, for example, 1 ml/l of the mercury(II) chloride solution (6.5). Add the same amount of the toxic substance two weeks after the start of the test.

Use suitable glass vessels to obtain a final test volume of, for example, 3 l. Other final test volumes are possible, adapt in such a case all relevant parameters and the calculation of test results. The total volume to be chosen is dependent on the number of samples to be taken for DOC or COD determination and the volumes necessary for the analytical procedure. Prepare the test mixtures as indicated in Table 1.

Add about 2/3 of the required test water (6.1) to the vessels. Then add the test compound solution or the wastewater (8.1.1) to obtain a DOC concentration of 50 mg/l to 400 mg/l or a COD concentration of 100 mg/l to 1000 mg/l in the final mixture to the respective vessels F_T. Take a sample (time t_0) and determine the DOC or COD concentration. Relate the measured value to the final test volume and use this value to calculate the total elimination based on t_0 [see equation (2) in 9.1].

Add the solutions 6.2.1 in the required amount to obtain the test medium in the final test volume. Measure the pH value and adjust if necessary to pH $7 \pm 0{,}5$ with an inorganic acid (see 6.4) or alkaline solution (see 6.3). Add activated sludge (8.2) as the inoculum. Adjust the sludge concentration to the initial concentration of the test compound. For 50 mg/l DOC use 0,2 g/l of suspended solids, and for 400 mg/l DOC use 1 g/l in the final mixture. For concentrations between 50 mg/l and 400 mg/l use sludge concentrations in between. Fill with the water (6.1) to the final volume and mix the content of the vessels separately.

Table 1 — Final distribution of test and reference compounds

Vessel	**Test medium** (6.2)	**Test compound** (8.1.1)	**Reference compound** (8.1.2)	**Inoculum** (8.2)
Test compound F_T	+	+	–	+
Blank F_B	+	–	–	+
Inoculum check F_C	+	–	+	+
Abiotic elimination check F_S (optional)	+	+	–	–

Set up the blank vessel F_B and the reference compound (8.1.2) in vessel F_C to operate in parallel with each test series. F_B contains only the inoculum with the same concentration of suspended solids and the same total volume as the test vessel. The reference compound shall be tested at the same concentration as the test compound.

If the test compound can be eliminated by abiotic processes, especially by air stripping, set up a vessel for abiotic elimination control (F_S) (optional), use no inoculum and add 10 ml/l of the mercury(II) chloride solution (6.5) or another suitable inorganic toxic compound.

To start the test, agitate the vessels using stirrers, aerate and incubate at the desired test temperature (see clause 5). Throughout the test, ensure that the sludge is well aerated and does not settle. In order to compensate for water losses by evaporation, check the volume of the medium in the vessels before each sampling and, if necessary,

make up with the water (6.1) to the volume or mass measured after the preceding sampling. Check the pH value at regular intervals, especially if the sludge nitrifies (e.g. when a sample for analysis is taken) and adjust to pH 7,0 ± 0,5 if necessary.

Take minimum sample volumes of the test mixtures, centrifuge at about 40 000 m/s^2 for 15 min or filter these portions through a carefully washed paper filter (7.3). Instead of paper filters, especially if the filtrate is not clear, filter the samples through membrane filters (7.3). Measure the DOC or COD concentrations in these samples at least in duplicate. If primary biodegradation is to be followed, use substance-specific analysis

Perform all the analyses as soon as possible. When measurements have to be postponed for up to 48 h, keep the samples at 4 °C in the dark and in tightly stoppered bottles. If the samples have to be stored for more then 48 h, add either 20 ml/l of the mercury(II) chloride solution (6.5) or another inorganic toxic substance, to prevent microbial activity and store at 4 °C. If chloride ions are added, the COD measurements at low concentrations have to be performed with special care. Instead of adding a toxic substance, store the samples at −18 °C.

Take samples at (3 ± 0,5) h after starting the test (time t_1), on two consecutive measurements at the end of the test (normally d 27 and d 28) and on at least at three intermediate time intervals (e.g. 7 d, 14 d and 21 d). Usually the maximum test period shall not exceed 28 d. If a sufficient level of DOC removal is attained, the plateau phase is reached and no further elimination is expected, the test can be considered to be completed. If degradation has obviously started but has not reached a plateau, extend the test by one to two weeks until this is the case.

If biodegradation with pre-exposed inoculum is to be determined, the test may be repeated using the existing inoculum from a preceding test. In this case, wash the remaining activated sludge, mix if necessary with fresh sludge to obtain the required concentration of suspended solids, and repeat the test.

9 Calculation and expression of results

9.1 Calculation

Calculate the percentage of DOC or COD removal in each vessel using equation (1). Use the concentration measured at t_1 (3 ± 0,5) h to determine biodegradation D_t. This value, obtained after addition of the salts of the test medium (6.2.1), neutralization and addition of the inoculum, considers the amount eliminated by adsorption as after this time adsorption and desorption are in most cases in equilibrium.

$$D_t = \left(1 - \frac{\rho_{cTt} - \rho_{cBt}}{\rho_{cT1} - \rho_{cB1}}\right) \times 100 \qquad (1)$$

where

ρ_{cT1} is the DOC concentration, in milligrams per litre, at time t_1 in vessel F_T;

ρ_{cB1} is the DOC concentration, in milligrams per litre, at time t_1 in vessel F_B;

ρ_{cTt} is the DOC concentration, in milligrams per litre, at time t in vessel F_T;

ρ_{cBt} is the DOC concentration, in milligrams per litre, at time t in vessel F_B.

Use the same equation if COD was measured. Calculate in the same way the biodegradation of the reference compound F_C and, if included, of the abiotic elimination check F_S (without considering ρ_{cB1} and ρ_{cBt}).

In the case of adsorbing substances the DOC or COD concentration at (t_1) after (3 ± 0,5) h can be significantly (>20 %) less than the value at time t_0. In this case, the static test cannot differentiate between biodegradation and adsorption. Calculate then, or as additional information, the total elimination D_e using equation (2).

$$D_e = \left(1 - \frac{\rho_{cTt} - \rho_{cBt}}{\rho_{cT0}}\right) \times 100 \qquad (2)$$

where

ρ_{cT0} is the DOC concentration, in milligrams per litre, at time t_0 in vessel F_T.

When specific analyses of the test compound are performed, calculate the percentage of primary biodegradation D_S of the test compound compared to the amount in vessel F_S using equation (3).

$$D_S = \frac{\rho_S - \rho_T}{\rho_S} \times 100 \quad (3)$$

where

ρ_T is the concentration of the test compound, in milligrams per litre, in vessel F_T at time t;

ρ_S is the concentration of the test compound, in milligrams per litre, in vessel F_S at time t.

9.2 Expression of results

Compile a table of measured values and the percentages of elimination for each measuring interval and each test vessel. Plot a biodegradation curve based on D_t and D_s and/or an elimination curve based on D_e in percent as a function of time (see example annex A). Indicate lag phase and degradation phase in days. Plot in the same way a biodegradation curve of the reference compound F_C and, if included, an elimination curve of the abiotic elimination check F_S.

Determine the mean value of percent biodegradation (or elimination, see 9.3) in the plateau phase or use the highest value, e.g. when the curve decreases in the plateau phase, and indicate this maximum level of biodegradation or elimination as "degree of biodegradation (or elimination) of the test compound" in the test report.

9.3 Indication for biodegradation

If the result of analysis of the first sample (t_1) after ($3 \pm 0,5$) h is significantly different from the value t_0, report the amount of deficient DOC or COD as "adsorbed by the activated sludge in the static test". If in vessel F_S (abiotic elimination check if included) a significant amount (>20 %) of DOC or COD loss is observed, further abiotic elimination processes may have taken place.

If the adsorption is low (e.g. less than 20 %), no significant abiotic elimination has taken place (e.g. less than 20 % in vessel F_S), a typical biodegradation curve with lag, degradation and plateau phase is obtained (see example annex A) or other information on the biodegradability of the test compound is available, assign the measured elimination of the test compound to biodegradation. If high initial adsorption takes place, the static test cannot differentiate between biological and abiotic elimination processes.

NOTE To obtain definitive information on biodegradability for test compounds in such an unclear case, it is recommended to perform a test based on oxygen consumption, for example, the respirometer test ISO 9408 or on carbon dioxide production such as ISO 9439 or any other suitable test (see ISO/TR 15462). In such a case, the adapted inoculum of this static test (ISO 9888) should preferably be used, where available.

10 Validity of results

The test is considered valid if the percentage degradation in vessel F_C (inoculum check) is greater than 70 % on the 14th day. If this is not fulfilled, the test should be repeated, for example using another inoculum.

11 Test report

The test report shall contain at least the following information:

a) a reference to this International Standard;

b) all necessary information for the identification of the test compound or the wastewater;

c) the concentration of the test compound in the test vessel and the DOC or COD content of this concentration;

d) the source, the concentration of suspended solids and any pretreatment of the activated sludge;

e) the incubation temperature of the test;

f) the analytical parameter used (DOC, COD, substance specific analysis), and the method of determination;

g) all the measured and calculated data (for example in tabular form) obtained and the degradation curve of the test compound;

h) the amount of biodegradation, total elimination and adsorption, expressed as a percentage, of the test compound;

i) the name of the reference compound used, the degradation curve and percentage obtained with this compound;

j) the amount of abiotic elimination in vessel F_S, if it was included;

k) the reasons in the event of rejection of the test;

l) any alteration of the standard procedure or any circumstances that may have affected the results.

Annex A
(informative)

Example of a biodegradation curve

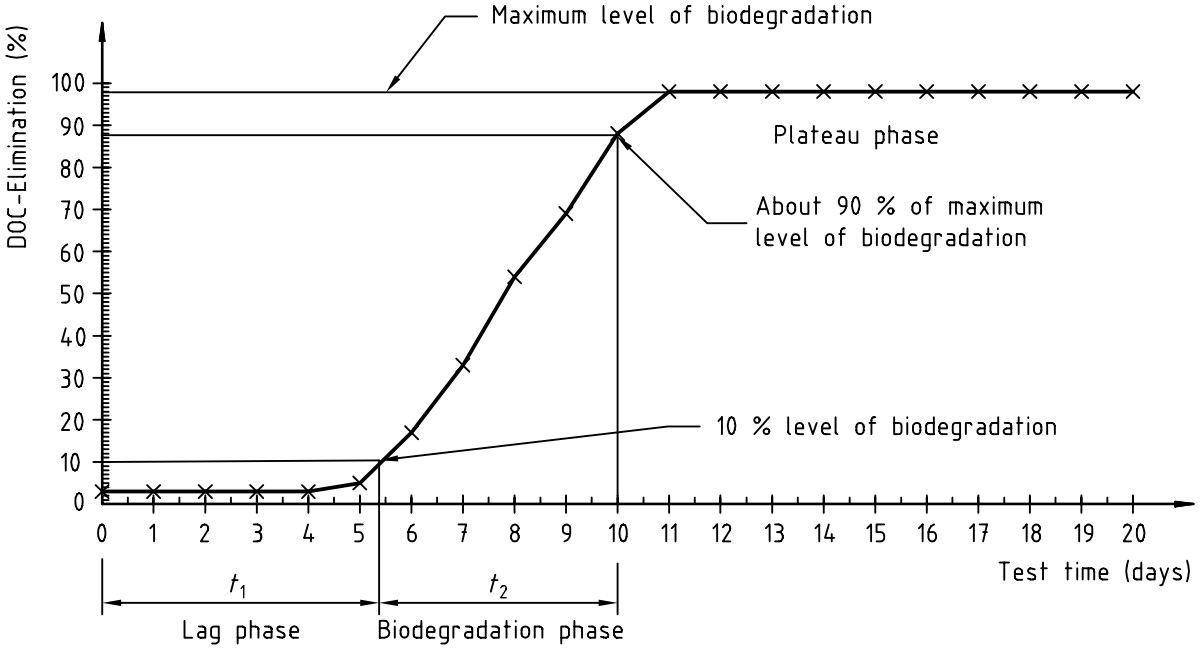

Figure A.1 — Biodegradation of diethylene glycol in the static test

Bibliography

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[2] ISO 8192:1986, *Water quality — Test for inhibition of oxygen consumption by activated sludge*.

[3] ISO 9408, *Water quality — Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium by determination of oxygen demand in a closed respirometer*.

[4] ISO 9439, *Water quality — Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium — Carbon dioxide evolution test*.

[5] ISO 9887, *Water quality — Evaluation of the aerobic biodegradability of organic compounds in an aqueous medium — Semi-continuous activated sludge method (SCAS)*.

[6] ISO 11733:1995, *Water quality — Evaluation of the elimination and biodegradability of organic compounds in an aqueous medium — Activated sludge simulation test.*

[7] ISO 11923:1997, *Water quality — Determination of suspended solids by filtration through glass-fibre filters.*

[8] ISO/TR 15462:1997, *Water quality — Selection of tests for biodegradability.*

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