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Water quality — Determination of the inhibitory effect of water constituents on the growth of activated sludge microorganisms

Qualité de l'eau — Détermination de l'effet inhibiteur des constituants de l'eau sur la croissance des micro-organismes de boues activées

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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 15522 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Annexes A, B and C of this International Standard are for information only.

Introduction

Information generated by this method may be helpful in estimating the effect of a test material on mixed bacterial communities in aerobic biological wastewater treatment systems and in choosing suitable initial concentrations for aerobic biodegradability tests

The results of this test should be considered only as a guide to the likely toxicity of the test material, since activated sludge from different sources, or even from the same source taken at different times, may differ in bacterial composition and concentration. Also, laboratory tests cannot truly simulate environmental conditions. For example, no account is taken of longer term adaptation of the microorganisms to the test material or of materials which may adsorb onto biofilm or activated sludge in subsequent wastewater treatment and build up to a toxic concentration over a longer period of time.

Water quality — Determination of the inhibitory effect of water constituents on the growth of activated sludge microorganisms

WARNING — Take appropriate precautions when handling sewage, as it may contain potentially pathogenic organisms. Handle with care all toxic test materials or those whose properties are unknown.

1 Scope

This International Standard specifies a method for assessing the potential toxicity of a test material to the growth of aerobic bacteria present in activated sludge. The inhibitory effect is restricted to those microorganisms capable of growth on the chosen organic test medium.

This method gives information on inhibitory effects on the microorganisms over incubation periods up to 6 h.

This method is applicable to water, wastewater and chemical substances which are soluble under the conditions of the test. Special care is needed with volatile or coloured materials and materials which form turbid suspensions or dispersions.

NOTE 1 Results with volatile test material should be interpreted with caution and are likely to underestimate any inhibitory effect because of the difficulties in maintaining the initial concentration in the test flasks.

NOTE 2 Coloured materials and materials of low water-solubility which form turbid suspensions or dispersions can be tested in many cases using colour/turbidity controls as blanks (see 9.4).

NOTE 3 Inhibitory effects on activated sludge microorganisms of test materials for which this test is not applicable can be determined by using an inhibition test based on respirometric measurements (see ISO 8192).

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 5667-16, *Water quality — Sampling — Part 16: General guidance for the biotesting of water, waste water, and water ingredients — Sampling, pretreatment, performance and evaluation.*

ISO 8192, *Water quality — Test for inhibition of oxygen consumption of activated sludge.*

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

3 Terms and definitions

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

3.1
growth
increase in the number of microbial cells during the test period, assessed by measurement of the biomass of a culture

NOTE Biomass can be determined by any suitable method, for example as turbidity [optical density (OD) or absorbance] with a spectrophotometer at 530 nm and expressed in relative turbidity units (OD_{530}).

3.2
growth curve
measured biomass growth graphically plotted against the incubation time

3.3
specific growth rate
μ
doubling of biomass (x) per time unit (t)

$\mu = 1/x \cdot dx/dt$

NOTE Growth rate is usually expressed in reciprocal hours (h^{-1}).

3.4
growth inhibition
difference in the growth at the end of the incubation time in the presence of organic test medium and test material, compared with that in a similar mixture without test material

NOTE Growth inhibition is expressed as a percentage.

3.5
inhibition curve
growth inhibition, in percent, plotted against the logarithm of the test material concentration

3.6
effective concentration
EC value
concentration of the test material giving a calculated growth inhibition, or interpolated from the inhibition curve, of 50 % (EC_{50}), 20 % (EC_{20}) or 80 % (EC_{80}) compared with that of a similar mixture without test material

4 Principle

Flasks containing organic test medium and test material are inoculated with an overnight culture of activated sludge microorganisms and incubated on a shaking device at 22 °C ± 2 °C. The total test duration is normally 6 h, including an exposure time of about 4,5 h. The biomass of these cultures and of blank controls without test material is determined using an appropriate method. Measurement of turbidity in a spectrophotometer at a wavelength of 530 nm and expression in relative units (OD_{530}) is recommended. The growth inhibition, in percent, at the end of incubation is calculated by comparison with blank controls and is plotted, for example in a semilogarithmic curve against the test material concentration, to derive the EC values. The sensitivity of the activated sludge microorganisms can be checked against a reference substance (see 6.7).

5 Test environment

Incubation shall take place in the dark or in diffuse light in an enclosure which is maintained at 22 °C ± 2 °C and which is free from vapours toxic to microorganisms.

6 Reagents

Use only reagents of recognized analytical grade.

6.1 Distilled or deionized water.

6.2 Organic 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
Water (6.1) quantity necessary to make up to	1 000 ml

In order to check this buffer solution, measurement of the pH, which should be about 7,4, is recommended. If this is not the case, prepare a new solution.

6.2.1.2 Solution B

Dissolve 22,5 g of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) in 1 000 ml of water (6.1).

6.2.1.3 Solution C

Dissolve 36,4 g of calcium chloride dihydrate ($CaCl_2 \cdot H_2O$) in 1 000 ml of water (6.1).

6.2.1.4 Solution D

Dissolve 0,25 g of iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) in 1 000 ml of water (6.1). Prepare this solution freshly before use.

NOTE It is not necessary to prepare this solution just before use if a drop of concentrated hydrochloric acid (HCl) is added.

6.2.1.5 Solution E

The addition of the following trace elements to improve aerobic growth is recommended.

Boric acid, H_3BO_3	50 mg
Cobalt chloride hexahydrate, $CoCl_2 \cdot 6H_2O$	50 mg
Manganese sulfate monohydrate, $MnSO_4 \cdot H_2O$	15 mg
Disodium molybdate dihydrate, $Na_2MoO_4 \cdot 2H_2O$	15 mg
Nickel chloride hexahydrate, $NiCl_2 \cdot 6H_2O$	10 mg
Zinc sulfate heptahydrate, $ZnSO_4 \cdot 7H_2O$	50 mg

Dissolve reagents in 1 000 ml of water (6.1)

6.2.1.6 Solution F (organic substrate)

Dissolve 80 g of dry extract of nutrient broth (commercially available mixture of beef extract and peptone) and 60 g of sodium acetate in 1 000 ml of water (6.1), or use the organic components of the synthetic sewage in accordance with ISO 11733.

6.2.2 Preparation

For 1 000 ml of test medium, add to about 800 ml of the water (6.1):

— 10 ml of solution A;

— 1 ml of each of solutions B to E (solution E is optional, but recommended);

— 25 ml of solution F.

Make up to 1 000 ml with the water (6.1).

6.3 Sodium azide solution (optional).

Dissolve 100 g of sodium azide (NaN_3) in 1 000 ml of water (6.1); this solution is used as a preservative.

6.4 Sodium hydroxide solution.

Dissolve 40 g sodium hydroxide (NaOH) in 1 000 ml of water (6.1); this solution is used for pH adjustment.

6.5 Sulfuric acid solution.

Dissolve 98 g of sulfuric acid (H_2SO_4) in 1 000 ml of water (6.1); this solution is used for pH adjustment.

6.6 Solution of test material.

Use water and wastewater samples directly or, if necessary, appropriately diluted. Dissolve a suitable amount of a water-soluble test material, e.g. 1 000 mg in 1 000 ml of water (6.1) as a stock solution. Determine the pH of the stock solution and the wastewater and adjust to pH 7,0 ± 0,2 with solutions 6.4 or 6.5 if necessary. Do not adjust the pH if the acid or alkaline effect is to be determined. For preparation see also ISO 5667-16. Samples with significant turbidity may influence the determination of turbidity. In this case use ISO 8192.

6.7 Stock solution of reference substance.

Dissolve 1 000 mg of 3,5-dichlorophenol in 1 000 ml of water (6.1) and adjust to pH 7,0 ± 0,2 with solutions 6.4 or 6.5 if necessary.

7 Inoculum

Collect activated sludge from the aeration basin of a wastewater treatment plant. Normally, wastewater treatment plants treating predominantly domestic sewage are sampled. If possible inhibitory effects shall not be determined for general purpose, but rather for a specific plant, using sludge from this plant. Let the sludge flocs settle for about 15 min, or longer if required, and use the supernatant for inoculation. Use the supernatant fresh or if necessary store up to 24 h at about 4 °C.

8 Apparatus

Ensure that all glassware is thoroughly cleaned and, in particular, free from organic or toxic matter. Usual laboratory equipment is required and the following apparatus.

8.1 Test flasks, such as 1 000 ml and 100 ml Erlenmeyer flasks, preferably with one baffle, closed e.g. with cotton plugs.

8.2 Shaking device for Erlenmeyer flasks, with a shaking speed of about 150 r/min.

8.3 Room with constant temperature or incubator at 22 °C ± 2 °C.

8.4 UV/visible spectrophotometer and matched cells, preferably with 1 cm or 4 cm optical pathlength, or any other instrument to determine biomass.

8.5 pH meter.

9 Procedure

9.1 Test design

Various experimental designs may be applied (see annex A). In the case of a water-soluble test material, for example, use

a) a preliminary test with test material at usually 1 mg/l, 10 mg/l and 100 mg/l and controls, to estimate the range of concentrations needed for the definitive test;

b) a definitive test using at least five concentrations in a geometric series in the expected toxicity range and controls. To establish concentrations use information from the preliminary test.

c) a test using only one concentration and controls to demonstrate that no toxic effect is to be expected up to the chosen concentration. Use, as a realistic upper concentration, 100 mg/l test material.

9.2 Test and control flasks

Prepare a sufficient number of labelled 100 ml Erlenmeyer flasks in order to have:

— at least two test flasks, F_T, for each concentration of test material (6.6);

— at least two flasks, F_R, for each desired concentration of the reference substance (6.7);

— at least two flasks, F_B, as blank controls without test material;

— at least one flask, F_C, as a colour/turbidity control at each test concentration, containing organic test medium and test material but no inoculum. Use this as a control only if coloured or turbid materials are tested.

NOTE If the measured data are to be treated statistically, more replicate flasks and controls will be required. For general details see ISO 5667-16.

9.3 Preculture

About 16 h to 20 h prior to commencement of the test, prepare a preculture of sufficient volume to ensure that there will be enough biomass to start the main culture. Use a concentration of inoculum (clause 7) of 0,5 ml per 20 ml organic test medium (6.2) in Erlenmeyer flasks (8.1). If no experience of the quality of the inoculum (clause 7) is yet available, inoculate some flasks with lower and some with higher volumes of the inoculum. Close the flasks, for example with cotton plugs, place them on the shaker and incubate for 16 h to 20 h at a shaking speed of about 150 r/min at the incubation temperature (clause 5).

After the preculture period, take a sample from each flask and measure the biomass (9.4). Cultures having turbidities of $> 0{,}6$ in cells with 1 cm optical pathlength or $> 1{,}5$ in cells with 4 cm optical pathlength will have a sufficiently high population growing in the logarithmic phase to be used as a source of inoculum in the ensuing test.

9.4 Measurement of biomass

Use for the measurement of growth any suitable technique of biomass determination. Measurement of turbidity (optical density, absorbance) in a spectrophotometer at a wavelength of 530 nm or at any other suitable wavelength is recommended. Express the results in relative turbidity units (OD_{530}). Take, for example in the case of turbidity measurement, 1 ml sample for 1 cm cuvettes and up to 5 ml for 4 cm cuvettes from the flasks and transfer it to appropriate storage vials containing 20 µl of the sodium azide solution (6.3) and mix thoroughly. These samples may be stored up to 24 h at room temperature until measurement. Measure turbidity in the cuvettes against non-inoculated fresh organic test medium (6.2) or, in the case of turbid or coloured test materials, against the corresponding control F_C (9.2) containing 20 µl of the sodium azide solution (6.3) as well.

NOTE 1 Turbidity can also be determined using techniques described in ISO 7027, for example, in formazine nephelometric units (FNU). Investigations have shown that there is a direct correlation between turbidity and biomass expressed as bacterial cell concentration.

NOTE 2 The addition of the sodium azide solution may be omitted when the measurement of biomass is performed immediately after taking of the samples. Addition is however strongly recommended, as it allows a collection of the samples and a combined measurement at the end of the test.

9.5 Preparation of main culture

For a main culture, prepare a sufficient amount of organic test medium (6.2) (experience has shown that in most cases 400 ml are sufficient) in a 1 000 ml Erlenmeyer flask, inoculate with 3 ml per 100 ml test medium of a well-grown preculture (9.3) and incubate under the same conditions as indicated for the precultures. Withdraw samples at regular intervals and determine the biomass (9.4). After 1 h to 3 h of incubation, the main culture usually reaches the early exponential growth phase (e.g. turbidity of 0,1 to 0,15 OD_{530} in 1 cm optical pathlength cells). At this time divide the main culture and continue as described in 9.6.

9.6 Test procedure

Add to the prepared flasks (9.2 and annex A) 20 ml of the pre-incubated main culture (9.5) and the desired concentrations of test material and reference substance. Make up with water (6.1) to 25 ml volume and take a sample for determination of biomass (see 9.4). Use sufficient test volume in the case of 4 cm optical pathlength cuvettes for turbidity measurements. Continue the incubation of the microorganisms exposed to the test material. Withdraw samples for biomass determination at regular intervals (for example each hour) or at least at the end of the incubation period. The total incubation period should not exceed 6 h to guarantee an exponential growth of the bacteria during the test period. The exposure time is less (about 4,5 h).

It is advised to determine growth inhibition by measuring the biomass at the end of the incubation period. It is recommended that biomass determinations be made at regular intervals, for example each hour, to obtain growth curves. The shape of a growth curve may give useful information about the irregularities in growth and the character of inhibition processes. In the case of some toxic chemicals, growth curves may show an initial lag phase followed by an increase in growth rate, indicating a rapid acclimatization or tolerance by some of the test organisms towards the test material.

It is recommended to determine in pretests the growth of the inoculum in the blank control, and to conclude from the measured data the appropriate duration of pre-exposure and total test duration to ensure exponential growth within the test.

10 Expression of results

10.1 Growth curves

The test result is calculated at the end of the exponential growth period. To obtain information on the normal growth of the microorganisms, plot the logarithm of biomass measurements [e.g. optical density, OD_{530} (see 9.4)] against time for each concentration of test substance, the blanks and the reference substance (see 9.2), using mean values to obtain growth curves. An example of typical growth curves is given in annex B.

10.2 Calculation of growth inhibition

Calculate the percentage of growth inhibition, I, at each concentration as follows:

$$I = \frac{B_c - B_t}{B_c - B_a} \times 100$$

where

B_c is the mean value of measured turbidity (OD_{530}) at the end of the incubation period in the blank control flasks F_B;

B_t is the mean value of measured turbidity (OD_{530}) at the end of the incubation period in the test flasks F_T;

B_a is the mean value of measured turbidity (OD_{530}) when the main culture is divided and the test material added to the flasks F_T.

Use the data from the preliminary and definitive tests to plot percentage inhibition against the logarithm of the concentration of test material, to obtain an inhibition curve and to calculate or interpolate from the graph the EC_{50} as that concentration which inhibits the growth by 50 % compared with the blank control. If sufficient data are available, the 95 % confidence limit of the EC_{50} and additional EC values can be determined. EC_{20} marks the beginning and EC_{80} the end of the inhibition range.

Measure in the same way the growth inhibition by the reference substance in flasks F_R and determine the EC values.

In view of the variability often observed in the results, it may in many cases be sufficient for the results to be expressed in orders of magnitude, e.g.:

EC_{50} < 1 mg/l

1 mg/l to 10 mg/l

10 mg/l to 100 mg/l

$>$ 100 mg/l.

In other cases, it may be sufficient to show that no toxic effects ($< EC_{20}$) are observed up to a realistic upper concentration (e.g. 100 mg/l), or the lowest ineffective dilution (LID) may be expressed, in accordance with annex C.

11 Interpretation of results

The results from this test can be used to select a concentration of test material for use in biodegradability tests. A suitable concentration would be that at which no growth inhibition occurs ($< EC_{20}$). The results also give an indication of the likely effect of a test material on biological sewage treatment processes. Even so, because of possible adsorption effects and possible reactions with other chemicals present in sewage, as well as biodegradation after a period of acclimatization, a final judgement of the effects of chemicals on sewage treatment cannot be made until a simulation test has been carried out.

The sensitivity of the activated sludge microorganisms is checked by the reference substance. The EC_{50} of 3,5-dichlorophenol should lie in the range 4 mg/l to 12 mg/l. Sufficient biomass should have been available. If biomass in the blank control flasks F_B (see 9.2) was determined by turbidity measurement in cells of 1 cm optical pathlength, an end value of at least 0,8 OD_{530} (see 9.4) should be observed at the end of the incubation period.

If this is not the case, the test should be repeated with an inoculum from another source.

12 Reproducibility

An international ring test with 23 participating laboratories was carried out in 1995 based on the test described in this International Standard (see [5]). The results obtained with the substances 3,5-dichlorophenol and potassium cyanide are shown in Table 1. All data were considered which were obtained according to the ring test procedure (identical to this International Standard) and which fulfilled the validity criteria.

Table 1 — Results of a ring test

Test material	Parameter	No. of results	Mean values	Standard deviation	Coefficient of variation
3,5-Dichlorophenol	EC_{20} (mg/l)	29	4,2	1,5	36 %
	EC_{50} (mg/l)	33	8,1	2,4	29 %
	EC_{80} (mg/l)	29	15,9	5,2	33 %
Potassium cyanide	EC_{20} (mg/l)	18	5,5	3,4	62 %
	EC_{50} (mg/l)	17	12,3	5,3	43 %
	EC_{80} (mg/l)	13	27,7	13,6	49 %
Growth parameters of the blanks	Specific growth rate (h^{-1})	69	0,56	0,19	34 %
	End turbidity (OD_{530})	69	1,04	0,47	45 %

13 Test report

The test report should contain at least the following information:

a) a reference to this International Standard;

b) all necessary information for identification of the test material;

c) source, and any pretreatment of the activated sludge microorganisms;

d) test temperature and measured pH values;

e) biomass at the end of the incubation period of the blank control;

f) name of the reference substance and the EC_{50};

g) measured data of biomass, the growth curves, the inhibition curve, the EC_{50} and, if possible, EC_{20} and EC_{80} and statistical data, if required, or the maximum concentration without inhibition of the test material;

h) all observations and deviations from the standard procedure which could have influenced the results.

Annex A
(informative)

Examples of contents of test and control flasks

A.1 Preliminary test

Flask	Symbol	Content	Test material/ ref. subst. concentration mg/l	Test material/ ref. subst. stock solution[a] ml	Inoculated test medium (9.5) ml	Uninoculated test medium (6.2) ml	Water (6.1) ml	Total volume ml
1 2	F_T F_T	test material	1	0,025	20	0	5	25
3 4	F_T F_T	test material	10	0,25	20	0	4,75	25
5 6	F_T F_T	test material	100	2,5	20	0	2,5	25
7 8	F_R F_R	reference substance	5	0,125	20	0	5	25
9 10	F_B F_B	blank control	0	0	20	0	5	25
11	F_C	colour/ turbidity control	1	0,025	0	20	5	25
12	F_C	colour/ turbidity control	10	0,25	0	20	4,75	25
13	F_C	colour/ turbidity control	100	2,5	0	20	2,5	25

[a] The concentration of the stock solution of the test material and the reference substance is in this example 1 g/l (see 6.6 and 6.7). Other stock solutions and volumes may be chosen as well.

A.2 Definitive test

For a definitive test, use for F_T and F_C the following concentrations:

2 mg/l; 4 mg/l; 8 mg/l; 16 mg/l; 32 mg/l;

and in addition the flasks or data of the preliminary test.

Annex B
(informative)

Typical growth curve using 3,5-dichlorophenol as test material

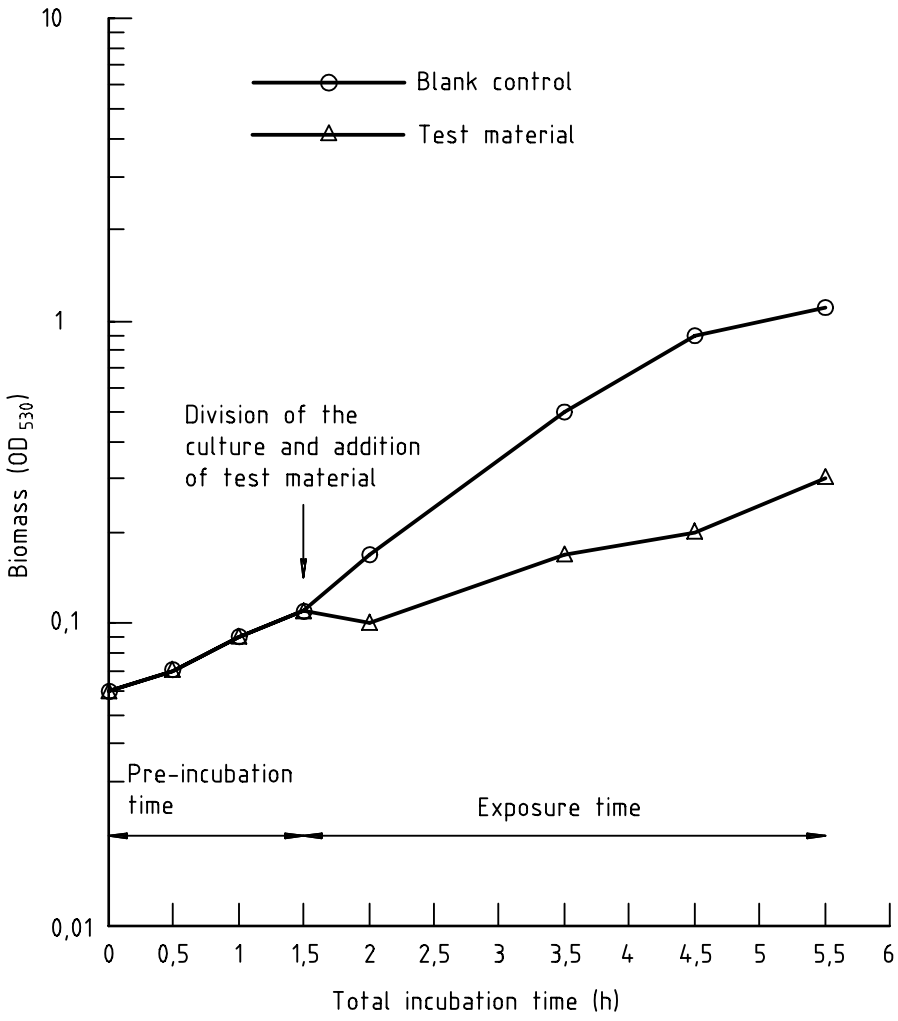

Figure B.1 — Typical growth curve using 3,5-dichlorophenol as test material

Annex C
(informative)

Determination of lowest ineffective dilution of wastewater

Where testing wastewater by means of a graduated dilution (D), the most concentrated test batch at which an inhibition $<$ 20 % is observed is termed the "Lowest Ineffective Dilution (LID)". This dilution is expressed as the reciprocal value of the volume fraction of wastewater in the test batch [e.g. if the wastewater content is 1 part in 4 (25 % volume fraction), the dilution level is D = 4].

In the bacteria growth inhibition test, the inorganic test medium prepared from solutions 6.2.1.1 to 6.2.1.5 and mixed according to 6.2.2 can be used to dilute the wastewater. Add, however, to each test flask and the blank control the same amounts of organic substrate (solution 6.2.1.6 according to 6.2.2) and inoculum. To obtain sufficient wastewater in the final mixture at the lowest dilution level of 2, use for pre-incubation of the main culture only 10 ml (see 9.6). Normally as a minimum dilution equal volumes of wastewater and the residual components of the test mixture are used. Therefore the dilution levels within a dilution series are $D \geqslant 2$ as a rule.

Use, for example, a composition of wastewater and the residual components of the test mixture to obtain a total test volume of 25 ml, as shown in Table C.1.

Table C.1

Parts of wastewater in the total mixture	Dilution level	Wastewater ml	Residual components ml
1 in 2	2	12,5	12,5
1 in 3	3	8,3	16,7
1 in 4	4	6,2	18,8
1 in 6	6	4,2	20,8
1 in 8	8	3,1	21,9
1 in 12	12	2,1	22,9
1 in 16	16	1,4	23,6
1 in 24	24	0,9	24,1
1 in 32	32	0,8	24,2
Blank control	0	0	25

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