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Nitrifikationshæmning for kemikalier og spildevand
Bestemmelse med aktiveret slam

Method for assessing the inhibition of nitrification of activated sludge micro-organisms by chemicals and waste water

WARNING – Activated sludges may contain pathogenic organisms, therefore take appropriate precautions when handling them.

Handle toxic test substances and those with unknown properties with care.

0 Introduction

This method is based on DS/EN ISO 9509:1996. The method has been revised compared to DS/EN ISO 9509 with the purpose of improving comparability of test results obtained at different laboratories. The major changes are:

- Increased aeration to prevent inhibition from low oxygen content.
- Control of ammonium/ammonia concentration taking the content in the test portion into account.
- Requirement of a minimum concentration of ammonium/ammonia at the end of test.
- Adjustment of pH
- Duplicate determinations (DS/EN ISO 9509 has single determination).
- Control of sludge concentration, measured as mixed liquor suspended solids.
- Sampling from test glasses 2-3 times during test (DS/EN ISO 9509 prescribes sampling once).
- Requirements for handling of samples for nitrate + nitrite analyses.

1 Scope

1.1 This Standard specifies a method for assessing the short-term inhibitory effects of test substances on nitrifying bacteria in activated sludge and is based on the International Standard ISO 9509 (1989). The inhibitory effects are estimated over an exposure period of 3 h.

1.2 The method is suitable for use with nitrifying activated sludge from domestic sewage. It is also possible to use nitrifying sludges derived from synthetic sewage.

1.3 The method is applicable to non-volatile chemical substances, which are soluble in water and also to waste waters. It is possible to use insoluble substances, if care is taken to ensure as much homogeneity as possible.

1.4 It is important to stress that sludges from different sources respond differently to a given concentration of an inhibitor and this is probably due, at least in part, to reaction between the inhibitor and components of the sludge resulting in a partial nullifying of the toxic effect. Also, since the test lasts only 3 h, it must be borne in mind that any inhibitory effects may diminish, or increase, over a longer period, e.g. in the continuous activated sludge system.

2 Normative references

The following standards contain provisions, which, through reference in this text, constitute provisions of the International Standard ISO 9509. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on the International Standard ISO 9509 are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Member of IEC and ISO maintain registers of currently valid International Standards.

ISO 6107-1: 1996, *Water quality – Vocabulary – Part 1.*

ISO 6107-3: 1993, *Water quality – Vocabulary – Part 3.*

ISO 5667-16: 1998, *Water quality – Sampling – Part 16*

3 Definitions

For the purposes of this Standard, the following definitions apply.

3.1 Test substance: Pure chemicals, mixtures, chemical products and waste waters.

3.2 Activated sludge: Accumulated biological mass (floc) produced in the treatment of waste water by the growth of bacteria and other micro-organisms in the presence of dissolved oxygen. [ISO 6107-1.]

3.3 Mixed liquor suspended solids (MLSS): The concentration of solids, expressed in a specified dried form, in the mixed liquor. [ISO 6107-3.]

NOTE – In this Standard, the MLSS are determined after filtration of a known volume and drying at about 100℃. The MLSS are expressed in milligrams per liter or grams per liter.

3.4 EC50 and EC20: Concentration of test substance giving a calculated or interpolated inhibition of nitrification of 50 % and 20 %, respectively, compared with a control containing no test material.

3.5 Nitrification: The oxidation of ammonium salts by bacteria. Usually, the end product of such an oxidation is nitrate. [ISO 6170-1.]

NOTE – Nitrites may be formed as intermediate products.

4 Principle

Performance of the test at a constant temperature, usually between 20℃ and 25℃, in an atmosphere free from dust and toxic vapors. Parallel aeration of a nitrifying sludge in the presence and absence of test material and assessment of the difference in concentration of oxidized nitrogen (nitrite N plus nitrate N) produced by the oxidation of ammonium salts. Calculation of the inhibition of nitrification of activated sludge microorganisms by the test material.

5 Reagents and materials

5.1 Water

Deionized or distilled.

5.2 Nitrifying activated sludge

Obtain a portion of sludge from a nitrifying treatment plant receiving domestic sewage or from a laboratory-scale plant treating domestic or synthetic sewage. Maintain the sludge in an aerobic condition and preferably use within 24 h of collection.

Before use, centrifuge the sludge (e.g. 1 100 g during 5 min) and discard the supernatant liquid. Wash the residue with an equal volume of water **(5.1)**, dilute the resulting mixture with ten times the volume of medium **(5.3)**, re-centrifuge and again discard the supernatant liquid. Finally, resuspend the sludge in an appropriate volume of water **(5.1)** to give the required concentration of mixed liquor suspended solids (e.g. 4 g/l) and aerate until use.

5.3 Medium

Dissolve 5.04 g of sodium hydrogencarbonate ($NaHCO_3$) and 2.65 g of ammonium sulfate [$(NH_4)_2SO_4$] in 1 liter of water **(5.1)**.

NOTE – This medium - when diluted 1 : 10, contains 56 mg/l of N and has a pH value of about 7.6. It allows the production of at least 25 mg/l of oxidized nitrogen without changing the pH.

5.4 Reference inhibitor

3,5-dichlorophenol may be used as reference inhibitor. An interlaboratory testing based on the test described in this method was carried out in 2003. The results obtained with 3,5 dichlorophenol are shown in Annex A.

NOTE – Another reference inhibitor may be used e.g. phenol

5.5 Stock solution of test substance

Prepare a stock solution or suspension of test substance in water (**5.1**) at a suitable concentration, e.g. 1 g/l or 10 g/l. It is

possible to use waste water without dilution.

6 Apparatus

6.1 Cylindrical glasses with a capacity of approx. 500 ml (e.g. with an internal diameter of approx. 5 cm and a height of approx. 34 cm).

6.2 Pasteur pipettes or other aeration device. The device shall admit an aeration intensity (approx. 600-800 ml air/min) ensuring a homogeneous distribution of the sludge in the test mixture.

6.3 Compressed air supply humidified by passage through a wash bottle containing water.

6.4 Apparatus necessary for analytical determination of ammonia and oxidized nitrogen in solution.

6.5 Filtration apparatus.

6.6 Filter made of glass fibre or paper, which does not release nitrogen.

6.7 pH meter and thermometer for measuring of pH and temperature during the test.

6.8 Oxygen meter for control of the oxygen concentration in the test mixture during the test.

6.9 Equipment: for determination of mixed liquid suspended solids (MLSS). Filtering is made on GF/A glass fibre filters (e.g. Whatman).

7 Sampling of waste water

Sampling shall be conducted in chemically inert, clean containers in accordance with ISO 5667-16. Fill the containers completely and seal them. Test the samples as soon as possible after collection. Where necessary, store the samples at a temperature of 2°to 5 ℃ in the dark for no longer than 48 h. For longer periods, store at –18℃. Test should preferable be performed within 1 month of sampling. Do not use chemicals to preserve the samples. Perform the necessary pH adjustment just before testing.

8 Procedure

8.1 Use sludges with specific nitrification rates between 1.5 mg of N/(g·h) and 6.5 mg of N/(g·h). If the rate is outside this **range**, it is essential to modify the procedure (see clause 9).

8.2 The test should be performed in duplicate, i.e. duplicates are prepared of each test concentration, the control and the reference.

High concentration of ammonium/ammonia may inhibit the nitrification. The amount of ammonium/ammonia nitrogen in the test substance is therefore measured and recorded before the testing. If the test substance contains a relatively large amount of ammonium/ammonia nitrogen, the ammonium/ammonia nitrogen concentrations in the test medium dosed to glasses with test substance are adjusted in order to ensure that the concentration in the final test mixture does not exceed 75 mg NH_4^+/NH_3-N/l.

pH of the test substance is measured and recorded. The pH shall be within the interval of 7.6 and 8.0 and is adjusted with hydrochloric/sulphuric acid or a solution of sodium hydroxide if necessary. The amount used of acid/base is recorded.

Water **(5.1)**, medium [usually 25 ml **(5.3)**] and test substance **(5.5)** are added to each cylindrical glass in selected volume ratios. The volume of the water is regulated to make up a final total volume of test mixture of 250 ml. The test substance is usually tested in a range of concentrations (often five). The aeration is turned on and it is measured that the oxygen concentration is 6-8 mg O_2/l. The measured O_2 concentrations are recorded.

Include control and reference glasses without test solution. The reference inhibitor 3,5-dichlorophenol **(5.4)** may be tested in five concentrations or in a single concentration e.g. 2 mg/l.

The test is started by adding the necessary amount of washed nitrifying sludge **(5.2)** to

each glass. Use intervals of e.g. 1 minute between addition to successive test glasses. The time of sludge addition is recorded. Add equal volumes of the sludge to the series of glasses so that the final concentration of mixed liquor suspended solids will be approx. 2.0 g MLSS/l (2.0 ± 0.5 g MLSS/l) (see Table A.1). The concentration of ammonium/ammonia nitrogen in the test glasses shall be ≥ 4 mg N/l at the termination of the test.

NOTE - It is allowed to use another sludge concentration when the purpose of the test is to assess the effect on a specific wastewater treatment plant (WWTP) at the actual sludge concentration or if the nitrification rate in the sludge complicates test with the recommended sludge concentration of 2.0 g SS/l. However, it should be noted that the sludge concentration might effect the test results.

8.3 Incubate all glasses for 3 hours at a constant temperature **(4)** and ordinary room lighting. pH is measured in one of the replicate test glass within the first 60 minutes and at the termination of the test. The temperature and the pH are measured in each glass simultaneously. Measurements of the temperature may also be performed in selected glasses using thermologgers.

Measure and record the oxygen concentration in one of the replicate test glass within the first 30 minutes and at the termination of the test. Keep the oxygen concentration within 6-8 mg O_2/l.

8.4 After 10 to 30, 90 and 180 minutes (the exact sampling time is noted), take a suitable volume of samples, e.g. 5 ml from the controls for analysis of oxidized nitrogen (NO_2^- + NO_3^- - N). Samples from the remaining glasses are taking after 10 to 30 and 180 minutes. The concentration of ammonium/ammonia nitrogen is analyzed in the samples taken after 180 minutes. A sample from one of the control glasses is analyzed as a minimum.

Filter the samples through a glass fibre filter or a paper filter **(6.6)**. Freeze the samples if analysis is not performed immediately. Thaw out frozen samples at room temperature. It is also possible to thaw out in cold or tepid water. The time of thawing shall be as short as possible and the samples shall be kept cool until the initiation of the analysis. Frozen samples should be analyzed within a week. Thawed samples shall not be re-frozen with the purpose of repeating the analysis. Samples to be analyzed on the day of the test shall be placed in a water bad with ice until initiation of the analysis.

8.5 Take samples (e.g. 20-25 ml) from each glass in the last period of the test (i.e. between 90 and 180 minutes) and determine the concentration of mixed liquor suspended solids in the glasses. Correction in the content of MLSS shall be made if the test substance contains significant amounts of suspended solids. Determine the concentration of MLSS of the test substance and correct the concentration before calculation of the nitrification rate.

8.6 An example of the volume required for setting up the test is shown in Annex B.

9 Calculation and expression of results

9.1 Calculate the nitrification rate by means of linear regression using the analyzed concentration of NO_2^- + NO_3^- - N. Express the rate in mg NO_2^- + NO_3^- - N produced per g MLSS (or g SS) per hour.

Calculate the percentage of inhibition of formation of oxidized nitrogen N as follows:

$$\%\,\text{inhibition} = \frac{N_C - N_T}{N_C} \cdot 100$$

where

N_C is the nitrification rate in controls [mg NO_2^- +NO_3^--N /(g SS · hour)]

N_T is the nitrification rate in glasses with test substance or ATU [mg NO_2^- + NO_3^--N/(g SS · hour)]

NOTE – Although the measurement of oxidized nitrogen is preferable, the percentage inhibition of ammonium/ammonia removal may be based on NH_4^+/NH_3-N concentration. However, this disappearance of ammonium/ammonia is not necessarily due to nitrification. Furthermore, ammonium/ammonia may be produced by degradation of nitrogen-containing substances.

9.2 Plot a graph of the percentage inhibition against the log of the concentration of test substance and interpolate the EC50 (and potentially EC20) from this

Alternative, use a linear regression program to estimate the EC50 (and potentially EC20).

10 Validity of results

The production of NO_2^- + NO_3^- in controls shall be linear in time.

The variations in the temperature measured in the test glasses must be within ± 1℃ during the whole test period.

The measured pH values must be within the interval of 7.5-9.0 (preferable 7.5-8.5).

The measured oxygen concentrations in the test glasses must be at least 6 mg O_2/l.

The result of the reference inhibitor is compared with results of interlaboratory test and it is assessed whether the result is within the expected interval, i.e. mean value ± 2 · standard deviation found in the interlaboratory test (see Annex A).

It is important that the nitrification has taken place in the control, but it is essential that sufficient ammonium salt (i.e. ≥ 4 mg N/l) is left at the end of the test period to ensure that the substrate was not rate limiting. Nitrification rates between 1.5 mg N/(g SS · h) and 6.5 mg N/(g SS · h) have been found suitable for this procedure for assessment of inhibition. If the rate of nitrification is lower than 1.5 mg N/(g SS · h), use a sludge from another source, or increase the proportion of nitrifiers in the sludge, e.g. by culturing the sludge for a few weeks with synthetic sewage at a suitable retention time (e.g. 6 h) in a laboratory-activated sludge plant.

If the nitrification rate is higher than 6.5 mg N/(g SS · h), use either a shorter incubation period or a larger volume of concentrated medium **(5.3)** to ensure that the concentration of ammonium salt does not become rate-limiting and that the pH does not fall. If necessary, carry out a preliminary test to ascertain the appropriate volume of medium to use.

NOTE – It should be noted, however, that with a larger proportion of nitrifiers, the nitrifier to inhibitor ration will be changed and a different EC50 may result.

11 Test report

The test report shall refer to this Standard and the International Standard ISO 9509. The report shall contain the following information:

a) Identity of the test substance, including sampling, storage time and conditions;

b) The specific nitrification rate of the activated sludge;

c) The source, date of sampling, concentration and pretreatment method of the activated sludge;

d) Measured data: Temperature, pH, O_2-concentration, MLSS measurements and analytical results of NO_2^- + NO_3^- - N and NH_4^+/NH_3-N;

e) The test results: The nitrification rate and the percentage of inhibition for each test glass are stated together with the concentration of the test substance in the glass. The inhibition curve is plotted and the EC50 (and possibly the EC20) is stated. The 95%-confidence interval of EC50 (and EC20) is given when possible;

f) The inhibition caused by the reference inhibitor;

g) Any other facts, not specified in this Standard, that are relevant concerning the procedure followed.

Annex A
(informative)

Interlaboratory test results

Table A.1 – Test results of 3,5 dichlorophenol (3,5-DCP)

Test organisms	Participants	Number of test series	Outliers	Parameter	Mean value	Standard deviation
Nitrifying activated sludge from different WWTPs	5	8	3	EC50	1,71 mg/l	0,50 mg/l
				Inhibition of 2 mg 3,5-DCP/l	55%	8%

Annex B
(informative)

Example for preparation of the test

Table B.1

Glass No.	1 + 2	3 + 4	4 + 5	6 + 7	8 + 9	10 +11	12 + 13
Medium (ml)	25	25	25	25	25	25	25
Activated sludge (ml)	125	125	125	125	125	125	125
Reference inhibitor[1)] (ml)	0	0	0	0	0	0	5
Water (ml)	100	99.75	99.2	97.5	92	75	95
Stock solution[2)] of test substance (ml)	0	0.25	0.8	2.5	8.0	25	0
Concentration of test substance (mg/l)	0	1	3.2	10	32	100	0
Total volume (ml)	250	250	250	250	250	250	250

Concentration of activated sludge = 4.0 g MLSS/l

1) Stock solution: 100 mg of reference inhibitor per litre
2) Stock solution: 1 g of test substance per litre

NOTE - Undiluted test substance must not be allowed to come into contact with the sludge.