ENVIRONMENT AGENCY

Determination of the inhibitory effect of chemicals, industrial and other waste waters on nitrification by *Nitrosomonas* bacteria (2006)

Methods for the Examination of Waters and Associated Materials

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This method describes procedures for determining the inhibitory effects on nitrification of environmental samples with *Nitrosomonas* bacteria.

Whilst this booklet reports details of the materials actually used, this does not constitute an endorsement of these products. Equivalent products are available and it should be understood that the performance characteristics of the method might differ when other materials are used. It is left to users to evaluate this method in their own laboratories. Only limited performance data are provided. Details of this method are included for guidance purposes only, as information on the routine use of this method would be welcomed to assess its full capabilities.

Contents

About this series

Introduction

This booklet is part of a series intended to provide authoritative guidance on methods of sampling and analysis for determining the quality of drinking water, ground water, river water and sea water, wastewater and effluents as well as sewage sludges, sediments, soil (including contaminated land) and biota. In addition, short reviews of the most important analytical techniques of interest to the water and sewage industries are included.

Performance of methods

Ideally, all methods should be fully evaluated with results from performance tests. These methods should be capable of establishing, within specified or pre-determined and acceptable limits of deviation and detection, whether or not any sample contains concentrations of parameters above those of interest.

For a method to be considered fully evaluated, individual results encompassing at least ten degrees of freedom from at least three laboratories should be reported. The specifications of performance generally relate to maximum tolerable values for total error (random and systematic errors) systematic error (bias) total standard deviation and limit of detection. Often, full evaluation is not possible and only limited performance data may be available. An indication of the status of the method is shown at the front of this publication on whether the method has undergone full performance testing.

In addition, good laboratory practice and analytical quality control are essential if satisfactory results are to be achieved.

Standing Committee of Analysts

The preparation of booklets within the series "Methods for the Examination of Waters and Associated Materials" and their continuing revision is the responsibility of the Standing Committee of Analysts. This committee was established in 1972 by the Department of the Environment and is now managed by the Environment Agency. At present, there are nine working groups, each responsible for one section or aspect of water quality analysis. They are

1 General principles of sampling and accuracy of results
2 Microbiological methods
3 Empirical and physical methods
4 Metals and metalloids
5 General non-metallic substances
6 Organic impurities
7 Biological methods
8 Biodegradability and inhibition methods
9 Radiochemical methods

The actual methods and reviews are produced by smaller panels of experts in the appropriate field, in co-operation with the working group and main committee. The names of those members principally associated with this method are listed at the back of the booklet.

Publication of new or revised methods will be notified to the technical press. If users wish to receive copies or advanced notice of forthcoming publications, or obtain details of the index of methods then contact the Secretary on the Agency's internet web-page (www.environment-agency.gov.uk/nls) or by post.

Every effort is made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary.

Dr D Westwood
Secretary

September 2003

Warning to users

The analytical procedures described in this booklet should only be carried out under the proper supervision of competent, trained analysts in properly equipped laboratories.

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc Act 1974 and all regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 2002 (SI 2002/2677). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted.

Numerous publications are available giving practical details on first aid and laboratory safety. These should be consulted and be readily accessible to all analysts. Amongst such publications are; "Safe Practices in Chemical Laboratories" and "Hazards in the Chemical Laboratory", 1992, produced by the Royal Society of Chemistry; "Guidelines for Microbiological Safety", 1986, Portland Press, Colchester, produced by Member Societies of the Microbiological Consultative Committee; and "Safety Precautions, Notes for Guidance" produced by the Public Health Laboratory Service. Another useful publication is "Good Laboratory Practice" produced by the Department of Health.

Glossary

In the context of this method, the following terms and definitions are used.

Ammonia/ammonium	These terms are used interchangeably and when determined, concentrations are normally expressed in milligrams (as nitrogen) per litre of sample (mg l^{-1}-N).
AQC	Analytical quality control.
EC_{50}	The EC_{50} concentration is the effective (or inhibitory) concentration of a test material that inhibits (relative to a non-toxic control) the measured microbial function by 50 %. For samples such as waste waters, the dilution of the sample is used, not its concentration. For example, if 100 ml of waste water are used and diluted (as described in the method) to 110 ml, this is equivalent to a dilution of 1 in 1.1 (or sample concentration of 90.9 %). The EC_{50} value may be determined from suitable plots (see Annex B) of the concentration (or dilution) of the test material versus the percentage inhibition.
Inhibition	Inhibition of the nitrification process manifests itself by preventing the sequential oxidation of ammonium to nitrite and then nitrate. The inhibitory effect of a test material is compared relative to a non-toxic control sample and expressed as percent nitrification inhibition (% NI) at a stated concentration, or dilution, of the test material. Inhibition can also be expressed as an EC_{50} value (see above).
Nitrification	Nitrification is a sequential process (each stage being carried out by different organisms) whereby ammonium is oxidized, first to nitrite, and then to nitrate. It is the removal of the ammonium ion from solution. Oxidation is accomplished using appropriate nitrifying bacteria. The nitrification rate is expressed as the concentration of ammonium removed from the test sample in unit time, for example, milligrams of ammonium per litre per hour. The nitrification rate for activated sludge is typically expressed as milligrams of ammonium per gram of dried sludge per hour.
Non-toxic control	In the presence of a non-toxic control, nitrification proceeds without any adverse effects, i.e. ammonium is sequentially oxidized to nitrite and then to nitrate. Only on rare occasions does the process stop at the first stage. Samples comprising non-toxic controls are analysed at the same time as samples under investigation. The non-toxic control material should be, wherever possible, of a similar matrix to the test material. For example, for chemical substances, a non-toxic control may comprise water; for waste waters and effluents, the non-toxic control may comprise a sample of effluent taken from a treatment works known to process only domestic sewage.
Test material	A pure chemical or compound, formulation, mixture, chemical product, waste water, other chemical substance (for example metabolite product) or suspected toxicant.
Toxicity	The adverse effect caused by a test material on nitrifying micro-organisms.

Determination of the inhibitory effect of chemicals, industrial and other waste waters on nitrification by *Nitrosomonas* bacteria

1 Introduction

This method enables the short-term inhibitory effects of a test material on nitrifying bacteria to be determined. The inhibitory effects are estimated over an exposure period of 4 hours at a temperature of 20 ± 2 ^{o}C.

This method should be read in conjunction with others[1, 2, 3] which describe techniques using activated sludge for measuring the rate of ammonia removal. Assessing the degree of inhibition to nitrification brought about by a waste water or by a specific compound is useful in defining the conditions of acceptance of trade wastes into sewers and for tracking compounds back to their original sources[1] when investigating incidents.

Whilst this method is suitable for use with commercially available nitrifying bacterial cultures it is also possible to use nitrifying activated sludge derived from domestic or synthetic sewages[1, 3]. To ensure their suitability, nitrifying bacterial cultures or activated sludges from different sources should be tested in order to check their sensitivity to reference toxicants, and where applicable, to the test material under investigation.

The nitrification process may be assessed by determining the reduction in the ammonium concentration (or additionally or alternatively, the formation of oxidized-nitrogen concentrations). For oxidized-nitrogen determinations, nitrite concentrations need to be determined when *Nitrosomonas* cultures are used. Nitrite and nitrate concentrations need to be determined when mixed cultures of *Nitrosomonas* and *Nitrobacter* are used, or when nitrifying activated sludge is used. In some methods[1, 2, 3], the use of activated sludge is described where the determination of the oxidized-nitrogen concentration is carried out, since some of the ammonium may be oxidized (i.e. removed from solution) by heterotrophic bacteria and used for cell synthesis. The method described in this booklet describes procedures for determining ammonium concentrations, since *Nitrosomonas* cultures are presumed to contain few, if any, heterotrophic bacteria.

Using the procedures described in this booklet, the nitrification rate should be within a suitable range for the determination, by measuring ammonium concentrations. Alternatively, nitrite concentrations may be determined. A minimum ammonium concentration (as nitrogen) of 5 mg l^{-1}-N should remain at the end of the 4 hour test period. This is so that ammonium concentrations do not become a rate-limiting factor in the reaction.

Inhibitory or toxic effects may be estimated from the difference in the nitrification rates using nitrifying bacteria both in the presence, and in the absence, of a test material in the presence of a known amount of ammonium. If, during the course of the test, the concentration of ammonium does not decrease, i.e. there is no removal of ammonium from solution, this would indicate that the test material exerts a toxic effect on the micro-organisms used.

The proportional decrease in the nitrification process can be related to a series of dilutions of the test material under investigation where actual concentrations are unknown (for example waste water). The percentage nitrification inhibition (% NI) at a particular concentration of test material can be estimated by preliminary experiments, for example in three ranges of concentrations comprising 1 - 10 mg l^{-1}, 10 - 100 mg l^{-1} and 100 - 1000 mg l^{-1}. Other concentrations may be required for definitive testing. Alternatively, waste waters may be

tested, for example at 2-fold, 4-fold and 8-fold dilutions of the original waste water sample. Similarly, other dilutions may be required for definitive testing.

Ammonium concentrations (expressed as nitrogen) should be determined using an appropriate method, which should be sufficiently rapid to facilitate determination of the end of the nitrification process. The method should be capable of determining ammonium concentrations (expressed as nitrogen) within the range 2 - 60 mg l^{-1}-N, with a discrimination of 0.1 mg l^{-1}-N. The method should also be validated to ensure that the aliquots of the test material sub-samples taken for analysis are stable and undergo no further oxidation prior to the determination of ammonium.

2 Performance characteristics of the method

2.1	Property determined	The inhibitory effect of the test material on the oxidation of ammonium by *Nitrosomonas* bacteria.
2.2	Type of sample	This method is applicable to those materials soluble in water and possessing moderate volatility. Insoluble and volatile test materials may be assessed provided suitable precautions are taken, for example the test material and bacterial mixture is as homogeneous as possible[4, 5].
2.3	Basis of method	A culture of *Nitrosomonas* bacteria is incubated at 20 ± 2 °C for a known period of time and in the presence of a known amount of ammonium. This incubation should be carried out both in the presence and the absence of the test material. During the incubation period, aliquots of the reaction mixture are removed and ammonium concentrations determined. These values are compared with determinations carried out at the same time on samples containing a non-toxic control in place of the test material. The difference in values is expressed as a percent nitrification inhibition (% NI).
2.4	Range of application	Up to 100 % inhibition relative to a non-toxic control.
2.5	Performance data	More than one reference toxicant should be analysed, as sensitivity changes may occur between different batches of the nitrification bacteria, and the percentage change may be toxicant-dependent. This is shown by the performance data shown in Table 1.
2.6	Limit of detection	A nominal value of 10 % nitrification inhibition with a minimum sample dilution of 1 in 1.1 (maximum sample concentration 90.9 %).

2.7	Time required	Operator time of approximately 2.5 hours per batch of up to 20 test vessels. Excluding chemical analysis, total time is about 4 - 5.5 hours.

3 Principle

The percent inhibition of nitrification by a test material under investigation is calculated by assessing the response of the test material, relative to a non-toxic control tested in parallel, on nitrifying *Nitrosomonas* bacteria. The response, in terms of the concentration of ammonium (expressed as nitrogen) oxidized after aeration of nitrifying *Nitrosomonas* bacteria at a temperature of 20 ± 2 ^{o}C is determined in the presence, and absence, of test material.

4 Interferences

Substances other than the test material in the sample being tested may exhibit an adverse effect on the micro-organisms. Hence, care should be taken to ensure that these substances do not enter the test system. Glassware should be stringently washed to remove grease and other adsorbed material and should then be rinsed with water (6.1) and carefully stored before use.

Solutions containing test material with ammonium concentrations (expressed as nitrogen) greater than 65 mg l^{-1}-N may inhibit the nitrification process. Samples containing ammonium concentrations as nitrogen greater than 65 mg l^{-1}-N should therefore be diluted before the nitrifying bacteria are exposed to the sample.

Any substance, compound or component present in the test material that chemically reacts with ammonia, or ammonium ions, under the test conditions used may effectively remove the ammonia, or ammonium ions, from solution. This would have the same effect as that caused by the nitrification process. This apparent nitrification should be considered and taken into account, especially if a pure compound is being tested.

Any sample that contains a substance, compound or component (for example an amine) that interferes with the chemical determination of ammonium may not be suitable for testing using this method. Supporting data would need to be made available.

Solutions exhibiting extremes of pH may interfere with the nitrification process or the determination of ammonium concentrations. If the sample pH value is outside of the range 8.0 ± 1.5 then pH-adjustment with sulphuric acid solution (6.6) or sodium hydroxide solution (6.7) should be undertaken prior to testing. Buffer (6.5) and carbon dioxide (7.2) both of which are added in the test procedure, should maintain the pH to between 8.0 ± 0.2 (see sections 9.3 and 9.5). Additional testing without pH-adjustment may be carried out, if it is required that the test should be performed to assess the nitrification process as a function of the pH of the test material.

5 Hazards

Media, reagents and bacteria used in these methods are covered by the Control of Substances Hazardous to Health Regulations[6] and appropriate risk assessments should be made before using this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[7] in this series.

Sewages and derived activated sludges may contain potentially pathogenic organisms and appropriate precautions should be taken when handling these materials. Producers of bacterial cultures of *Nitrosomonas* cultures should be able to provide information relating to the absence of pathogenic organisms in the cultures supplied.

Test materials may be toxic and should be regarded as potentially hazardous. Appropriate precautions should be taken to avoid contact with skin and clothing, and inhalation of dusts or vapours. Sample bottles containing aerated sewages, activated sludges or test materials that may produce harmful vapours should occasionally be vented to reduce pressure build-up. In addition, sample bottles containing aerated sewages or activated sludges should be loosely covered to reduce aerosols or vapours escaping into the atmosphere. Carbon dioxide is an asphyxiant and appropriate precautions should be taken when handling this gas.

6 Reagents

All reagents should be of an analytical grade quality unless otherwise stated.

6.1 Water. Distilled water, reverse osmosis water and deionised water are suitable for use and may be used as a non-toxic control sample especially when screening industrial effluents.

6.2 Stock suspension of nitrifying bacterial culture. Commercially prepared cultures of nitrifying bacteria, predominantly *Nitrosomonas*, are available from various suppliers. These should be stored as recommended by the supplier. All cultures, and each batch of culture, should be assessed (see Annex A) to ensure it is suitable and fit for purpose. This testing should indicate the volume of stock suspension required for the working suspension (6.3) to be added to the test vessels. This volume (up to 4 ml per test vessel) may be changed from day to day according to the results of operational usage. *Nitrosomonas* bacteria from different sources may be acclimatized to, or possess different sensitivities to the test material and/or reference toxicant. If this is suspected, then the opportunity should be taken to repeat the test with *Nitrosomonas* bacteria from different sources. If activated sludge is used, this should be similarly tested.

6.3 Working suspension of nitrifying bacterial culture. In a suitably sized beaker, prepare in water (6.1) an appropriate amount of the stock suspension of nitrifying bacterial culture (6.2). Typically, each test vessel (7.4) requires 4.0 ± 0.1 ml of working suspension. Loosely cover the top of the beaker and stir (aerate) the suspension for at least one hour at 20 ± 2 ^{o}C until required. The working suspension of the nitrifying bacteria should be used within the working day of its preparation.

6.4 Standard ammonium chloride solution (1000 mg l^{-1} as nitrogen). Into a 1000-ml volumetric flask, weigh 3.819 ± 0.010 g of ammonium chloride. Add sufficient water (6.1) to dissolve the ammonium chloride and make to volume with water (6.1). Mix well. The solution may be stored between 2 - 8 ^{o}C for up to 1 month.

6.5 Potassium hydrogen carbonate (buffer).

6.6 Sulphuric acid solution (1 Molar). This solution may be stored for up to 12 months at 2 - 8 ^{o}C.

6.7 Sodium hydroxide solution (1 Molar). This solution may be stored for up to 12 months at 2 - 8 ^{o}C.

6.8 Reference toxicant stock solution (1000 mg l^{-1}). Weigh 1.000 ± 0.005 g of, for example, 3,5-dichlorophenol into a 1000 ml volumetric flask. Add sufficient water (6.1) to dissolve the 3,5-dichlorophenol, with stirring or sonication as required. Make to volume with water (6.1) and mix well. The pH of this solution should be between 6.5 - 8.5. The solution may be stored in the dark between 2 - 8 ^{o}C for up to 6 months. This solution is used to prepare a reference toxicant working stock solution, for example 10 mg l^{-1}. This working stock solution is used to prepare a series of dilutions to enable an EC_{50} value to be determined.

6.9 Control solutions. A blank (non-toxic) control solution is required for each type of sample matrix. Water (6.1) may be used as a non-toxic control for pure chemical compounds or industrial waste waters. Sewage samples to be tested should be compared with appropriate control sewage samples obtained from a sewage treatment works treating mainly domestic waste. Sewage samples for control purposes may be stored between -18 ^{o}C to -23 ^{o}C for up to 6 months. When the samples are thawed, they may be stored between 2 - 8 ^{o}C for up to 5 days.

7 Apparatus

Apparatus should be free from contamination before use. Normal laboratory glassware and apparatus is required in addition to the following.

7.1 Gas flow rig for nitrification system. This will depend on the specific manufacturer and different systems are available which are capable of mixing gases and aerating the test vessels. The addition of carbon dioxide to the bicarbonate-buffered solution helps maintain the pH within a narrow range during the test.

7.2 Air supply. This should be of a suitable type and capacity to supply air at a flow rate of 60 ml per minute to each test vessel required.

7.3 Carbon dioxide supply. This should be of suitable type and capacity to supply carbon dioxide gas at a flow rate of 3 ml per minute to each test vessel required.

7.4 Test vessels. Throughout these procedures, vessels of at least 110 ml capacity have been used. This allows for additional capacity, which may be required due to the aeration of the test solution.

7.5 pH meter.

7.6 Stopwatch.

8 Sample collection and preservation

Where appropriate, samples should be taken in glass bottles, and stored in the dark at 2 - 8 ^{o}C. Analysis should be carried out as soon as possible after sampling.

9 Analytical procedure

Step	Procedure	Note
9.1	Test samples	
9.1.1	Appropriate solutions of the test material under investigation should be prepared. For example, if the test material comprises a pure compound prepare in water (6.1) solutions containing 1 g l^{-1}, 10 g l^{-1} etc, as appropriate. If the test material comprises an effluent then prepare in water (6.1) appropriate dilutions, for example 5-fold, 10-fold dilutions etc, as appropriate, see note a.	(a) Consideration may need to be given to the range and spacing of the concentrations/dilutions so that an appropriate EC_{50} value can be calculated. All details should be recorded and an example of a typical work sheet is shown in Annex B. Appropriate AQC samples (for example standard and blank solutions) should also be prepared and analysed.
9.1.2	Determine and record the pH of an aliquot of the test material solution. Adjust the pH with acid (6.6) or alkali (6.7), where this is required (note b).	(b) If the effect of the test material is to be determined without recourse to pH adjustment, see section 4, then the test material solution need not be pH-adjusted.
9.1.3	Determine and record the ammonium concentration (expressed as nitrogen) of the test material solution (notes c and d).	(c) If the ammonium concentration (as nitrogen) is greater than 65 mg l^{-1}-N the test material solution should be appropriately diluted to provide 100 ml of diluted solution containing an ammonium concentration as nitrogen of 60 $\pm$ 5 mg l^{-1}-N, see section 4. (d) Where the solution contains ammonium (as nitrogen) at a concentration of less than 65 mg l^{-1}-N, calculate the amount of ammonium chloride solution (6.4) required to be added to 100 ml of test material solution to produce a test material solution containing ammonium (as nitrogen) of 6.0 $\pm$ 0.5 mg.

9.1.4 Prepare a series of dilutions of the reference toxicant solution (6.8) at suitable concentrations for testing.

9.1.5 Prepare a non-toxic control sample (6.9) for each matrix type.

9.2 Set up the gas flow system (see sections 7.1, 7.2 and 7.3) according to the manufacturer's instructions. Use one test vessel (7.4) for each solution or diluted solution of test material, non-toxic control sample and reference toxicant, as appropriate.

9.3 Add buffer to each test vessel, i. e. 1.0 ± 0.1 g of potassium hydrogen carbonate (6.5). Add 100.0 ± 0.5 ml of test mixture (see section 9.1 and note e) to appropriate test vessels (note f).

(e) This may be the original test material solution, pH-adjusted or diluted test mixture (see notes b and c).

(f) For greater precision, replicate test vessels of test material, blank (non-toxic) control samples and reference toxicant may be prepared and tested.

9.4 Add ammonium chloride solution (6.4) to those test vessels where the test material solution contains ammonium (as nitrogen) of less than 65 mg l^{-1}-N, see note d. Add water (6.1) to all test vessels such that the final volume of test material solution, whether pH-adjusted, diluted or containing added ammonium chloride solution is 106 ml (see note g).

(g) The volume of ammonium chloride (6.4) plus water (6.1) should be no greater than 6.0 ± 0.1 ml.

9.5 Aerate each test vessel to ensure dissolution of the potassium hydrogen carbonate buffer (note h). Ensure that there is a steady flow of mixed air and carbon dioxide (see sections 7.2 and 7.3) and that aeration is sufficient and even between test vessels. The pH value of each solution should be 8.0 ± 0.2.

(h) This usually takes several minutes.

9.6 Switch off the aeration system. Add 4.0 ± 0.1 ml of the working suspension of nitrifying bacterial culture (6.3) to each test vessel (see Annex A). Mix the suspension thoroughly. Immediately (note i) remove a suitable aliquot (note j) from each test vessel. Transfer this aliquot to a suitable container. Start the stopwatch (note i) to time the test for 4 hours (note k) and recommence aeration of

(i) The removal of the aliquot and starting of the stopwatch should be carried out at the same time. As many as 30 samples may need to be treated in this manner.

(j) The same volume should be removed from each test vessel

	the buffered bacterial test mixture to begin the test.	and should be sufficient to carry out all chemical determinations required. Replicate testing of the aliquots improves the precision of the analysis. (k) At this point, time t = 0.
9.7	For each aliquot removed, determine the initial ammonium concentration as nitrogen (i.e. $N_{t=0}$) (notes f and l).	(l) Due to the test volume being made to 110 ml, the initial ammonium concentration as nitrogen ($N_{t=0}$) should be 54.5 $\pm$ 5.0 mg l^{-1}-N.
9.8	Periodically during the nitrification process, check the gas flow rates through the test vessels and adjust accordingly (see sections 7.1 to 7.3).	
9.9	After a suitable time-period during the nitrification process, switch off the aeration system, remove a suitable aliquot from each test vessel containing the blank (non-toxic) control sample and transfer this aliquot to a suitable container. Recommence aeration of the test mixtures to continue the test. Determine the ammonium concentration in each aliquot removed (notes f and m).	(m) If the loss of ammonium in the blank non-toxic control aliquot is greater than expected (for example 80 % removal by 4 hours) then consideration should be given to terminating the test. If the ammonium concentration (as nitrogen) falls below 5 mg l^{-1}-N then the test should be aborted.
9.10	After 4 hours ± 5 minutes from the start of the test (note n) switch off the aeration system, remove a suitable aliquot from each test vessel and transfer this aliquot to a suitable container. Recommence aeration of the test vessels to continue the test in case nitrification is less than 60 % in the controls. Determine the ammonium concentration expressed as nitrogen ($N_{t=4}$) in each aliquot removed (notes f and o).	(n) At this point, time t = 4. (o) Normally, the total duration of the test should be 4 hours, with the loss of ammonium calculated from data generated between the start of the test period (time t = 0) and the end of the test period (time t = 4).
9.11	If the removal of ammonium (nitrification) in each test vessel containing blank (non-toxic) control sample is less than 60 % (see section 10.1 and note p) aeration of all the test vessels may be continued for up to 5.5 hours. Alternatively, the test may be continued until 80 % nitrification is achieved (note q). This will	(p) Where the ammonium concentration as nitrogen is ≥ 20 mg l^{-1}-N at 4 hours, then nitrification is less than 60 % and the test should be continued.

require further periodic removal of aliquots from each test vessel containing blank (non-toxic) control sample and the subsequent determination of ammonium concentrations until sufficient data are obtained.

(q) Usually, 80 % nitrification will give an ammonium concentration range (as nitrogen) of 10 - 12 mg l^{-1}-N.

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10 Calculation and expression of results

10.1 Removal of ammonia

The concentration of ammonium (expressed as nitrogen) removed in each test vessel during a normal four hour period is given by

$$C = N_{t=0} - N_{t=4} \quad \text{mg } l^{-1}\text{-N} \quad \text{.....(1)}$$

Where

C is the concentration of ammonium as nitrogen (mg l^{-1}-N) removed,

$N_{t=0}$ is the initial ammonium concentration as nitrogen (mg l^{-1}-N) at t = 0,

$N_{t=4}$ is the final ammonium concentration as nitrogen (mg l^{-1}-N) at the end of the test, normally t = 4. If a time other than 4 hours is used, the actual time should be reported.

10.2 Percent inhibition

The percentage inhibition, i.e. removal of ammonium in the presence of the test material as a percentage of that in the non-toxic control sample is calculated using

$$\% \text{ Inhibition (\% NI)} = 100 \times (C_c - C_s) / C_c \quad \text{.....(2)}$$

Where

C_c is the concentration of ammonium as nitrogen (mg l^{-1}-N) removed for the blank control sample,

C_s is the concentration of ammonium as nitrogen (mg l^{-1}-N) removed for the test material.

Where aliquots are analysed in replicate, the mean value should be used.

The calculations should be repeated for each control matrix, dilution or concentration of test material and reference toxicant. Where the maximum inhibition value is less than say 50 % (i.e. less than an EC_{50} value) report the highest % NI and corresponding test material dilution or concentration. For example, a 1.1 dilution (90.9% concentration) of waste water producing 30 % NI.

10.3 Calculation of EC_{50}

A graph of the percentage inhibition at the concentration or dilution of test material should be plotted against the concentration or dilution of test material (see Figures B1 and B2). From

these graphs, it is possible to estimate the EC_{50} value or other EC value by interpolation. Alternatively, in some cases, it may be preferable to plot a logarithmic relationship.

10.4 Interpretation

From the results obtained with an industrial waste water and knowledge of the volume of waste effluent discharged to the sewer, an assessment may be made as to whether the waste effluent will be inhibitory to nitrification in the treatment plant[(2)]. This assumes that there is no degradation of the compounds present in the waste effluent or acclimatization of the organisms.

11 Sources of error

If it is suspected that the test material under investigation contains a significant concentration of nitrifying bacteria, which would produce a measurable nitrification rate, thus preventing the detection of inhibition, it may be necessary to remove or inhibit their action, for example by sterilisation of the test material. However, it is very likely that sterilisation by filtration, chemical action or heat treatment might change the sample toxicity and hence adversely affect the results of the test. To ascertain if this is a likely scenario, other controls may be investigated, for example the addition of no nitrifying bacteria to the test vessel may indicate whether nitrification occurs and hence, ascertain if the test material contains bacteria that may produce significant nitrification.

12 Validity of results

The tests may be considered valid if the amount of ammonium (expressed as nitrogen) removed in the blank (non-toxic) control sample is for example within the range 80 ± 10 % of that amount originally present. This corresponds to an ammonium concentration (expressed as nitrogen) of 5 - 12 mg l^{-1}-N. In addition, the tests may be considered valid if the EC_{50} value of the reference toxicant is within an acceptable range.

13 References

1. Standing Committee of Analysts, The Assessment of the Nitrifying Ability of Activated Sludge 1980 (Tentative Methods*). Methods for the Examination of Waters and Associated Materials*, in this series, ISBN 0117516554.

2. Standing Committee of Analysts, Methods for Assessing the Treatability of Chemicals and Industrial Wastewaters and their Toxicity to Sewage Treatment Processes 1982. *Methods for the Examination of Waters and Associated Materials*, in this series, ISBN 0117519596.

3. Water Quality - Method for assessing the inhibition of nitrification of nitrifying organisms by chemicals and wastewaters. ISO 9509:1989 (E).

4. International Standards Organisation (ISO), ISO 10634:1995 Water Quality – Guidance for the preparation and treatment of poorly water-soluble organic compounds for the subsequent evaluation of their biodegradability in an aqueous medium.

5. US EPA Draft Ecological Effects Test Guidelines OPPTS 850.6800 “Modified activated sludge, respiration inhibition test for sparingly soluble chemicals”

6. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.

7. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and Procedures for Laboratories. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

Table 1 Performance data

The data comprise an amalgamation of mean EC_{50} results from different batches of bacteria used on different days.

Reference toxicant	Mean (mg l^{-1})	Relative standard deviation (%)	Number of results	Number of batches	Number of results per batch
3,5-dichlorophenol	1	25.5	58	10	5-6
methanol	155	39.4	27	10	1-5
sodium diethyldithiocarbamate	4.2	34.9	26	9	1-5
2,2'-dipyridyl	58.1	22.7	27	10	1-5
allylthiourea	0.1	18.2	18	6	1-6

Data provided by ALcontrol Laboratories, Rotherham.

Annex A Calibration of nitrifying bacteria

For each batch of nitrifying bacterial culture (6.2) the level of nitrifying activity should to be assessed. Several different volumes of the culture should be tested and the volume that utilizes 80 % of the ammonium concentration in water (6.1) over the 4-hour test period should be used in the subsequent tests. The volumes of bacterial culture to be tested will vary from batch to batch and the example volumes given are provided solely as guidance.

Prepare in a suitable beaker a sufficient volume of bacterial culture to assess the volume of culture required for the test. Loosely cover the beaker and stir the suspension for at least one hour at a temperature of 20 ± 2 ^{o}C prior to use.

Set up the aeration apparatus (7.1) to provide aeration for four or more test vessels (7.4) as required.

Add to each test vessel 1.0 ± 0.1 g of potassium hydrogen carbonate and 100.0 ± 1.0 ml of water (6.1).

Add 6.0 ± 0.1 ml of ammonium chloride solution (6.4) to each test vessel. Mix well.

Aerate each test vessel (see sections 7.1, 7.2 and 7.3) to ensure dissolution of the potassium hydrogen carbonate and mixing of the ammonium chloride.

Switch off the aeration system. Add to each test vessel, the appropriate volumes of prepared nitrifying bacterial culture (6.2) and water (6.1). For example, see Table A1. Mix the suspensions thoroughly. Immediately remove a suitable aliquot from each test vessel and transfer each aliquot to separate suitable containers. Start the stopwatch to time the test for 4 hours and recommence aeration of the buffered bacterial test solution to begin the test.

Table A1 Volumes of nitrifying bacterial culture and water

Volume of nitrifying bacterial culture (ml)	Volume of water (ml)
0.5	3.5
1.0	3.0
1.5	2.5
2.0	2.0

For each aliquot removed determine the initial (i.e. $N_{t=0}$) ammonium concentration as nitrogen. This should be in the range 54.5 $\pm$ 5.0 mg l^{-1}-N.

Periodically during the nitrification process, check the gas flow rates through the test vessels and adjust accordingly (see sections 7.1, 7.2 and 7.3).

After 3 hours ± 5 minutes, switch off the aeration system, remove a suitable aliquot from the test vessel containing the largest volume of nitrifying bacterial culture and transfer this aliquot to a suitable container. Recommence aeration of the buffered bacterial test solution to continue the test. Determine the ammonium concentration ($N_{t=3}$) expressed as nitrogen in the aliquot removed. If 80 % of the ammonium in this test vessel has been removed (corresponding to an ammonium concentration as nitrogen in the range

20 - 24 mg l^{-1}-N) terminate the test and repeat the test using different volumes of culture. Record the test period as time t = 3.

If 80 % of the ammonium in this test vessel, at time t = 3, has not been removed, then after 3.5 hours ± 5 minutes, switch off the aeration system. Remove a suitable aliquot from the test vessel containing the largest volume of nitrifying bacterial culture. Transfer this aliquot to a suitable container. Recommence aeration of the buffered bacterial test solution to continue the test. Determine the ammonium concentration ($N_{t=3.5}$) expressed as nitrogen in the aliquot removed. If 80 % of the ammonium in this test vessel (at time t = 3.5) has been removed (corresponding to an ammonium concentration as nitrogen in the range 10 - 12 mg l^{-1}-N) then stop the test. Record the test period as time t = 3.5.

If 80 % of the ammonium in this test vessel, at time t = 3.5, has not been removed, then after 4.0 hours ± 5 minutes, switch off the aeration system. Remove a suitable aliquot from each test vessel and transfer these aliquots to separate suitable containers. Determine the ammonium concentration as nitrogen ($N_{t=4}$) in the aliquot removed.

For each test vessel, calculate the percent removal of ammonium, % R, using the following equation:

$$\% R = 100 (N_{t=0} - N_{t=4}) / N_{t=0} \quad \text{.....(3)}$$

Where:

% R is the percentage ammonium removed in 4 hours.

$N_{t=0}$ is the initial ammonium concentration as nitrogen at time t = 0.

$N_{t=4}$ is the final ammonium concentration as nitrogen at time t = 4.

Plot % R for each test vessel (along the y-axis) against the volume of nitrifying bacterial culture added to the test vessel (along the x-axis). The volume of bacterial culture (to the nearest 0.1 ml) equivalent to 80 % ammonium removal after four hours should be used for the working suspension of nitrifying bacterial culture (6.3). This should be used for determining the inhibitory effects of test materials.

Annex B Examples of calculations

Table B1 Example of a typical worksheet

Date Analyst

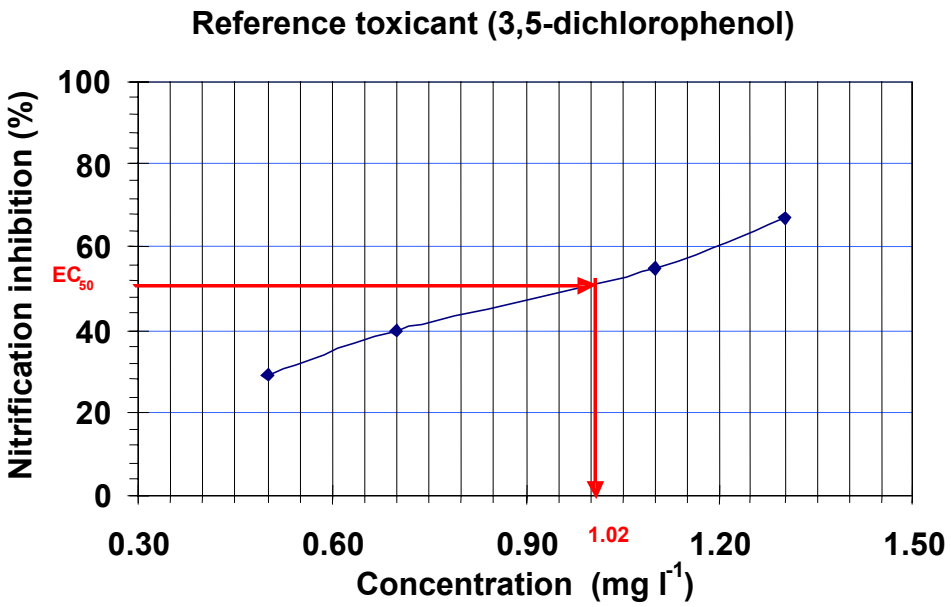
Nitrification inhibition worksheet

Test vessel number	Sample code	Sample name/details	Initial concentration or dilution of sample	Final concentration or dilution of sample	Nitrogen in test vessel (mg l^{-1}-N)	Volume of water to add (ml)	Volume of ammonium chloride solution to add (ml)	$N_{t=0}$ (mg l^{-1}-N)	$N_{t=4}$ (mg l^{-1}-N)	Nitrogen removal* (mg l^{-1}-N) *** ($C = N_{t=0} - N_{t=4}$)	Nitrification inhibition (%)** (% NI = 100 x (C_c - C_s) / C_c)
1		Blank water			0	0	6	54.9	7.4	47.5	
2		Blank water			0	0	6	54.2	8.6	45.6	
3		3,5-dichlorophenol AQC	0.055 mg l^{-1}	0.05 mg l^{-1}	0	0	6	54.0	21.1	32.9	29.3
4		3,5-dichlorophenol AQC	0.11 mg l^{-1}	0.10 mg l^{-1}	0	0	6	53.5	40.7	12.8	72.5
5		3,5-dichlorophenol AQC	0.22 mg l^{-1}	0.20 mg l^{-1}	0	0	6	54.2	48.0	6.2	86.7
6		3,5-dichlorophenol AQC	0.44 mg l^{-1}	0.40 mg l^{-1}	0	0	6	53.8	50.8	3.0	93.6
7		Industrial discharge A	1 to 1	1 to 1.1	0	0	6	54.6	16.8	37.8	18.8
8		Industrial discharge B	1 to 1	1 to 1.1	0.3	0	6	54.7	16.3	38.4	17.5
9		Industrial discharge C	1 to 1	1 to 1.1	2.1	0.2	5.8	55.6	35.2	20.4	56.2
10		Industrial discharge D	1 to 250	1 to 275	0.4	0	6	54.8	40.7	14.1	69.7
11		Industrial discharge D	1 to 500	1 to 550	0.2	0	6	56.2	31.0	25.2	45.9
12		Industrial discharge D	1 to 1000	1 to 1100	0.1	0	6	54.3	19.9	34.4	26.1
13		Industrial discharge E##	1 to 1	1 to 1.1	55.8	5.6	0.4	51.8	42.2	9.6	79.4
14		Industrial discharge F	1 to 1.5	1 to 1.65	60	6	0	55.7	18.4	37.3	19.9
										46.55#	

* obtained using equation 1
** obtained using equation 2 and mean value for blank water

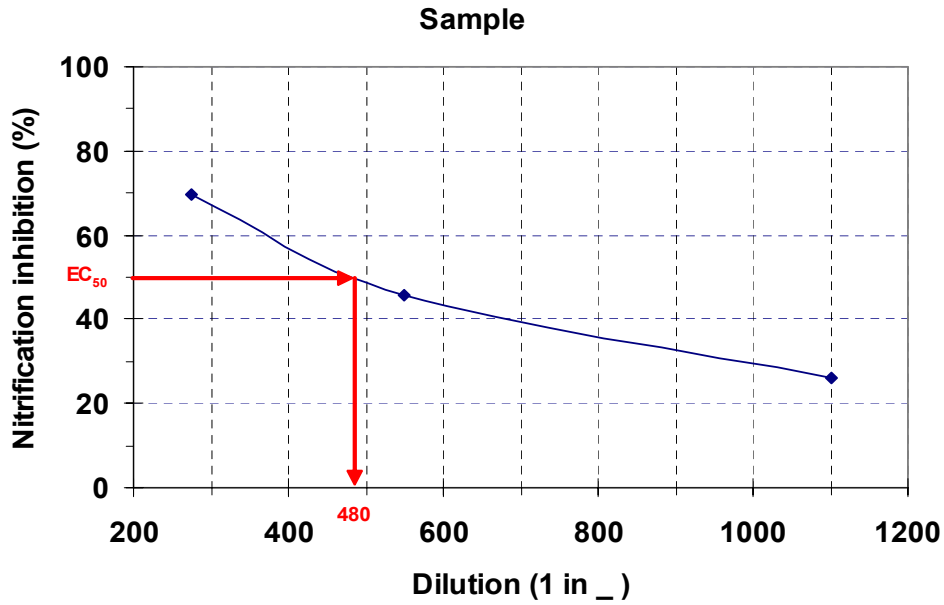
mean of 47.5 and 45.6 for blank water
a pH neutralised sample
Volume of culture per test vessel [1.2] x number of test vessels [14+2] = [19.2] total (ml)
Volume of water per test vessel [2.8] x number of test vessels [14+2] = [44.8] total (ml)
Culture batch number
*** Appropriate AQC samples should also be analysed

Figure B1 Example of EC_{50} interpolation using sample concentration (reference toxicant)



From this graph, the EC_{50} value is 1.02.

Figure B2 Example of EC_{50} interpolation using sample dilution



From this graph, the EC_{50} value is approximately 480-fold dilution.

Address for correspondence

However well procedures may be tested, there is always the possibility of discovering hitherto unknown problems. Analysts with such information are requested to contact the Secretary of the Standing Committee of Analysts at the address given below. In addition, if users would like to receive advanced notice of forthcoming publications please contact the Secretary on the Agency's web-page or by post.

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