

**OECD GUIDELINES FOR THE TESTING OF CHEMICALS**

**Proposal for a revised Guideline 209**

**Inhibition of respiration of activated sludge (carbon and/or ammonium oxidation)**

**INTRODUCTION**

1. This guideline describes a method to determine the effects of a substance on micro-organisms from activated sludge (largely bacteria) by measuring their respiration rate (carbon and/or ammonium oxidation) under defined conditions in the presence of different concentrations of the test substance. The method is based on the ETAD ( Ecological and Toxicological Association of the Dyestuffs Manufacturing industry) test (1, 2) , on the existing OECD Test Guideline 209 (3) and on the revised ISO Standard 8192 (4). The purpose of the test is to provide a rapid screening method to assess the effects of substances on the microorganisms of the activated sludge of the biological (aerobic) stage of waste-water treatment plants . The results of the test may also serve as an indicator of suitable non-inhibitory concentrations of test substances to be used in biodegradability tests (for example OECD 301 series, OECD 310, OECD 302 series and OECD 303).

2. Overall, the method seems to have been applied successfully since it was first published, but on some occasions spurious results were reported (e.g. 2, 5). Concentration related respiration curves are sometimes bi-phasic, dose-response plots have been distorted and EC<sub>50</sub> values have been unexpectedly low (5). Investigations showed that such results are obtained when the activated sludge used in the test nitrifies significantly and the test substance has a greater effect on the oxidation of ammonium than on general heterotrophic oxidation. Therefore, these spurious results may be overcome by performing additional testing using a specific inhibitor of nitrification. By measuring the oxygen uptake rates in the presence and absence of such an inhibitor, e.g. N-allylthiourea (ATU), the separate total heterotrophic and nitrification oxygen uptake rates can be calculated ( 4,7,8). Thus, the inhibitory effects of a test substance on the two processes may be determined and the EC<sub>50</sub> values for both the oxidation of organic carbon (heterotrophic) and ammonium oxidation (nitrification) may be calculated in the usual way.

**NOTE:** In some, rare, cases the inhibitory effect of N-allylthiourea may be partially or completely nullified as a result of complexation with test substances or medium supplements e.g. Cu<sup>++</sup> ions (6) which are essential for *Nitrosomonas*, but are toxic in higher concentration.

3. The need for nitrification in the aerobic treatment of wastewaters, as a necessary step in the process of removing nitrogen compounds from wastewaters by denitrification to gaseous products, has become more

urgent at least in European countries since the EU has now set lower limits for the concentration of nitrogen in treated effluents discharged to receiving waters.

4. For most purposes, the method to assess the effect on organic carbon oxidation processes alone is adequate. An examination of the effect on nitrification alone, or on both nitrification and organic carbon oxidation separately, are required to be known if interpretation issues arise because of the level of nitrification in the sludge.

### **PRINCIPLE OF THE TEST**

5. The respiration rates of samples of activated sludge fed with synthetic activated wastewater are measured in an enclosed cell containing an oxygen electrode after a contact time of 3 hours. Under consideration of the realistic exposure scenario longer contact times could be appropriate. An additional exposure period of 30 minutes is included if the test substance is rapidly degraded, e.g., abiotically via hydrolysis, or is volatile and the dose cannot be adequately maintained. The sensitivity of the activated sludge must be checked with a suitable reference substance at each exposure period.

6. The inhibition of oxygen uptake by organic carbon oxidising micro-organisms may be separately expressed from that by micro-organisms oxidising ammonium by measurement of the rates of uptake of oxygen in the absence and presence of N-allylthiourea, a specific inhibitor of the oxidation to nitrite by the first-stage nitrifying bacteria. The percentage inhibition of the rate of oxygen uptake is calculated by comparison of the rate of oxygen uptake in the presence of a test substance with the mean oxygen uptake rate of the corresponding controls containing no test substance, both in the presence and absence of the specific inhibitor, N-allylthiourea.

7. Any oxygen uptake arising from abiotic processes may be detected by determining the rate in mixtures of test substance, synthetic medium and water, omitting activated sludge. The test is typically used to determine the fifty percent effect concentration ( $EC_{50}$ ) of the test substance and/or the no-observed effect concentration (NOEC).

### **INFORMATION OF THE TEST SUBSTANCE**

8. The identification (preferably CAS number), name (IUPAC), purity, water solubility, vapour pressure, volatility and adsorption characteristics of the test substance should be known to enable correct

interpretation of results to be made. Normally, volatile substances cannot be tested adequately unless special precautions are taken (see paragraphs 16 and 17).

### **APPLICABILITY OF THE METHOD**

9. The test may be applied to water-soluble, poorly soluble and volatile substances. However, it may not always be possible to obtain EC<sub>50</sub> values with chemicals of limited solubility and valid results with volatile chemicals may only be obtained providing that the bulk (say >80%) of the test substance remains in the reaction mixture at the end of the exposure period(s). Analytical support data are required to refine the EC<sub>x</sub> concentration when there is any uncertainty regarding the stability of the test substance or its volatility.

### **REFERENCE SUBSTANCES**

10. Reference substances may be tested periodically in order to assure that the test protocol and test conditions are reliable, and to check the sensitivity of each batch of activated sludge used as microbial inoculums for that working day. The chemical 3,5-dichlorophenol (3,5-DCP) is recommended as the reference inhibitory substance, since it is a known inhibitor of respiration and is used in many types of test for inhibition/toxicity (4). Also copper (II) sulphate pentahydrate can be used as a reference substance for the inhibition of total respiration (9). N-methylaniline can be used as a specific reference inhibitor of nitrification (4).

### **VALIDITY CRITERIA AND REPRODUCIBILITY**

11. The total blank control (without the test substance or reference substance) oxygen uptake rate should be more than 20 mg/l per hour, and the coefficient of variation of oxygen uptake rate in control replicates should not be more than 30% at the end of definitive test.

12. In an international ring test 2004 organized by ISO (4) using activated sludge derived from domestic sewage, the EC<sub>50</sub> of 3,5-DCP was found to lie in the range 2mg/l to 25mg/l for total respiration, 5mg/l to 40mg/l for heterotrophic respiration and 0.1mg/l to 10mg/l for nitrification respiration. If the EC<sub>50</sub> of 3,5-DCP does not lie in the expected range, repeat the test with activated sludge from another source. The EC<sub>50</sub> of copper (II) sulphate pentahydrate lies in the range of 53-155 mg/L (9).

## **DESCRIPTION OF THE TEST METHOD**

### **Test vessels and apparatus**

13. Usual laboratory equipment and the following are required:
- (a) Test vessels – for example, 1000 ml beakers to contain 500 ml of reaction mixture (see 5 in Fig.1).
  - (b) Cell and attachments for measuring concentration of dissolved oxygen. A suitable oxygen electrode, an enclosed cell to contain the sample with no headspace and a recorder (e.g: 4, 7, 8 and in Fig.1). Alternatively, a BOD bottle may be used with a suitable sleeve adaptor for sealing the oxygen electrode against the neck of the bottle (see Fig. 2). To avoid loss of displaced liquid on insertion of the oxygen electrode, it is advisable first to insert a funnel or glass tube through the sleeve, or to use vessels with flared-out rims. In both cases a magnetic stirrer or alternative stirrer method, e.g. self-stirring probe, is also required.
  - (c) Magnetic stirrers and followers, covered with inert material, for use in the test vessels.
  - (d) Aeration device.

If necessary, pass compressed air through an appropriate filter to remove dust and oil and through wash bottles containing water to humidify the air. Aerate the contents of vessels with Pasteur pipettes, or other aeration devices, which do not adsorb chemicals. An orbital shaker operated at orbiting speeds between 150 and 250 rpm with flasks of, for example 2000 ml capacity, can be used to satisfy the oxygen demand for the sludge and overcome difficulties with substances that produce excessive foam, are volatile and therefore lost or are difficult to disperse when aerated by air sparging.

The test system is typically, a number of beakers aerated continuously and sequentially established (e.g., at *ca.* 10 – 15 minute intervals) then analysed in a sequential manner. Validated instrumentation that allows the simultaneous aeration and measurement of the oxygen consumption rate in the mixtures may also be used.

- (e) pH-meter.
- (f) Centrifuge, general bench-top centrifuge for sludge capable of 10,000 m/s<sup>2</sup>.

### **Reagents**

14. Use analytical grade reagents throughout.

### **Water**

15. Distilled or deionised water, containing less than 1mg/L DOC, except where chlorine free tap water is specified.

**Test medium – (100-fold strength OECD synthetic sewage)**

16. Prepare the medium to contain the following constituents at the stated amounts:

peptone	16g
meat extract (or a comparable vegetable extract)	11g
urea	3g
sodium chloride (NaCl)	0.7g
calcium chloride dihydrate (CaCl <sub>2</sub> , 2H <sub>2</sub> O)	0.4g
magnesium sulphate heptahydrate (MgSO <sub>4</sub> , 7H <sub>2</sub> O)	0.2g
anhydrous potassium monohydrogen phosphate (K <sub>2</sub> HPO <sub>4</sub> )	2.8g
distilled or deionized water	to 1 litre

The pH of this solution shall be  $7.5 \pm 0.5$ .

If the prepared medium is not used immediately, store it in the dark at 0°C to 4°C, for no longer than 1 week or under conditions, that do not produce any change in the composition.

17. Alternatively, sterilize components of the medium individually prior to storage, or add the peptone and meat extract shortly before carrying out the test. Prior to use, ensure that the medium is thoroughly mixed and adjust the pH as necessary.

**Test substance**

18. A stock solution should only be prepared for readily water soluble test substances and up to the maximum water solubility (precipitations are not acceptable) only. Poorly water soluble substances, mixtures with components of different water solubility and adsorptive substances have to be directly weighted into the test vessels. In these cases use of stock solutions may be an alternative if dissolved concentrations of the test substances are analytical determined in the test vessels (prior to adding activated sludge). If preparing WAFs (water accommodated fractions) an analytical determination of the dissolved concentrations of the test substances in the test vessels is essential, too. Ultrasonication, organic solvents, dispersants/emulsifiers to improve solubility should be avoided. Pre-stirring suspensions, e.g. overnight is possible when there is adequate information available concerning the stability of the substance under such conditions.

19. The test substance may adversely affect pH control within the test system. Determine the pH of the test substance treated mixtures prior to the test set up in a preliminary trial to ascertain whether pH adjustment will be necessary prior the main test and again on the day of the main test. Neutralise solutions/ suspensions of test substance in water prior to inoculum addition if necessary. However, since neutralisation

may change the chemical properties of the substance, further testing could be necessary to assess the effect of the test substance on the sludge without pH adjustment.

20. The toxic effects of volatile substances, especially in tests in which air is bubbled through the system can result in variable effect levels occurring, owing to losses of the substance during the exposure period. Exercise caution with such substances by performing substance specific analysis of control mixtures containing the substance and modifying the aeration regime.

### **Reference substance**

21. If 3,5-dichlorophenol is used as reference substance, prepare a solution of 1.00g of 3,5-dichlorophenol in 1000ml of water. Use warm water and/or ultrasonication to accelerate the dissolution and make the solution up to volume when it has cooled to room temperature. Check the pH of the solution and adjust, if necessary, with NaOH or H<sub>2</sub>SO<sub>4</sub> to pH 7 – 8.

If copper(II)sulphate pentahydrate is used as a reference substance, concentrations of 58 mg/l, 100 mg/l and 180 mg/l (a factor of 1.8) are used. The substance is weighed in directly into the test vessels (29 – 50 – 90 mg for 500 ml total volume). It is then dissolved with 284 ml of autoclaved tap water. Copper(II)sulphate pentahydrate is easily soluble. When the test is started, 16 ml of synthetic sewage and 200 ml of activated sludge are added.

### **Specific inhibitor of nitrification**

22. Dissolve 2.32 g N-allylthiourea (ATU) in 1000ml of water (paragraph 13). The addition of 2.5 ml of this stock solution to an incubation mixture of final volume of 500 ml results in a final concentration of 11.6 mg ATU/l (10<sup>-4</sup>mol/l) which is sufficient to cause 100% inhibition of nitrification in a nitrifying activated sludge containing 1500 mg suspended solids/l.

### **Abiotic control**

23. Under some, rare conditions, a test substance with strong reducing properties may cause measurable abiotic oxygen consumption. In such cases, abiotic controls are necessary to discriminate between abiotic oxygen uptake by the test substance and microbial respiration. Abiotic controls may be prepared by omitting the inoculum and synthetic medium from the test mixtures. Similarly, abiotic controls without inoculum may be included when supporting analytical measurements are performed to determine the achieved dose during the exposure phase of the test, e.g. when using stock solutions of poorly water soluble substances or mixtures with components with different water solubility. In other cases it may be necessary to prepare an abiotic

control with sterilized inoculum. Some substances may produce or consume oxygen if only the surface area is big enough for reaction, even if they normally need a much higher temperature or pressure to do so. In this respect special attention should be given to peroxy substances. A sterilized inoculum will provide a big surface area.

### **Inoculum**

24. For general use, collect activated sludge from the exit of the aeration tank of a well-operated wastewater treatment works receiving predominantly domestic sewage. Depending on the purpose of the test, other adequate types or sources of activated sludge, e.g. sludge grown in the laboratory, may also be used at suitable suspended solids concentrations of 2 g/l to 4 g/l. However, sludges from different treatment plants are likely to exhibit different characteristics and sensitivities.

25. The sludge may be used as collected but coarse particles have been removed by settling for a short period, e.g. 15 minutes and decanting the upper layer of finer solids for use or sieving (e.g., 1 mm<sup>2</sup> mesh). Alternatively, the sludge may be homogenized in a blender for a *ca.* 15 seconds or longer, but caution is needed regarding the temperature change which might occur for long periods of blending.

26. Washing the sludge can be necessary, e.g., if the endogenous respiration rate is low. First centrifuge for a period to produce a clear supernatant and pellet of sewage solids e.g., 10 minutes at *ca.* 10,000 m/s<sup>2</sup>. Discard the supernatant liquid and re-suspend the sludge in chlorine-free tap water, with shaking, and then remove the wash-water by re-centrifuging and discarding again. Repeat the washing and centrifuging process, if necessary. Determine the dry mass of a known volume of the re-suspended sludge and concentrate by removing liquor or dilute further in chlorine-free tap water to obtain the required sludge solids concentration of 3 g/l. Continuously aerate the activated sludge (e.g. 2l/minute) at the test temperature and, where possible use it on day of collection. If this is not possible, it has been the practice to feed the sludge daily with the test medium (50 ml medium/l activated sludge) for two additional days. The sludge is then used for the test and the results are accepted as valid, provided that no significant change in its activity, by assessment of its endogenous heterotrophic and nitrification respiration rate, has occurred.

27. Difficulties can arise if foaming occurs during the incubation to the extent that the foam and the sludge solids carried on it, are expelled from the aeration vessels. Occasionally, foaming may simply result from the presence of the synthetic sewage, but foaming should be anticipated if the test substance is, or contains, a surfactant. Loss of sludge solids from the test mixtures will result in artificially lowered respiration rates that could mistakenly be interpreted as a result of inhibition. In addition, aeration of surfactant solution concentrates the surfactant in the foam layer; loss of foam from the test system will lower the exposure concentrations. If foaming occurs add a surfactant-free silicone emulsion antifoam agent and/or use the shake flask aeration method. If the problem is associated with the presence of the synthetic sewage,

modify the sewage concentration by including an antifoam reagent at a rate of e.g., 50 µl/l. If foaming is caused by the test substance, determine the quantity needed for abatement at the maximum test concentration, then treat all individual aeration vessels identically (including those, e.g. blank controls and reference vessels where foam is absent).

## **TEST PROCEDURE**

28. The inhibition of three different oxygen uptakes may be determined, namely, total, only heterotrophic and that due to nitrification. For certain purposes the total measurement of total oxygen uptake inhibition could be adequate. The effects on heterotrophic oxygen uptake from the oxidation of organic carbon and those due to the oxidation of ammonium are required to be known, when there is a specific requirement for such two separate end-points for a particular substance or to fulfil a regulatory requirement.

### **Test conditions**

29. Perform the test at a temperature within the range  $20 \pm 2^\circ\text{C}$  and in an atmosphere free from dust and toxic vapours.

### **Test mixtures**

30. Prepare test vessel mixtures ( $F_T$ ) containing dilution water, synthetic medium and stock solutions of the test substance to obtain different known concentrations of the test substance. (See Table 1 for example of volumes of constituents). Adjust the pH to  $7.5 \pm 0.5$ , if required, dilute with water and add the inoculum to obtain equal final volumes in the vessels and to begin the aeration. Do not adjust the pH if the inhibitory effect of pH is to be tested or the effect of the test substance on the sludge without pH adjustment.

### **Reference mixtures**

31. Prepare mixtures ( $F_R$ ) with the reference compound e.g. 3,5-dichlorophenol in place of the test substance in the same way as the test mixtures.

### **Blank controls**

32. Prepare blank controls ( $F_B$ ) at the beginning and end of the exposure period in tests in which the test beakers are set up sequentially at intervals. In tests performed using validated equipment which allows simultaneous measurements of oxygen consumption to be made, at least one blank control should be included in each batch of simultaneous analysis. Blank controls contain an equal volume of activated sludge

and synthetic medium but not test or reference substance. Dilute with water to the same volume as the test and reference mixtures.

### **Abiotic control**

33. If required, for example if a test substance is known or suspected to have strong reducing properties, prepare a mixture  $F_A$  to measure the abiotic oxygen consumption. The mixture will have the same amounts of test substance alone and the same volume as the test mixtures, but no activated sludge.

### **General procedure and measurements**

34. Test mixtures, reference mixtures and the blank and abiotic controls are incubated at the test temperature under conditions of forced aeration (*ca.* 1 L air/minute) to keep the dissolved oxygen concentration above 60 – 70% saturation and to maintain the sludge flocs in suspension. Stirring the cultures is also necessary to maintain sludge flocs in suspension. The incubation is considered to begin with the initial contact of the activated sludge inoculum with the other constituents of the final mixture. At the end of incubation after the specified exposure times of usually 3 hours samples are withdrawn to measure the rate of decrease of the concentration of dissolved oxygen in the cell designed for the purpose (Fig.1) or in a completely filled BOD bottle. The manner in which the incubations begin also depends on the capacity of the equipment used to measure oxygen consumption rates. For example, if it comprises a single oxygen probe, the measurements are made individually. In this case, prepare the various mixtures required for the test in synthetic sewage but withhold the inoculum and then add the requisite portions of sludge to each vessel of the series and start each incubation in turn, at conveniently timed intervals of e.g. 10 to 15 minutes. Alternatively, the measuring system may comprise multiple probes that facilitate multiple simultaneous measurements; in this case, inoculum may be added at the same time to appropriate groups of vessels.

35. The activated sludge concentration in all test, reference and blank (but not abiotic control) mixtures is nominally 1500 mg/l of suspended solids. Measure the oxygen consumption after 3 hours of exposure. Perform additional 30-minute exposure measurements as required and previously described.

### **Nitrification potential of sludge**

36. In order to decide whether a sludge nitrifies and, if so, at what rate, prepare mixtures ( $F_B$ ) as in the blank control and additional ‘control’ mixtures ( $F_N$ ) but which also contain N-allylthiourea at 11.6 mg/l. Aerate and incubate at  $20^{\circ}\pm 2^{\circ}\text{C}$  for 3 hours and then measure the rates of oxygen uptake and calculate the rate of oxygen uptake due to nitrification.

## Test designs

### *Range-finding test*

37. A preliminary test is used when necessary to estimate the range of concentrations of the test substance needed in a definitive test for determining the inhibition of oxygen consumption. Alternatively, the absence of inhibition of oxygen consumption by the test substance in a preliminary test may demonstrate that a definitive test is unnecessary, but triplicates at the highest tested concentration of the preliminary test (typically 1000 mg/l, but dependent on the data requirement) should be included.

Table 1      Examples of mixtures for the preliminary test

<b>Reagent</b>	<b>Original Concentration</b>				
Test substance stock solution	10 g/l				
Synthetic medium stock solution	See paragraph 17				
Activated sludge stock suspension	3 g/l of suspended solids				
Components of mixtures	Dosing into test vessels (a)				
	F <sub>T1</sub>	F <sub>T2</sub>	F <sub>T3-5</sub>	F <sub>B1-2</sub>	F <sub>A</sub>
Test substance stock solution (ml) (paragraphs 19, 20)	0.5	5	50	0	50
Synthetic medium stock solution (ml) (paragraph 17)	16	16	16	16	16
Activated sludge suspension (ml) (paragraphs 25, 26, 27)	250	250	250	250	0
Water (paragraph 16)	233.5	229	184	234	434
Total volume of mixtures (ml)	500	500	500	500	500
Concentrations in the mixture					
Test suspension (mg/l)	10	100	1000	0	1000
Activated sludge (suspended solids) (mg/l)	1500	1500	1500	1500	0
(a) The same procedure should be followed with the reference substance, to give flasks F <sub>R1-3</sub>					

Perform the test using at least three concentrations of the test substance, for example, 10 mg/l, 100 mg/l and 1000 mg/l with a blank control and, if necessary, an abiotic control with the highest concentrations of the test substance (see as example Table 1). Ideally the lowest concentration should have no effect on oxygen consumption. Calculate the rates of oxygen uptake and the rate of nitrification if relevant and then calculate

the percentage inhibition. Depending on the purpose of the test, it is also possible simply to determine the toxicity of a limit concentration, e.g. 1000 mg/l. If no significant toxic effect occurs at this concentration, further testing at higher or lower concentrations is not necessary.

### ***Definitive test***

#### **Inhibition of total oxygen uptake**

38. Carry out the test using a range of concentrations deduced from the preliminary test. Use at least five concentrations in a logarithmic series and include blank controls as described. The abiotic control does not need to be repeated if there was no oxygen uptake in the preliminary test, but if significant uptake occurs include abiotic controls for each concentration of test substance. The sensitivity of the sludge must be checked using the reference substance 3,5-dichlorophenol. Check the sludge sensitivity for each test series, since the sensitivity is known to fluctuate. In all cases, samples are withdrawn from the test vessels after 3 hours for measurement of the rate of oxygen uptake in the oxygen electrode cell. From the data collected, the specific respiration rate of the control and test mixtures is calculated; the percentage inhibition is then calculated from equation 7, below.

#### **Differentiation between inhibition of heterotrophic respiration and nitrification**

39. The use of the specific nitrification inhibitor, ATU, enables the direct assessment of the inhibitory effects of test substances on heterotrophic oxidation and by subtracting the oxygen uptake rate in the presence of ATU from the total uptake rate (no ATU present), the effects on the rate of nitrification may be calculated. Prepare two sets of reaction mixtures, comprising at least five concentrations of the test substance, but to one set add, additionally, ATU to each mixture at a final concentration of 11.6 mg/l, which has been shown to inhibit nitrification completely in sludges with suspended solids concentrations of up to 3000 mg/l. Measure the oxygen uptake rates after the exposure period; these direct values represent heterotrophic respiration only and the differences between these and the corresponding total respiration rates represent nitrification. The various degrees of inhibition are then calculated.

### **Measurements**

40. After the exposure period(s) transfer a sample from the first aeration vessel to the oxygen electrode cell (Fig.1) and immediately measure the concentration of dissolved oxygen. If a multiple electrode system is available, then the measurements may be made simultaneously. Stirring (by means of a covered magnet) is essential at the same rate as when the electrode is calibrated to ensure, that the probe responds with minimal delay to changing oxygen concentrations, and to allow regular and reproducible oxygen measurement in the measuring vessel. Usually, the self-stirring probe system of some oxygen electrodes is adequate. The cell must be rinsed with tap water between measurements. Alternatively, use the sample to fill a BOD bottle (Fig.2) fitted with a magnetic stirrer. Insert an oxygen probe with a sleeve adaptor into the neck of the bottle

and start the magnetic stirrer. In both cases measure and record continuously the concentration of dissolved oxygen for a period, usually 5 to 10 minutes or until the oxygen concentration falls below 2 mg/l. Remove the electrode, return the mixture to the aeration vessel and continue aerating and stirring.

### **Verification of the test substance concentration**

41. For some purposes it might be needed to measure the concentration of the test substance in the test vessels during the test (e.g. for PNEC calculations). If using stock solutions of poorly water soluble substances, of mixtures with components with different water solubility or of concentrations of good water soluble substances about maximum water solubility analytical estimation of the test substance concentrations in the test vessels is obligatory.

## **DATA AND REPORTING**

### **Calculation of oxygen uptake rates**

42. Calculate the oxygen uptake rates from the measured values, e.g. from the linear part of the graphs of oxygen concentration versus time, limiting the calculations to oxygen concentrations between 2.0 mg/l and 7.0 mg/l, since higher and lower concentrations may themselves influence rates of consumption. Excursion into concentration bands below or above these values is occasionally unavoidable and necessary, for example, when respiration is heavily suppressed and consequently very slow or if a particular activated sludge respire very quickly. This is acceptable provided the extended sections of the uptake graph are straight and their gradients do not change as they pass through the 2.0 mg/l or 7.0 mg/l O<sub>2</sub> boundaries. Any curved sections of the graph indicate that the measurement system is stabilising or the uptake rate is changing and should not be used for the calculation of respiration rates. Express the oxygen uptake rate in milligrams per litre per hour (mg/lh) or milligrams per gram dry sludge per hour (mg/gh). The oxygen consumption rate, R, in mg/lh, may be calculated or interpolated from the linear part of the recorded oxygen decrease graph according to Equation 1:

$$R = (Q_1 - Q_2)/\Delta t \cdot 60 \quad (1)$$

where Q<sub>1</sub> is the oxygen concentration at the beginning of the selected section of the linear phase (mg/l);

Q<sub>2</sub> is the oxygen concentration at the end of the selected section of the linear phase (mg/l);

Δt is the time interval between these two measurements (min.).

The specific respiration rate (R<sub>s</sub>) is expressed as the amount of oxygen consumed per g dry weight of sludge per hour (mg/gh) according to Equation 2:

$$R_s = R/SS \quad (2)$$

where SS is the concentration of suspended solids in the test mixture (g/l).

The different indices of R which may be combined are:-

S	specific rate
T	total respiration rate
N	rate due to nitrification respiration
H	rate due to heterotrophic respiration
A	rate due to abiotic processes
B	rate based on blank assays (mean)

### **Calculation of oxygen uptake rate due to nitrification**

43. The coherence of total respiration ( $R_T$ ), nitrification respiration ( $R_N$ ) and heterotrophic respiration ( $R_H$ ) is given by Equation 3:

$$R_N = R_T - R_H \quad (3)$$

where  $R_N$  is the rate of oxygen uptake due to nitrification (mg/lh);

$R_T$  is the measured rate of oxygen uptake by the blank control (no ATU;  $F_B$ ) (mg/lh).

$R_H$  is the measured rate of oxygen uptake of the blank control with added ATU ( $F_N$ ) (mg/lh).

This coherence is valid for blank values ( $R_{NB}$ ,  $R_{TB}$ ,  $R_{HB}$ ), abiotic controls ( $R_{NA}$ ,  $R_{TA}$ ,  $R_{HA}$ ) and assays with test substances ( $R_{NS}$ ,  $R_{TS}$ ,  $R_{HS}$ ) (mg/gh). Specific respiration rates are calculated from:-

$$R_{NS} = R_N/SS \quad (4)$$

$$R_{TS} = R_T/SS \quad (5)$$

$$R_{HS} = R_H/SS \quad (6)$$

If  $R_N$  is insignificant (e.g. < 5% of  $R_T$  in blank controls) in a preliminary test, it may be assumed that the heterotrophic oxygen uptake equals the total uptake and that no nitrification is occurring. An alternative source of activated sludge would be needed if the tests were to consider effects on heterotrophic and nitrifying micro-organisms. A definitive test is performed if there is evidence of suppressed oxygen uptake rates with different test substance concentrations.

### **Calculation of percentage of inhibition**

44. The percentage inhibition,  $I_T$ , of total oxygen consumption at each concentration of test substance is given by Equation 7:

$$I_T = [1 - (R_T - R_{TA})/R_{TB}] \cdot 100\% \quad (7)$$

Similarly, the percentage inhibition of heterotrophic oxygen uptake,  $I_H$ , at each concentration of test substance is given by Equation 8:

$$I_H = [1 - (R_H - R_{HA}) / R_{HB}] / 100\% \quad (8)$$

Finally, the inhibition of oxygen uptake due to nitrification,  $I_N$ , at each concentration is given by Equation 9: -

$$I_N = [1 - (R_T - R_H) / (R_{TB} - R_{HB})] \cdot 100\% \quad (9)$$

Plot the percentage inhibition of oxygen uptake against logarithm of the test substance concentration (inhibition curve, see Fig.3 Annex 3). Inhibition curves are plotted for each aeration period, for example after 30 min and 3h. Calculate or interpolate from the graph the concentration of test substance which inhibits the oxygen uptake by 50% ( $EC_{50}$ ). If suitable data are available, the 95% confidence limits of the  $EC_{50}$ , the slope of the curve and suitable values to mark the beginning of inhibition (for example,  $EC_{10}$  or  $EC_{20}$ ) and the end of the inhibition range (for example,  $EC_{80}$  or  $EC_{90}$ ) may be calculated or interpolated.

NOTE: In view of the variability often observed in the results, it may in many cases be sufficient to express the results additionally in order of magnitude, for example:

$$EC_{50} < 1\text{mg/l}$$

$$EC_{50} \quad 1\text{mg/l to } 10\text{mg/l}$$

$$EC_{50} \quad 10\text{mg/l to } 100\text{mg/l}$$

$$EC_{50} > 100\text{mg/l}$$

### **Interpretation of results**

#### ***ECx***

45.  $EC_x$ -values including their associated lower and upper 95% confidence limits for the parameter are calculated using appropriate statistical methods (e.g. probit analysis, logistic or Weibull function, trimmed Spearman-Kärber method or simple interpolation). An  $EC_x$  is obtained by inserting a value corresponding to  $x\%$  of the control mean into the equation found. To compute the  $EC_{50}$  or any other  $EC_x$ , the per-treatment means ( $X$ ) should be subjected to regression analysis.

#### ***NOEC estimation***

46. If a statistical analysis is intended to determine the NOEC/LOEC, per-vessel statistics (individual vessels are considered as replicates) are necessary. Appropriate statistical methods should be used according to OECD Document 54 on the Current Approaches in the Statistical Analysis of Ecotoxicity Data: a Guidance to Application [11]. In general, adverse effects of the test substance compared to the control are investigated using one-tailed (smaller) hypothesis testing at  $p \leq 0.05$ .

NOTE: When nitrifying sludges are used, complications may arise due to the occasionally disparate sensitivity of nitrifying micro-organism. Thus, for example, the respiration curves for inhibition of total oxygen uptake may be biphasic, e.g. EC<sub>50</sub> values may be significantly lower and inhibition-concentration plots may be distorted. Additionally, changes may take place in the activity of the nitrifying population of a sludge sample between a preliminary assessment and a subsequent definitive investigation giving rise to two apparently contradictory sets of results within the same study. Such interferences may be clarified by measuring the rate of oxygen uptake in the presence and absence of a specific inhibitor of nitrification added deliberately to the test mixtures. This has several benefits for interpreting results. First, it confirms whether or not the sludge sample is nitrifying. Secondly, it quantifies the contribution of nitrification (if present) to the overall oxygen uptake, and, third, it facilitates an assessment within the one test of toxicity both to heterotrophic and autotrophic oxygen uptake.

### **Test report**

47. The test report must include the following information:

#### ***Test substance:***

- common name, chemical name, CAS number, purity;
- physico-chemical properties of the test substance (e.g. log K<sub>ow</sub>, water solubility, vapour pressure, Henry's constant (H) and possible information on the fate of the test substance e.g. adsorption to activated sludge).
- 

#### ***Test system:***

- source, conditions of operation of the wastewater treatment plant and influent it receives, concentration, pre-treatment and maintenance of the activated sludge;

#### ***Test conditions:***

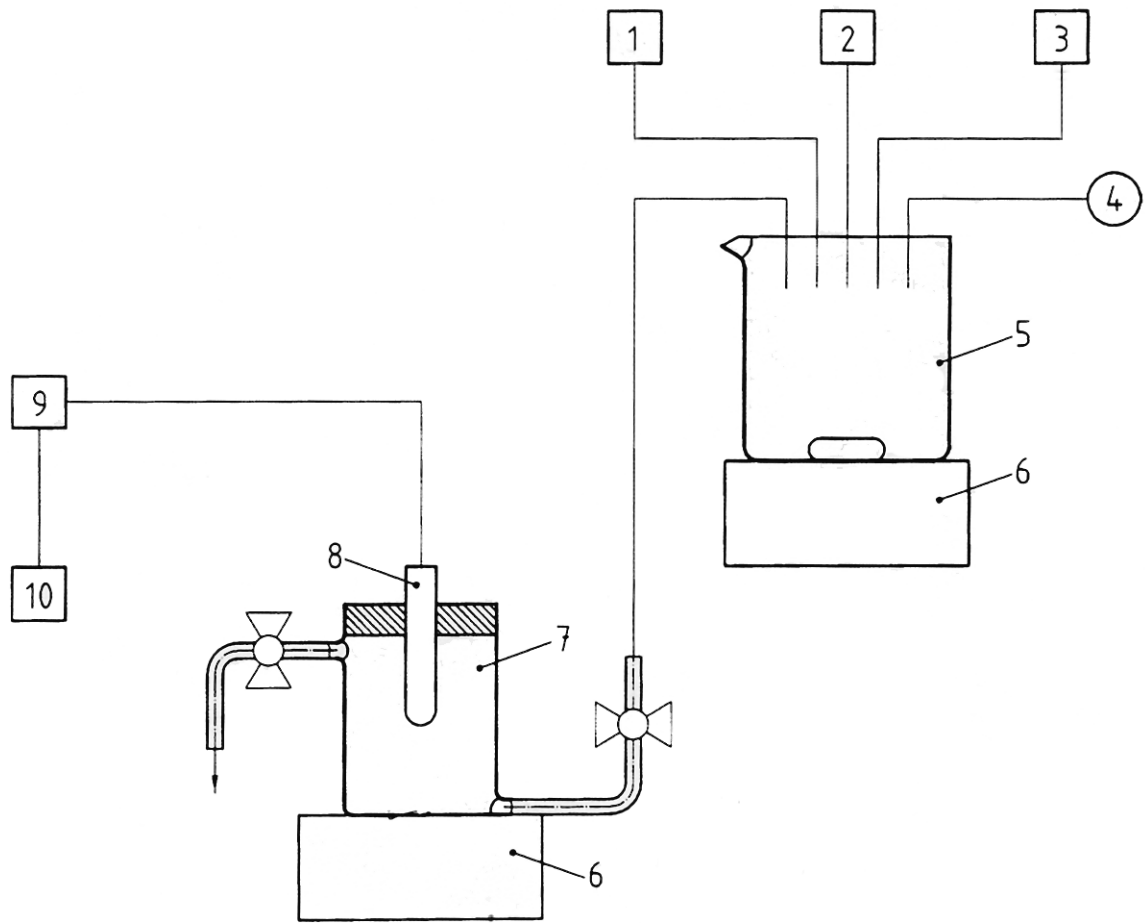
- test temperature, pH during the test and duration of the exposure phase(s),

#### ***Results:***

- all measured data, inhibition curve(s) and method for calculation of EC<sub>50</sub>;
- EC<sub>50</sub> and, if possible, 95 per cent confidence limits, possibly EC<sub>20</sub>, EC<sub>80</sub>; possibly NOEC and the used statistical methods, no observed adverse effect concentration (NOAEC) if the EC<sub>50</sub> cannot be determined.
- indicate results for total, heterotrophic and nitrification inhibition;
- abiotic oxygen uptake in the physico-chemical control (if used);
- name of the reference substance and results with this substance;
- all observations and deviations from the standard procedure which could have influenced the result.

## LITERATURE

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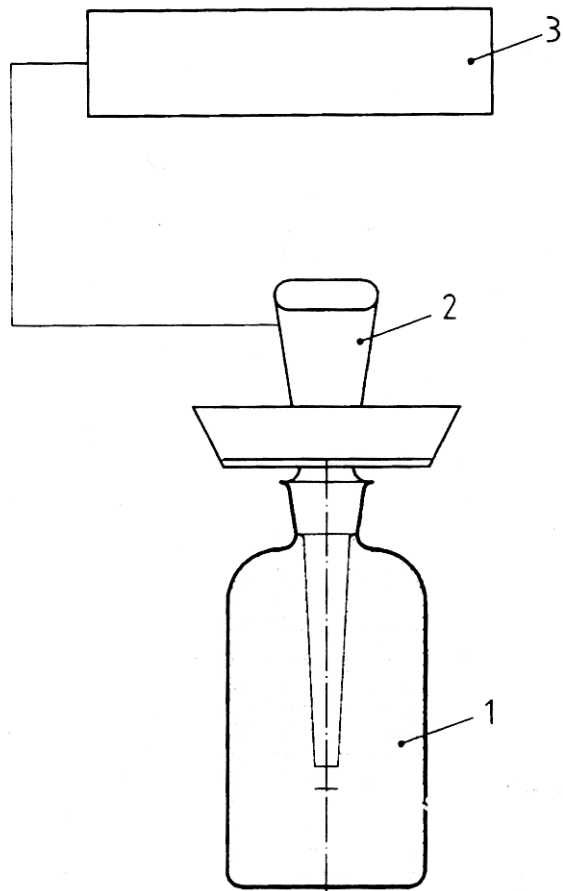
**Annex 1**

**Key**

- 1 activated sludge
- 2 synthetic medium
- 3 test material
- 4 air
- 5 mixing vessel
- 6 magnetic stirrer
- 7 oxygen measuring cell
- 8 oxygen electrode
- 9 oxygen measuring instrument
- 10 recorder

Fig. 1 Examples for measuring unit

Annex 2

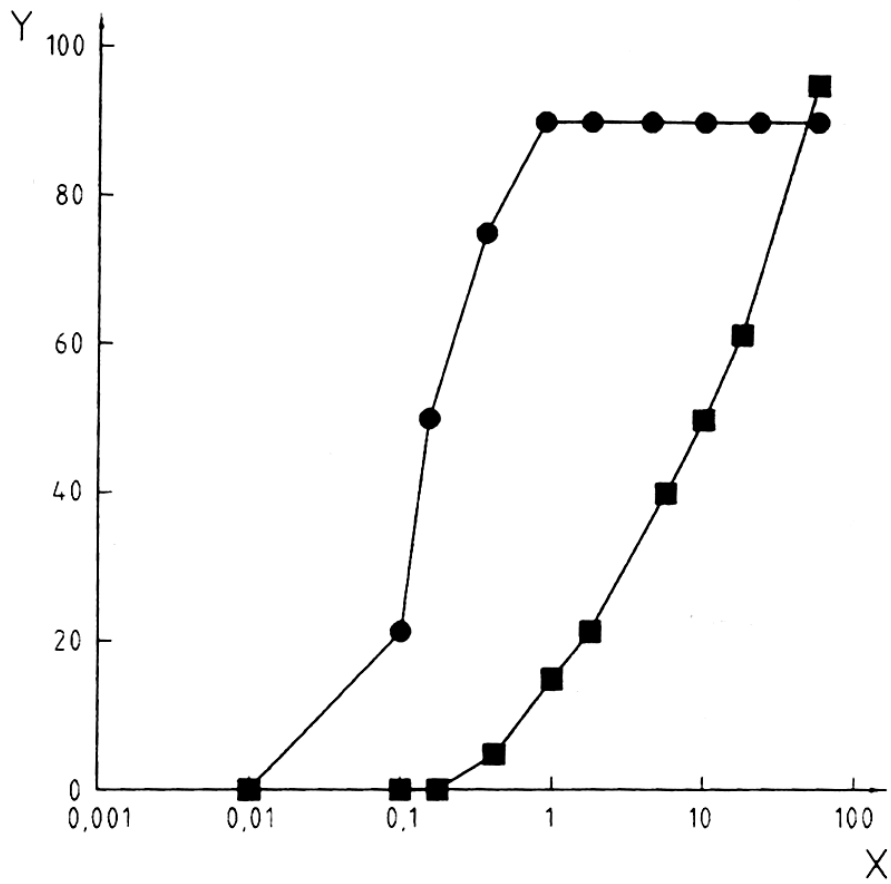


Key

- 1 Test vessel
- 2 oxygen electrode
- 3 oxygen measuring instrument

Fig 2. Example of measuring unit, using a BOD bottle

Annex 3



Key

X concentration of 3,5-dichlorophenol (mg/l)

Y inhibition (%)

—■— inhibition heterotrophic respiration

—●— inhibition nitrification

} using a nitrifying sludge

Fig. 3 Example of inhibition curves