Determination of the ready biodegradeblity of chemicals according to OECD 301F

Application Report: “Manometric Respirometry”

Measurement methods:
Respirometry, differential pressure measurement

Measurement range:
0–100% biodegradability; pressure: 500–1,350 hPa

Measuring equipment:
- **Pressure Transducers**
  Note: The pressure transducers used must be capable of outputting differential pressure. This is not the case with all systems. Please check with your device manufacturer regarding this capability before taking a measurement.
- **pH meter:** Minimum resolution of the pH reading display: 0.01
- **pH measurement chain:**
  Membrane: cylindrical, spherical, or conical membrane
  Diaphragm: split ring or platinum wire

Accessories:
- Magnetic stirring platform for sample vials
- Brown sample bottles with a nominal volume of 510 ml (minimum of 4 bottles)
- Stirrer bars with a stirrer bar remover
- Thermostatic cabinet (Temp. = 22 °C ± 1 °C or a user-defined temperature)
  Note: *22 ± 2 °C is the allowed range for incubation. The temperature maintenance should be ± 1 K or better.*
- Rubber quiver
- Opaque container for aerating the biological inoculum (V ≈ 1 l)
- Filter or decanter
- Laboratory scale
- Aeration pump with a strainer
- possibly a centrifuge for processing a sludge sample
- Variable-volume pipettes with a volume of 20 ml and additional tips as needed
- 1,000-ml measuring flasks (7 pieces)

Reagents:
- Sodium hydroxide pellets
- 0.1 M NaOH solution
- 0.1 M HCl solution
- HCl Concentrate
- NaEDTA
- Potassium dihydrogen phosphate
- Dipotassium hydrogen phosphate
- Disodium hydrogen phosphate dihydrate
- Ammonium chloride
- Magnesium sulfate heptahydrate
- Calcium chloride
- Iron(III) chloride hexahydrate
- Possibly excipients to make the test substance water-soluble
- Technical buffer solutions with pH values of 7.00 and 10.01

Description of the Measurement Procedure

The substance to be tested for biodegradability is dissolved in a diluent containing microbial inoculum and incubated at a constant temperature for 28 days. The decomposition processes caused by the bacteria oxidize the test substance in the test solution, consuming a portion of the oxygen from the gas phase of the test bottle. The carbon dioxide produced in this process is absorbed by an absorbent (usually NaOH pellets) and thus removed from the gas phase of the bottle. The negative pressure that builds up slowly in this manner is directly proportional to the oxygen consumed and is therefore a characteristic parameter for the degradation performance of the microorganisms used. By calculating the theoretically expected oxygen consumption for the oxidation of the entire test substance added, the degradation performance of the microorganisms over the observed period can be expressed as a percentage of the theoretical complete degradation. If it is not possible to calculate the theoretical oxygen demand, the less meaningful ratio of the actual measured oxygen consumption to the COD of the test solution can still be reported as biodegradability. However, this value must be specifically identified by the addition of "x% of the COD."

Requirements for deionized water or distilled water used to prepare the dilution water

Only deionized or distilled water that is free of toxic substances such as copper or solvents is suitable for preparing the dilution water. Such toxic contaminants may already be present in the raw water from which the deionized or distilled water is produced, or they may be introduced into the dilution water during the processing, such as filtration through an ion exchanger. In cases of highly variable results and unusually low degradation rates, the water should therefore also be analyzed using appropriate analytical methods if in doubt. Furthermore, the organic carbon content in the dilution water must not exceed 10% of the organic carbon content introduced by the test substance. To verify compliance with this limit, it is recommended to perform a measurement using the LC-OCD method (Liquid Chromatography – Organic Carbon Detection). In any case, water from the same process batch should always be used for each test series.

Definitions

- **Test substance:** A substance whose biodegradability is to be tested.
- **Dilution water:** A salt solution prepared according to the specified formula and used to adjust the osmotic conditions for the microorganisms. This solution is required for diluting all cultures.
- **Inoculum:** A solution that can be prepared from a wide variety of sources, such as activated sludge or surface water samples, and that contains the microorganisms necessary for degradation.
- **Measurement solution:** A mixture of diluent, inoculum, and stock solution that is poured into the measuring flasks and in which the actual measurement takes place.
- **Stock solution:** A solution of the test substance in diluent water, which serves to ensure the homogeneous introduction of the test substance into the test solution.
- **Blank solution:** A solution consisting of diluent and inoculum, which is used to mathematically compensate for oxygen consumption caused by any organic impurities present in the inoculum and also serves as a medium for completely insoluble substances.

Preliminary Work

Inoculum:

The biological starter material can be obtained from a wide variety of sources. A sludge sample from the biological stage of a wastewater treatment plant is just as feasible as a sample from surface water. The only important thing is that the sample is adequately aerated during transport and during extended storage periods. When using sludge, it is also important to ensure that it is filtered or decanted before inoculation to remove as much organic material as possible, which could skew the measurement results.

For the recommended procedure described here, in which 1 l of test solution contains 20 ml of inoculum, the concentration of organic matter in the inoculum must not exceed 1.5 g/l. If solutions with a different composition are used, the concentration of organic matter in the test solution must be limited to 30 mg/l. If this cannot be clearly guaranteed after filtration or decanting, the sludge must be dried, the solid content weighed, and the inoculum diluted with dilution water in accordance with the specifications before being added to the test solution. Reference [1] describes a wide range of treatment options for a variety of sources of biological inoculum. In particular, specific procedural instructions are provided here for treatment when inhibitory or toxic substances are suspected, for concentration and dilution, and for washing, centrifugation, homogenization, and conditioning of sludges to meet the test conditions. If your sample requires specific pretreatment due to its characteristics

(e.g., too viscous, contaminated, or not adapted to the measurement conditions), please refer to this literature for the relevant procedural instructions.

Conditioning in Biology:
To acclimate the microorganisms to the osmotic conditions in the test solution, it may be necessary to condition them before use. This is done by preparing a solution with a concentration of approximately 3–5 g of inoculum per 1 liter of diluent. This mixture is aerated for 5–7 days in an opaque container and can then be used directly as a conditioned inoculum solution to inoculate the dilution solution used for the measurement. If sludge is used for this preparation, the solution must be diluted once more by a factor of 5 before inoculation to ensure that the solids content in the measurement solution is definitely below 30 mg/l. In this case, the determination of dry matter can be omitted, since the solids content has already been reduced accordingly due to the high dilution. In certain cases, conditioning can also serve to increase the accuracy of the entire procedure if significant statistical deviations are observed during the evaluation.

Amount of dilution water:
Four salt solutions are needed to prepare the dilution water.
Note: When preparing the following salt solutions, use only water that meets the requirements listed above and analytical-grade reagents.

Solution 1: Phosphate buffer solution with a pH of 7.4

- 8.5 g potassium dihydrogen phosphate (KH_2PO_4)
- 21.75 g dipotassium hydrogen phosphate (K_2HPO_4)
- 33.4 g disodium hydrogen phosphate dihydrate ($Na_2 HPO_4 *2H_2O$)
- 0.5 g ammonium chloride (NH_4Cl)

Pour into a 1000-ml volumetric flask containing approximately 500 ml of water, then mix by shaking gently. Fill to the calibration mark and shake again.
Note: The pH of this buffer solution should be 7.4 ± 0.2 without any further adjustment.

Solution 2: Calcium chloride, 27.5 g/l solution

- 27.5 g of anhydrous calcium chloride ($CaCl_2$)
  (or an equivalent amount if the hydrate is used (e.g., 36.4 g of $CaCl_2 *2H_2O$))

Pour into a 1000-ml volumetric flask containing approximately 500 ml of water, then mix by shaking gently. Fill to the calibration mark and shake again.

Solution 3: Magnesium sulfate heptahydrate, 22.5 g/l solution

- 22.5 g magnesium sulfate heptahydrate ($MgSO_4 \cdot *7H_2O$)

Pour into a 1000-ml volumetric flask containing approximately 500 ml of water, then mix by gently shaking. Fill to the calibration mark and shake again.

Solution 4: Iron(III) chloride hexahydrate, 0.25 g/l solution

- 0.25 g of iron(III) chloride hexahydrate ($FeCl_3 *6H_2O$)

Pour into a 1000-ml volumetric flask containing approximately 500 ml of water, then mix by shaking gently. Fill to the calibration mark and shake again.
Note: To ensure the long-term stability of Solution 4, add one drop of concentrated HCl or 0.4 g of NaEDTA.

To prepare the dilution water, first fill a 1000-ml volumetric flask with approximately 800 ml of water, pipette 10 ml of Solution 1 into it, and mix the solution by gently shaking the flask. Then add 1 ml each of Solutions 2–4 and fill the volumetric flask with water up to the calibration mark.

Stock solution of the test substance:
If possible, the test substance should be dissolved in a stock solution before being added to the test solution to ensure homogeneous distribution in the test solution. Particularly when testing mixtures of solids in which the concentration of the test substance is low, care must be taken to ensure thorough homogenization before preparing the stock solution. Furthermore, all other components in a mixture must be tested separately for biodegradability to ensure that oxygen consumption results solely from the degradation of the test substance.

Emulsifiers and other additives that ensure the solubility of the test substance may be used provided they are neither biodegradable nor toxic to living organisms and do not cause any further interference with the measurement process, such as excessive foaming or overheating due to exothermic reactions. The use of solid carriers for solid test substances is not recommended; however, for oily test substances, the use of such aids may be appropriate. In any case, when using an additive, an additional sample must be added to the test series that contains only the additive in inoculated diluent water. If multiple additives are used, each one must be tested individually. Additives are permitted only if they show no degradability in these blank values.

Using the permitted mixing methods, an aqueous (with diluent water) stock solution of the test substance with a concentration of approximately 1–10 g/l is prepared; this value should be understood only as a rough guideline, since lower concentrations may also be possible, depending, for example, on the solubility of the test substance. If only a small amount of the test substance is available, correspondingly small amounts of the stock solution should be prepared. In any case, however, the exact concentration of the test substance in the stock solution must be known. If the test substance is completely insoluble in water even with permitted additives, proceed according to the instructions in the section “Procedure in Case of Complete Insolubility of the Test Substance.”

Calculation of the Theoretical Oxygen Demand (ThSB):
The theoretical oxygen demand can only be calculated if the chemical composition of the test substance is known. If it is not known, the COD of the test substance must be determined [2], and the calculations described above must be performed using this value instead of the ThSB. In this case, the result must be labeled “x % COD.” For a substance of the form $C_c\ H_h\ Cl_{cl}\ N_n\ Na_{na}\ O_o\ P_p\ S_s$, the ThSB is calculated using the formulas given below, where the uppercase letters represent the respective chemical elements and the lowercase subscript letters represent the number of atoms in a molecule of the test substance. If a particular type of atom is not present in the molecule, the value of its subscript must be set to zero.
M stands for the molar mass of the test substance in $\frac{\mathrm{g}}{\mathrm{mol}}$

The information in square brackets indicates the units of the values and is included for the sake of completeness. The unit of the ThSB should be interpreted as mg of oxygen per mg of test substance used.
The following applies to oxidation without nitrification:

$$\mathrm{ThOD} = \frac{16\left[\frac{\mathrm{g}}{\mathrm{mol}}\right] \times \left(2\mathrm{c} + \frac{1}{2}(\mathrm{h} - \mathrm{cl} - 3\mathrm{n}) + 3\mathrm{s} + \frac{5}{2}\mathrm{p} + \frac{1}{2}\mathrm{na} - \mathrm{o}\right) \times \left[\frac{\mathrm{mg}}{\mathrm{mg}}\right]}{\mathrm{M}\left[\frac{\mathrm{g}}{\mathrm{mol}}\right]}$$

The following applies to oxidation with nitrification:

$$\mathrm{ThOD} = \frac{16\left[\frac{\mathrm{g}}{\mathrm{mol}}\right] \times \left(2\mathrm{c} + \frac{1}{2}(\mathrm{h} - \mathrm{cl}) + \frac{5}{2}\mathrm{n} + 3\mathrm{s} + \frac{5}{2}\mathrm{p} + \frac{1}{2}\mathrm{na} - \mathrm{o}\right) \times \left[\frac{\mathrm{mg}}{\mathrm{mg}}\right]}{\mathrm{M}\left[\frac{\mathrm{g}}{\mathrm{mol}}\right]}$$

Select the formula based on the expected degradation process.

Implementation

You must prepare 1 liter of a test solution consisting of inoculum, stock solution, and diluent water, and calculate the exact concentration of the test substance in the test solution. Based on the concentration of the stock solution you prepared, calculate the volume of this solution that must be pipetted into a 1000-ml volumetric flask to achieve a concentration of 100 mg/l of the test substance in the measuring solution.

Example:
The requirement is as follows: A concentration of 100 mg/l of the test substance must be prepared in a 1000-ml volumetric flask as part of the measurement procedure.

→ c = 100 mg/L and V = 1 L

Using this formula $\mathbf{c = \frac{m}{V}}$, the mass m is given by:

m = c × V = 100 mg/L × 1 L = 100 mg = 0.1 g.

Therefore, 0.1 g of the pure test substance must be added to the measuring solution. However, the test substance is present in the stock solution; thus, the corresponding volume of the stock solution must be pipetted into the measuring solution flask. If, for example, your stock solution has a concentration of c = 5 g/l, then the result is:

$$\mathbf{c = \frac{m}{V} \rightarrow V = \frac{m}{c} = \frac{0.1g}{5g} = 0.02l = 20ml}$$

In this case, you would need to pipette 20 mL of the stock solution into the 1-liter volumetric flask to obtain a concentration of 100 mg/l of the test substance in the test solution after filling to the mark. Place approximately 500 ml of diluent water in a 1-liter volumetric flask and pipette the calculated amount of stock solution into it. Then add 20 ml of the inoculum and fill the flask with diluent water to a level a few centimeters below the mark. Measure the pH of the solution. If necessary, adjust the pH to 7.4 ± 0.2 using a few drops of a 0.1 M NaOH solution or a 0.1 M HCl solution. Next, fill the flask with diluent water up to the calibration mark. Since a duplicate determination of the test solution is recommended for each measurement run, the prepared test solution must be divided between two measuring bottles. Fill two measuring bottles with 300 ml of the test solution each. This volume was chosen to ensure that, when the bottle is sealed, there is still a sufficiently large volume of gas above the test solution.

Note: If the abiotic degradation of the test substance is to be investigated, the test solution must be spiked with a substance toxic to microorganisms - such as copper ions - in sufficient concentration prior to incubation. In this case, it is, of course, recommended to omit the inoculum material when preparing the test solution.

To account for any oxygen uptake resulting from the degradation of organic matter in the inoculum during the calculation, a blank solution must be prepared. Here, too, duplicate determinations are recommended. Place 20 mL of the inoculum in a 1-liter volumetric flask and fill it with diluent water to a level a few centimeters below the mark. It is absolutely essential that the same inoculum material be used at this stage in the same quantity as when inoculating the test solution, since the concentration of microorganisms and organic contaminants is unknown. This blank value is subtracted from the oxygen consumption of the test solution and must reflect as accurately as possible the oxygen consumption of the organic substances from the inoculum material contained in the test solution. Now measure the pH of the blank solution. If necessary, adjust the pH to 7.4 ± 0.2 using a few drops of a 0.1 M NaOH solution or a 0.1 M HCl solution. Then fill the flask to the mark with diluent water. Fill two measuring bottles with 300 ml each of the blank solution. Place a rubber sleeve containing 2–3 NaOH tablets into the neck of each of the 4 measuring bottles and screw the measuring heads onto the bottles. Incubate the prepared measuring bottles at 22 ± 2°C for 28 days.

Note: Other measurement temperatures can be set as needed, but must be kept constant within a range of ±1°C during the measurement. OECD Guideline 301 specifies a temperature range of 20–24 °C; however, the operating temperature range of the sensor heads (see technical specifications) ultimately limits their use. For WTW® measurement heads, the permissible operating range is between +0°C and +55°C.

Procedure to follow when the test substance is completely insoluble

If the test substance cannot be prepared as an aqueous solution even with the addition of excipients, it must be weighed directly into inoculated diluent water to serve as the test solution. Here, too, it is recommended to perform duplicate determinations of the measured values. Prepare a spiked dilution water as described in the previous sections and calculate the required weight of the test substance to achieve the required concentration of 100 mg/l of test substance in the 300 ml of the measurement solution. Here, too, the potential introduction of solids from sludge must be limited to a maximum of 30 mg/l in the measurement solution. Dispense 300 ml of the blank solutions into each of two measuring flasks. Add the precisely weighed amount of the test substance directly into the measuring flask. If the test substances are contaminated, ensure that they are homogenized beforehand.

Seal the measuring bottle with a suitable cap and shake it several times to ensure that the test substance has been completely absorbed by the measuring solution and that no residue remains on the sides of the bottle. Again, set up a duplicate blank. Place a rubber quiver containing 2–3 NaOH pellets into the neck of each bottle and screw the measuring head onto the bottle. Incubate the prepared measuring bottles at the selected temperature within ± 1 K for 28 days.

Analysis

If no negative pressure has developed in the measuring bottle over the entire period, it can be assumed that the test substance is microbiologically toxic. If this is not plausible, the integrity of the measuring system should be checked.

As mentioned earlier under “Measuring Equipment,” the measuring head must be able to output the differential pressure as a measured value, from which oxygen consumption can be calculated. Only in this way is it possible to directly determine the oxygen consumption based on the to determine the weighed-in amount of test substance. The oxygen consumption (mass m_{02}) is calculated from the measured differential pressure (Δp) is calculated using the following formula:

$$\mathbf{m_{O2} = \frac{\Delta p \times V \times M}{R \times T}}$$

With:

V Volume of the gas phase above the test solution.

When using the brown standard volumetric flasks, the volume is calculated as follows:

V = Bottle volume – Volume of the test solution = 0.510 L – 0.3 L = 0.21 L

If you use a different measuring bottle, the volume of the gas phase must be calculated accordingly.

M Molar mass of oxygen = 32 g/mol

R Universal gas constant = 8.314472 $\frac{\mathbf{J}}{\mathbf{mol \times K}}$

T Absolute temperature during measurement

The recommended incubation temperature of 20°C corresponds to an absolute temperature of 293.15 K.
If you have selected a different incubation temperature, the absolute temperature is calculated using the following formula:
Calculate:

$\mathbf{T = \upsilon + 273{,}15K}$ where T = temperature in °C

Now use the formula provided to calculate the mass of oxygen consumed for each measured differential pressure. Then calculate the average of the two oxygen consumption values for the blank solutions.

$$\mathbf{b_m = \frac{b_1 + b_2}{2}}$$

No average values are calculated from the oxygen consumption values for the test solutions; instead, a BOD value is determined from each of the two solutions, and two different degradability values are calculated from this, both of which are reported as results.

The BOD is calculated using the following formula:

$$\mathbf{BOD = \frac{A}{m_{Sample}}}$$

The BOD [mg/mg] is defined here as mg of oxygen per mg of test substance used.

A Corrected oxygen consumption of the test solution:

A m_{O2} -b_m

$m_{O2:}$ Oxygen consumption value of the test solution

b_m Mean oxygen consumption of the blank solutions in [mg]

m_{Sample} Mass of the test substance in the measuring vial in [mg]

m_{Sample} = $c_{Solution} \times V_{MF}$

featuring:

$c_{Solution}$ Concentration of the test substance in the measurement solution (here, 100 mg/l)

V_{MF} Volume of the test solution in the test bottle (300 ml in this case)

For each of the two oxygen consumption values obtained from the double determination, calculate a BOD. So you get a BOD_1 and a BOD_2, which are on equal footing as long as they satisfy the condition:

$|BOD_1 - BOD_2| < 0.1 \times (BOD_1 + BOD_2)$

If this condition is not met, the measurement must be repeated.
Biodegradability D is calculated using the following formula:

$$\mathbf{D = \frac{BOD}{ThSB} \times 100[\%]}$$

Calculate the biodegradability for each of the two BOD values. The two biodegradability values are given equal weight listed side by side as the result.

Note on the Validity of the Measurement

The oxygen uptake of the blank solution typically ranges from 20 to 30 mg/L and should not exceed 60 mg/L within 28 days. Values above this range should be reviewed with a critical eye, taking into account the procedure and the data. In case of doubt, more intensive preparation of the inoculum according to [1] is recommended. If
the pH value after measurement is outside the range of pH 6–8.5, and at the same time, the degradability is less than 60%
If this is the case, the test must be repeated using a lower concentration of the test substance in the test solution. The absolute oxygen consumption must not exceed 900 mg during a single test run in a bottle. If the ThOD for the weighed amount of substance exceeds this value, a smaller amount must be used.

Bibliography

[1] OECD Guideline for the Testing of Chemicals: Ready Biodegradability, 301F Manometric Respirometry, paragraphs 9 through 15, 1992

Note

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