Manometric respiration tests according to OECD 301F with the OxiTop® Control measuring system under GLP conditions

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Note: This report was made by the use of the OxiTop® Control system. It can also easily be conducted with the OxiTop®-IDS system without any problem.

1 Introduction

The determination of the "ready biodegradability" characterizes substance properties concerning the possible long term effects of chemicals on the environment (cf. Beek et al., 2001). This determination, while following the OECD Guidelines 301F or 92/69 EEC, C.4-D by means of oxygen consumption due to mineralization under aerobic conditions, is described below. The manometric respiration test was carried out under GLP conditions with the pressure difference measuring system, OxiTop® Control.

2 Good Laboratory Practice

According to § 19a of the ChemG (German Chemicals Act), all non-clinical, health and environment relevant safety checks of substances or compositions have to be carried out following the Good Laboratory Practice (GLP) regulations if their results should enable the assessment of possible hazards for humans and the environment in a permit, admission, registration, application or information procedure.

The legal regulations of the GLP are specified in appendix 1 of the ChemG.

3 Manometric respiration test OECD 301 F

3.1 Basic information

The test determines the complete biodegradability of organic substances in an aqueous medium by determining the oxygen consumption in a closed respirometer. The test specimen is added to mineral dilution water as the single carbon source. The CO_2 developing due to oxygen consumption is absorbed by concentrated NaOH and manometrically measured as a negative pressure. The respirometer test is mainly suitable for the assessment of the biodegradability of water-soluble substances, especially for wastewater but also for volatile and hardly soluble substances. The standard test concentration is 100 mg/L, at least a theoretical oxygen demand (ThOD) of 100 mg/L.

3.2 Description of the OxiTop® Control measuring system

The measuring system consists of the OxiTop® OC110 controller, the OxiTop® -C measuring heads, the PF600 measuring bottles, the IS12 stirring unit and the TS606-6/4-i thermostat cabinet, all by the company WTW, Weilheim, Gemany. Due to oxygen consumption in the closed system, CO_2 develops which is removed from the head space by absorption and thus causes a negative pressure. The measuring heads record and save the pressure difference semi-continuously during the specified measuring period (with 28 days, one measurement every 112 minutes). The saved measurement data is transferred to the controller by infrared transmission, imported to a PC with the Achat OC program and evaluated with a company-specific Excel-sheet.

3.3 Execution

The dilution water consists of deionized water and four mineral nutrient solutions (cf. OECD 301 F in conjunction with OECD 301 A).

Activated sludge from a wastewater treatment plant with mainly municipal wastewater is used as the inoculum, corresponding to a concentration of 30 mg TSS/L.

In every test series, a reference substance (sodium acetate or sodium benzoate) is tested in a parallel series with a concentration of 100 mg/L.

A typical test design is as follows:
- 2–3 bottles with the test specimen and inoculum
- 2 bottles with the test specimen, the reference substance and inoculum (toxicity test, optional)
- 2–3 bottles with the reference substance
- 1–2 bottles with the test specimen without inoculum but with a biocide (abiotic check, optional)
- 2–3 bottles with inoculum (blank values)

The standard filling volume is 164 mL/bottle. This enables to determine BOD values up to 400 mg/L in the aqueous phase. Approx. 16.4 mg/bottle solid matter (TSS) is directly weighted into the reactors (corresponds to 100 mg/L), in the case of substances dissolved in water or wastewater samples a corresponding amount of the substance or COD. Optionally, the toxicity of the test substance towards activated sludge organisms can be determined by the inhibition of the degradability of the reference substance.
The abiotic check (optional) is poisoned with 1 mL of a cupric sulfate solution (10 g CuSO4 * 5H2O /L aqua deion.) in order to detect any abiotic effects during mineralization.
A magnetic stirring rod is put in every bottle, the rubber tubular is inserted, an NaOH tablet is inserted to absorb the released CO_2, and the measuring heads are screwed on tightly.

3.4 Evaluation

In derivation of the general law of gas and taking the solubility of oxygen in water into account, the following formula (already programmed in the OxiTop® Controller) applies to the calculation of the biochemical oxygen demand (BOD):

$$BOD = \frac{M}{R * T_m} * \left(V_t - V_l \right) + a \frac{T_m}{T_o} * p$$

where:

BOD	Oxygen demand of the test specimen in mg/l
M	Molecular weight of oxygen (32000 mg/mol)
R	Gas constant (83.144 l*mbar/(mol*K)
To	Reference temperature (273.15 K)
Tm	Incubation temperature (294.65 K)
Vt	Bottle volume in ml
Vl	Filling volume in ml
	Bunsen absorption coefficient (0.03103)
p	Difference of the oxygen partial pressure in hPa

The degradation rate is calculated according to the following formula:

According to OECD 301 Annex IV formulas, the theoretical oxygen demand (ThOD) is calculated with the empirical formula, which differentiates between the mineralization of nitrogen to ammonia ($ThOD_{NH4}$) and the nitrification of nitrogen to nitrate ($ThOD_{NO3}$). The standard evaluation method uses the $ThOD_{NH4}$; the results for both reference values are quoted if necessary. With wastewater samples, the Chemical Oxygen Demand (COD) of the sample is used as the reference point instead of the calculated ThOD. For the toxicity test solutions with the reference substance, the degradation values relate to the $ThOD_{total}$ (sum of $ThOD_{Reference\ substance}$ + $ThOD_{Test\ specimen}$).

Test specimens that develop an oxygen consumption of > 60% of the ThOD after 28 days are regarded as „readily biodegradable“. This value has to be reached within 10 days after the lag phase (degradation reaches 10% of the ThOD), (= 10 days window). The EU detergents directive does not apply the 10 days window and quotes the result as the “ultimate biodegradability”.
If the toxicity test solutions containing both the test sample and the reference sample develop a degradation rate of less than 25 % within 14 days (referring to the total ThOD), the test substance is regarded as toxic and the test should possibly be repeated with a lower test concentration and/or a higher inoculation concentration.

3.5 Validity criteria

A test is only accepted as valid if the following validity criteria are met:
- The self-consumption of O_2 should not exceed 60 mg/L after 28 days; usual values are between 20–30 mg/L.
- The reference substance has to be degraded by more than 60 % in the first 14 days.
- The difference of the degradation rates of the parallel solutions of the test substance should be below 20 % after the 10 days window has run out.
- If the pH value is not within the range 6.0 .... 8.5 after the test, the test should be repeated with a lower concentration of the test specimen.

4 Evaluation example

An example for the evaluation of an (anonymized) test specimen is given in the appendix in table 1 and 2 and in figure 1. It is a water-soluble, non-volatile test substance. The test substance has an ultimate degradability of 61.1 % after 28 days. At the end of the 10 days window, the biodegradation of the test substance is still under 60 %, which means it could not be classified as “readily biodegradable”. The reference substance meets the degradation criterion of > 60 % already after four days. The

$$\text{Degradation in \% of the ThOD} = \frac{\text{BOD}_{\text{Test solution}}[\text{mg/L}]\ \text{BOD}_{\text{Control solution}}[\text{mg/L}]}{\text{ThOD}[\text{mg/L}]} * 100$$

different solutions of a series develop very similarly, which can be seen in table 1. The criterion of self-consumption under 60 mg/L could well be met in the check solutions (blank samples).

5 Implementation of GLP regulations

5.1 Checking the measuring heads

The OxiTop® Control measuring system provides a GLP mode that monitors the times of the measuring heads. This is not an actual "Calibration" but just a "Check" of the measuring heads, as they can only by readjusted by the manufacturer. In the Hydrotox GmbH laboratory, the measuring heads are routinely checked with the OxiTop® PM test equipment every 12 months and after unexpected measuring results.

5.2 Test design and validity criteria

According to previous experiences, some test solutions have to be regarded as outliers. The reasons for this are: Leakiness between bottle and measuring head, different distribution of the inoculum (sludge flakes) or temperature fluctuations. To prevent individual outliers from endangering the entire test, for every series one or two additional bottles (up to four bottles with the test specimen) are prepared in our laboratory. According to OECD 301 F, two preparations are allowed as well. A bigger number of parallel solutions has the advantage of enabling a more reliable statement on the variability of the individual measurements.

The validity criteria themselves (cf. 3.5) include quality features concerning the variability of measurement results (20% criterion), the self-consumption of the inoculum and the development of the degradability of the reference substance. More quality features can be defined due to "Expert Knowledge". Examples: A degradation rate of the test or reference substance of clearly more than 100% or a reduction of already achieved degradation values during the period would not be acceptable but suggest irregularities or nitrification.

5.3 Temperature constancy

The OECD 301 F prescribes a temperature range of 22 ± 2°C. Within this range, the temperature may fluctuate by ± 1°C. These criteria are safely met in the WTW thermostat cabinets. Fluctuations of the temperature have a direct effect on the pressure in the bottles and thus on the measurement results. Under GLP conditions, the temperature has to be checked with a MIN/MAX thermometer during the entire test period.

5.4 Sample dataset

Quality assurance of computer-based systems is required principally. Pressure value recording using the measuring heads is regarded as a "Black Box System" and can be checked indirectly only, by means of calibration with the calibration tablets (cf. 5.1). After the data has been read out with the Achat OC program, it is simply copied into an Excel sheet. Every single measuring head and every test series are individually labeled, i.e. there is no danger of data loss or incorrect assignment. In the Hydrotox GmbH laboratory, individual measured values of each series are additionally read out, noted down and compared to the later printout of the Excel table as random checks. A write-protected validated Excel sheet is principally used for data evaluation. The program is validated by means of sample datasets that were checked by individual calculations. In addition to the hand-written records, all measurement data and calculation steps are archived as raw data, and the program validation and measuring head checks are archived as hardcopies to make sure the results can be checked later at any time.

6 Conclusions

The manometric respirometry test according to OECD 301 F can be carried out under GLP conditions with OxiTop® Control measuring system and is an easily carried out test for the assessment of the ready biodegradability of chemicals. Contrary to the other degradation tests of the OECD 301 series, this test does not require any handling during the entire test duration. We want to point out, however, that the selection of suitable degradation tests is determined by the substance characteristics (water solubility, volatility, nitrogen concentration, formation of foam etc.), the required final point (DOC, O_2, CO_2) and the potency of the inoculum, which means the use of other OECD tests continues to be justified.

7 List of references

BEEK, B.; BÖHLING, S.; FRANKE, C.; JÖHNCKE, U.; STUDINGER, G.; THUMM, E. (2001): The Assessment of Biodegradation and Persistence. In: Biodegradation and Persistence, ed. by B. Beek. The Handbook of Environmental Chemistry, Vol. 2, Reactions and Processes, Part K (O. Hutzinger, Editor-in-Chief); p. 291-320; ISBN 3-540-62576-3.

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OECD 301 F: Manometric Respirometry Test. Organisation for Economic Co-operation and Development Chemicals Testing - Guidelines. Adopted 17.07.1992

92/69 EEC, C.4-D: Manometric Respiratory Test (Appendix V of 67/548/EEC)

EC Regulation No. 648/2004 EC of the European Parliament and of the Council of 31 March 2004 on detergents. Official Journal of the European Union L 104, p. 1-35 of 8.4.2004

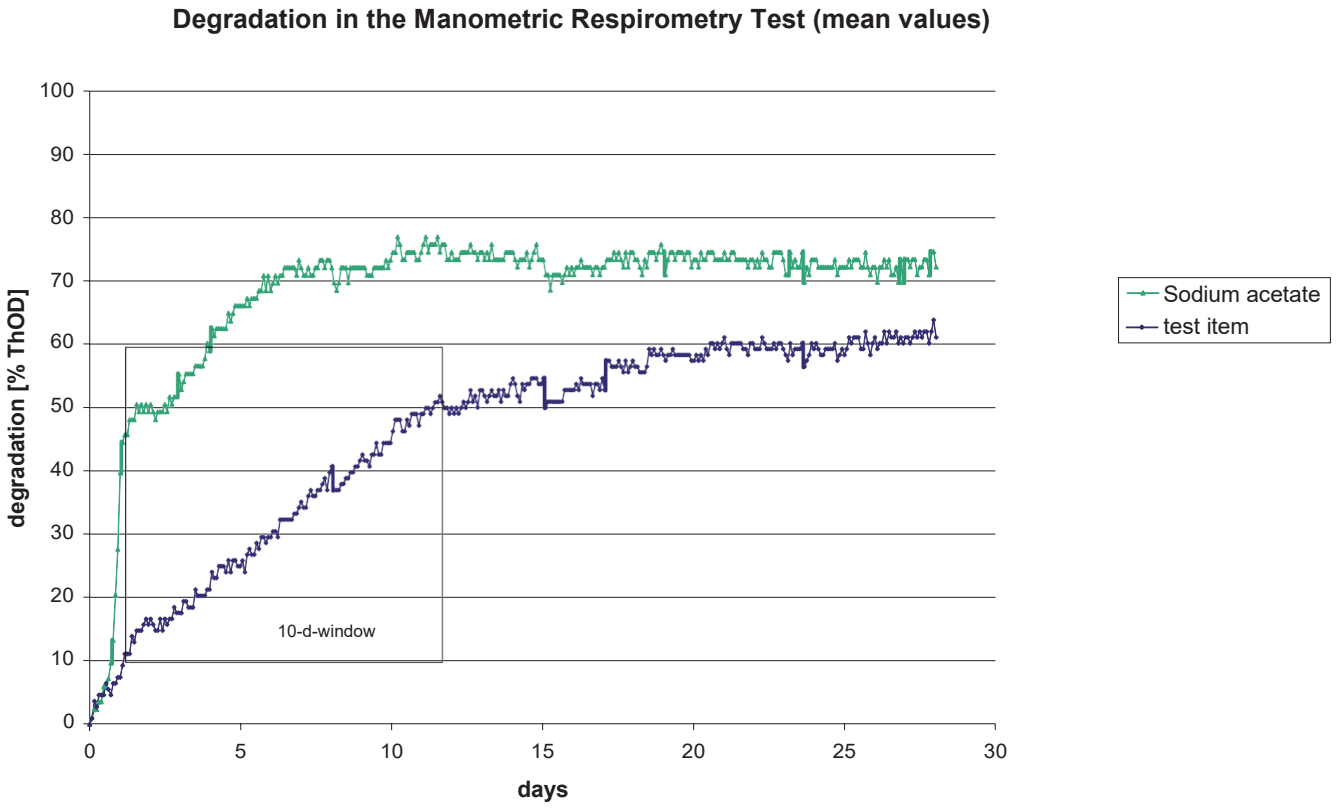
8 Appendix

Tab. 1 Measurement of oxygen consumption

	Test item			Sodium acetate			Blank		
Test item [ml/164 ml]* Reference item [mg/164 ml]	1,64	1,64	1,64						
				16,4	16,4	16,4			
ThOD [mg/l]	101,3	101,3	101,3	78,0	78,0	78,0			
day of measurement	oxygen consumption [mg O_2/l]								
0	0	0	0	0	0	0	0	0	0
4	30,9	33,8	36,6	59,1	59,1	56,3	11,3	8,4	8,4
8	53,5	56,3	59,1	76	67,5	70,3	16,9	14,1	14,1
12	67,5	70,3	73,2	84,4	73,2	76	19,7	19,7	19,7
16	73,2	76	73,2	81,6	73,2	73,2	22,5	22,5	16,9
20	81,6	84,4	84,4	90	78,8	78,8	25,3	25,3	25,3
24	87,2	87,2	87,2	92,8	78,8	78,8	28,1	28,1	22,5
28	90	90	90	92,8	78,8	78,8	28,1	30,9	25,3

Tab.2: Degradation in % of ThOD

	Test item			Sodium acetate		
Test item [ml/164 ml]* Reference item [mg/164 ml]	1,64	1,64	1,64			
				16,4	16,4	16,4
ThOD [mg/l]	101,3	101,3	101,3	78,0	78,0	78,0
day of measurement	degradation [%]					
0	0	0	0	0	0	0
4	21,3	24,1	26,9	63,8	63,8	60,2
8	38,0	40,7	43,5	78,2	67,3	70,9
12	47,2	49,9	52,8	82,9	68,6	72,2
16	51,9	54,6	51,9	78,2	67,4	67,4
20	55,6	58,3	58,3	82,9	68,6	68,6
24	60,2	60,2	60,2	85,3	67,4	67,4
28	61,1	61,1	61,1	82,9	65,0	68,6



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