Development and Evaluation of an Online CO₂ Evolution Test and a Multicomponent Biodegradation Test System

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Well-established biodegradation tests use biogenously evolved carbon dioxide (CO₂) as an analytical parameter to determine the ultimate biodegradability of substances. A newly developed analytical technique based on the continuous online measurement of conductivity showed its suitability over other techniques. It could be demonstrated that the method met all criteria of established biodegradation tests, gave continuous biodegradation curves, and was more reliable than other tests. In parallel experiments, only small variations in the biodegradation pattern occurred. When comparing the new online CO₂ method with existing CO₂ evolution tests, growth rates and lag periods were similar and only the final degree of biodegradation of aniline was slightly lower. A further test development was the unification and parallel measurement of all three important summary parameters for biodegradation—i.e., CO₂ evolution, determination of the biochemical oxygen demand (BOD), and removal of dissolved organic carbon (DOC)—in a multicomponent biodegradation test system (MCBTS). The practicability of this test method was demonstrated with aniline. This test system had advantages for poorly water-soluble and highly volatile compounds and allowed the determination of the carbon fraction integrated into biomass (heterotrophic yield). The integrated online measurements of CO₂ and BOD systems produced continuous degradation curves, which better met the stringent criteria of ready biodegradability (60% biodegradation in a 10-day window). Furthermore the data could be used to calculate maximal growth rates for the modeling of biodegradation processes.

Biodegradability has always been considered an important attribute for chemicals, but until recently it was rarely quantitatively incorporated into safety assessments. Concern about environmental quality and advances in models for predicting environmental concentrations have significantly increased a demand for reliable biodegradation data (9, 11, 31–36). Therefore, various laboratory test methods for investigating and monitoring biodegradation processes have been developed and standardized (2). Most efforts have been concentrated on biodegradation tests in the aerobic aquatic environment. Methods for measuring biodegradability can be divided into two principal groups: direct measurement of parent compound concentrations and indirect measurement of parent compound bioconversion, such as carbon dioxide production, decrease in dissolved organic carbon (DOC), cumulative oxygen consumption (biochemical oxygen demand [BOD]), and decrease in chemical oxygen demand (COD). Direct measurement can quantify the disappearance of the parent compound even at low concentrations and is frequently used for the determination of primary biodegradation by using substance-specific analytical methods. Otherwise, it can necessitate complicated analytical procedures depending on the molecule structure (e.g., polymers) and physicochemical properties (e.g., highly volatile or poorly water-soluble compounds). Furthermore, it is not possible to determine the mineralization of the parent compound if not all intermediate metabolites can be quanti-

fied. The indirect measurement of biodegradation by using summary parameters, such as BOD, COD, or DOC, is often easy and can be automated. In the case of DOC or COD measurements, it may be necessary to determine physical/chemical elimination processes, such as adsorption to biomass or stripping processes, to differentiate biodegradation from abiotic elimination. The assessment of CO₂ and BOD allows an accurate determination of biodegradation processes, and continuous methods can be used for analysis. Especially the determination of the end product carbon dioxide is an important parameter in the estimation of the mineralization of a test compound. However, the use of only one indirect biodegradation parameter for biodegradation may be misleading (13). For example, oxygen can be consumed during biochemical oxidation of ethanol to acetic acid without any decrease in DOC (40).

For practical and legislative purposes, a number of biodegradation test procedures have been standardized. An overview is given by ISO 15462 (15–22) and Pagga (38). The most important and most frequently used tests are those for ready biodegradability. These are the most stringent tests, offering only limited opportunities for biodegradation and acclimatization of the inoculum. On the other hand, they have a fairly high predictive value for the real environment. The ready biodegradability tests are based solely on the indirect summary parameters, such as the removal of DOC or COD, the production of the catabolic end product carbon dioxide, and the determination of the BOD. In recent years, there has been increasing interest in the determination of carbon dioxide. Especially for testing polymers in composting and soil tests or for testing the biodegradability of detergents, the determination of CO₂ production has proven to be an important parameter (7, 10, 39).

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The existing biodegradation methods based on CO_2 measurement and DOC often use discontinuous analytical techniques. However, continuous measurement of the BOD is possible with a respirometer (37). The major advantage of continuous measurement is not only the involvement of less manual work during the test but also the possibility of obtaining much better biodegradation curves, which is very advantageous for the determination of ready biodegradability. According to the Organisation for Economic Co-operation and Development (OECD) guidelines (31–36), a test substance is regarded as readily biodegradable if the degree of biodegradation based on DOC removal is $>70\%$. In the case of CO_2 production or BOD determination, 60% of the theoretical values have to be reached. These limit values have to be achieved within a period of 10 days (10-day window) during a test of 28 days. Methods that allow continuous measurement of the analytical parameter reduce the risk of missing the 10-day window.

After the development of an elegant new pressure-based respirometer, the Oxitop method (41), we used the continuous measurement of conductivity for an online CO_2 evolution test. This method used the direct linear relationship between CO_2 production and change of conductivity in a well-specified, calibrated system. Together with the ions of an aquatic potassium hydroxide (KOH) solution, the biogenously evolved CO_2 produced potassium carbonate (K_2CO_3). The carbonate was less dissociated and therefore showed less conductivity. The linear correlation between the amount of CO_2 liberated and the change in conductivity could be used to determine the formed CO_2 very accurately. The main purpose of this study was the establishment of a conductivity-based online CO_2 evolution test. This test fulfilled the requirements of standardized biodegradation tests and may serve as a basis for further development of biodegradation tests in different areas.

As a consequence of the development of the new Oxitop respirometer and the conductivity-based online CO_2 evolution test, we also developed a test with parallel measurement of all three important summary parameters for ultimate biodegradation (CO_2 , BOD, and DOC), the multicomponent biodegradation test system (MCBTS). Such a test is not required for the usual standard investigations but may have advantages for compounds that are poorly water soluble or volatile and show no clear results in any standard biodegradation test. In such a case, information obtained from three independent analytical parameters may lead to a better and more reliable prediction of biodegradability.

MATERIALS AND METHODS

Chemicals. Aniline, sodium benzoate, trace elements, and mineral salts were analytical-grade chemicals purchased from Merck, Darmstadt, Germany.

Activated sludge. Activated sludge was collected from a laboratory wastewater treatment plant fed with municipal sewage. The inoculum was preconditioned to reduce the endogenous CO_2 production rate. This was done by sieving the sludge with sieves with an 0.8-mm pore size to remove coarse particles, washing it once with tap water, bringing it to a concentration of 5 g of dry matter liter^{-1} , and finally aerating it for 2 days. The usual concentration of activated sludge in the tests was 30 mg of dry substance liter^{-1} .

Conventional CO_2 evolution test. The principle of the widely used CO_2 evolution test (OECD 301 B [32] and ISO 9439 [16]), also known as the Sturm test, was the determination of the ultimate biodegradability of organic compounds by aerobic microorganisms, using a static aqueous test system and the evolution of CO_2 as the analytical parameter. A 1.50-liter test mixture was prepared in 2-liter

FIG. 1. Schematic views of the test apparatus used for the MCBTS system (A) and the online CO_2 evolution test (B). The total volume of the two vessels was 2.5 liters, and the culture volume was 1.5 liter. The parts are labeled as follows: 1, test vessel; 2, CO_2 trap with NaOH or KOH; 3, conductivity electrode; 4, pressure sensor for BOD measurement; 5, sampling port; 6, aeration unit; and 7, de-aeration unit.

vessels containing an inorganic medium and the organic compound as the sole source of carbon at a concentration of 10 to 40 mg of organic carbon liter^{-1} . Usually activated sludge, obtained from a wastewater treatment plant or from another source in the environment, was used as a mixed inoculum. The vessels were aerated with 1 to 2 bubbles of CO_2 -free air per s (50 ml min^{-1}) and incubated at $20 \pm 2^\circ\text{C}$ for usually 28 days. Agitation was increased by stirring with a magnetic bar (length, 40 mm) at about 800 rpm. The biogenous CO_2 formed during the microbial degradation was trapped in two external adjacent vessels (volume, 200 ml) containing an aqueous sodium hydroxide (NaOH) solution (0.05 M, 100 ml). Samples were taken at regular intervals to determine the amount of dissolved inorganic carbon (DIC) and to calculate the amount of CO_2 produced. This evolved CO_2 was compared with the calculated theoretical amount (ThCO_2), and the degree of biodegradation was expressed as a percentage.

Online CO_2 evolution test. The degradation of chemical compounds in the online CO_2 evolution were based on the same principle as the conventional CO_2 evolution test. Test vessels were cylindrical bottles with a 2-liter volume containing the same mixture of inorganic medium, organic test substance, and mixed inoculum in a liquid volume of 1.5 liter. Incubation, aeration with CO_2 -free air, and agitation of the test mixture, blank, and control vessels were also comparable. The exhaust gas from the vessels was passed through a glass chamber (volume, 150 ml) that was immersed in the test mixture and filled with 50 ml of a 0.25 M aqueous NaOH or KOH solution. Here, CO_2 was trapped and continuously measured by changes in conductivity (Fig. 1B), using an electrode. The absorption solution was continuously stirred with a magnetic bar (length, 10 mm) at 400 to 500 rpm. After conversion of the measured conductivity (millisiemens per centimeter) to CO_2 (milligrams per liter) and subtracting the blank values, biodegradation was calculated and expressed as a percentage of the ThCO_2 . There was a linear correlation between the amount of carbon dioxide liberated and the change in conductivity. This correlation was determined for each volume and concentration of absorption solution used.

Respirometric biodegradation tests. The respirometric tests were carried out in the Sapromat and Oxitop systems. A major difference between the two systems was the supply of oxygen. The Sapromat was able to replace the oxygen that had been used for biodegradation from the electrochemical oxygen production unit.

In the Oxitop, such a replacement of oxygen was not necessary, but care had to be taken that the headspace over the liquid phase in the test vessels was large enough so that it contained sufficient oxygen and allowed complete oxidative biodegradation of the test compound (8, 13, 41). The concentration of inoculum was 30 mg of dry matter ml of activated sludge⁻¹, and the concentration of the test compound was 100 mg of substance liter⁻¹, or 50 to 100 mg liter⁻¹ of theoretical oxygen demand (ThOD). The test duration was 28 days, and the tests were performed at an incubation temperature of 20°C. The measured BOD was compared with the calculated ThOD to obtain the biodegradation degree of the test substance.

MCBTS. The system described for the online CO₂ evolution test was additionally equipped with an Oxitop pressure indicator (Fig. 1A). Vessels were fitted with a glass chamber (volume, 150 ml) immersed in the test mixture, filled with 50 ml of 0.25 M aqueous NaOH or KOH solution and containing a conductivity electrode. In the MCBTS, the electronic pressure indicator for BOD determinations was not equipped with an absorber unit as usual and integrated into the stopper. The bottles were filled with test mixtures and closed gastight. The biogenically evolved CO₂ passed through the absorber solution and changed the conductivity of the absorber. Thus, CO₂ was permanently removed from the atmosphere, resulting in a pressure decrease in the closed vessel. To the side of the test vessel, a neck with a diaphragm was located, which allowed liquid samples for DOC determination to be taken from the test mixture by a syringe (Fig. 1A). Using MCBTS, CO₂ and BOD could be recorded continuously and automatically and the removal of DOC of sufficiently water-soluble test substances could be measured, depending on the frequency of samples taken.

Determination of $\mu_{\max}$. The maximal growth rate ($\mu_{\max}$) was determined according to Blok and Struys (5) in the increasing exponential phase. Based on CO₂ production, BOD consumption, and DOC removal, the $\mu_{\max}$ was calculated according to the equation

$$\mu_{\max} = \ln(x_{t_2}/x_{t_1})/(t_2 - t_1)$$

where x_{t_1} and x_{t_2} were the appropriate parameters (CO₂ produced, oxygen consumed, and DOC removed) at times t_1 and t_2 in the increasing exponential phase.

RESULTS

Absorption of CO₂. Aqueous NaOH and KOH solutions were tested as absorbing materials in a concentration range of 0.25 to 1.0 M. KOH solutions proved to absorb CO₂ faster than NaOH solutions. Furthermore, an increasing absorbance pattern was observed with an increasing volume of the absorber. The initial pH of the absorber solution was 13.3, which decreased proportionally to the amount of CO₂ absorbed. To check the reliability of the system (absorber, 40 ml of 0.25 M KOH solution), sodium carbonate (Na₂CO₃) was added with a syringe as a concentrated stock solution ($c = 21.2$ g liter⁻¹) to simulate different CO₂ concentrations. At an initial total inorganic carbon (TIC) concentration of 20 mg liter⁻¹, the pH decreased by 0.3 to 0.4. At an initial TIC concentration of 40 mg liter⁻¹, it decreased by 2.4. For an initial TIC concentration of 40 mg liter⁻¹, the absorber volume was increased to 50 ml and the pH decreased by 0.9. During the test period of 28 days, we did not observe a significant diminution of the absorber volume due to evaporation. At an initial volume of 30 ml, the reduction was low (mean, 0.18 ml; coefficient of variation, 43%; 95% confidence interval of the mean, 0.10 to 0.26 ml). The total carbon dioxide binding capacity of the absorbing solution was calculated according to the equation

$$m(\text{CO}_2) = V(\text{OH}^-) \times c(\text{OH}^-) \times M_r(\text{OH}^-) \times 1.2941$$

where $m(\text{CO}_2)$ was the mass of carbon dioxide (grams), $m(\text{OH}^-)$ was the mass of hydroxide ions (grams), $V(\text{OH}^-)$ was the volume of the absorbing hydroxide ion solution (liters), $c(\text{OH}^-)$ was the concentration of the absorbing hydroxide ion

TABLE 1. Influence of the pH of the test medium on the liberation of CO₂^a

pH	Time (days) for liberation of		
	20% CO ₂	50% CO ₂	90% CO ₂
4.0	0.2	0.5	1.2
7.0	0.6	1.8	4.2
8.0	1.3	3.0	5.2

^a Carbon dioxide was added as Na₂CO₃ to get a final concentration of 2 mM.

solution (moles per liter), and $M_r(\text{OH}^-)$ was the relative molar mass of hydroxide ions (grams per mole). Therefore the total carbon dioxide binding capacities were 165, 220, and 275 mg of CO₂ for 30, 40, and 50 ml of a 0.25 M absorption solution (NaOH or KOH), respectively. The influence of the pH in the test medium on the liberation of CO₂ is shown in Table 1. As expected, the lower the pH the faster was the liberation of CO₂.

The long-term stability of six conductivity electrodes (WTW, Weilheim, Germany) was tested over a period of 19 months by monitoring an electrode-specific cell constant. During this period, three electrodes remained in a 0.25 M NaOH solution and three electrodes remained in a 1.00 M NaOH solution. During this period, the coefficient of variation of the cell constant varied between 1.26 and 1.31% in the 0.25 M solution and between 0.05 and 1.02% in the 1.00 M solution. According to technical information of the supplier, the cell constant should be 0.475 cm⁻¹ and variations should not exceed $\pm 1.5\%$. Therefore, the conductivity sensors tested were very suitable even for long-term studies.

The calibration of the conductivity electrodes occurred by direct addition of different amounts of gaseous CO₂ (1 to 160 mg) via a rubber septum into a closed stirred system with a total volume of 100 ml containing 40 or 50 ml of 0.25 M KOH as the absorption solution. After an equilibration period of 2 h, the difference in conductivity was recorded and a sample was withdrawn to determine the concentration of inorganic carbon (IC). The calibration functions for two different volumes of absorption solution are shown in Fig. 2.

Blank values. CO₂ production or cumulative BOD in the vessels for the blank values was due to endogenous oxidation activity of the microorganisms of the inoculum and took place without any addition of a biodegradable carbon source. In biodegradation tests, it was essential to determine this endogenous activity and to correct the CO₂ and BOD measured from the test assays with test substance by subtracting the blank values. In the online CO₂ test system, the CO₂ production in the blanks proved to be a continuous and linear process over the whole incubation period of 28 days. In 13 independent tests, the final concentration of CO₂ in blank vessels with an inoculum concentration of 30 mg of dry matter liter of suspended solids⁻¹ ranged from 22 to 36 mg liter⁻¹ with a mean value of 30 mg liter⁻¹ (coefficient of variation, 20.7%; 95% confidence interval of the mean, 24.8 to 35.2 mg liter⁻¹).

Variation of parallel measurements. The degrees of biodegradation measured in the plateau phase in parallel vessels were used to calculate the mean degree of biodegradation of the test substance. Therefore it was important to find out whether parallel measurements gave comparable plateaus. Eight differ-

FIG. 2. Calibration curves with 0.25 M KOH as an absorber. (A) Forty milliliters of absorption solution. (B) Fifty milliliters of absorption solution. The capacity of the absorber was 220 mg of CO_2 , with 40 ml of absorption solution and 275 mg of CO_2 with 50 ml of absorption solution. The number of replicate tests was six. The coefficients of variation were 6.4% for 40 ml of absorption solution and 7.2% for 50 ml of absorption solution.

ent parallel tests with aniline as a test compound were performed, and the differences between the plateaus were determined. The mean of the differences was 3.5%, with a coefficient of variation of 0.732%. The 95% confidence interval of the mean of the differences ranged from 1.36 to 5.64%. The mean of the final degree of biodegradation was 79.8% (coefficient of variation, 13.1%; 95% confidence interval of the mean, 74.3 to 85.4%).

Biodegradation patterns of aniline as a reference compound. Aniline is a reference compound for many OECD tests and therefore well characterized. It was used to compare the conventional CO_2 evolution test (OECD 302 B) with the new online CO_2 evolution test. The data obtained in the two test systems are summarized in Table 2. Maximal growth rates and lag periods were in a comparable range, whereas the degree of biodegradation was slightly lower in the online CO_2 evolution test.

Influence of aeration in the online CO_2 evolution test. The influence of air supply in the online CO_2 evolution test was tested by using aerated open systems (12 replicates) with about 50 ml of CO_2 -free air min^{-1} and comparing the results with those from nonaerated open systems (10 replicates). The total volume of the test vessels was 2.5 liters, and the volume of the test mixture was 1.5 liter. In the aerated and nonaerated systems, we found comparable degrees of degradation (75.9 and

78.1%, respectively). The lag periods (3.2 and 3.8 days, respectively) and the μ_{max} (2.4 day^{-1} in both systems) were also in the same range (Fig. 3). The coefficients of variation for all indicated parameters ranged between 10.6 and 22.9%. A prerequisite for these results was that sufficient oxygen was available in the nonaerated system. The total oxygen content in the system was about 270 mg. This exceeded the total oxygen demand in a test vessel containing a completely biodegradable organic test substance at a usual test concentration of 20 mg of carbon liter $^{-1}$ (ThOD, about 130 to 150 mg liter $^{-1}$ including

FIG. 3. Degradation of aniline in aerated and nonaerated CO_2 evolution test based on CO_2 production and in a CO_2 evolution test system with online measurement. The numbers of replicate tests were 12 for the aerated system, 10 for the nonaerated system, and 13 for the online system. The coefficients of variation of the mean final biodegradation were 10.6% for the aerated system, 6.3% for the nonaerated system, and 14.1% for the online system.

TABLE 2. Biodegradation of aniline in the modified CO_2 evolution test and the online CO_2 evolution test^a

CO_2 evolution test	Mean degree of biodegradation (%) ^b	Mean μ_{max} (day^{-1}) ^b	Lag period (days) ^b
Modified	86 (12.0)	2.4 (15.0)	3.8 (16.0)
Online	75 (14.1)	2.6 (18.0)	3.4 (18.0)

^a The test period was 28 days. The numbers of tests performed were 51 for the modified CO_2 evolution test and 13 for the online CO_2 evolution test.

^b Coefficients of variation (percent) are indicated in parentheses.

TABLE 3. Biodegradation parameters for aniline in the multicomponent biodegradation test system^a

Parameter ^b	DOC elimination		Oxygen consumption		CO ₂ production	
	Biodegradation (%)	$\mu_{\max}$ (day ⁻¹)	Biodegradation (%)	$\mu_{\max}$ (day ⁻¹)	Biodegradation (%)	$\mu_{\max}$ (day ⁻¹)
Mean value	96.0	6.0	82.5	3.0	83.0	1.7
CV (%)	5.4	13.2	18.0	32.4	10.4	22.1
95% CIM	83.0–100.0	4.1–8.0	70.2–95.3	2.2–3.9	77.5–90.6	1.4–2.0

^a The test period was 28 days, and the number of experiments performed was 6. Lag periods were as follows: 2.0 days for DOC elimination, 2.8 days for oxygen consumption, and 3.9 days for CO₂ production.

^b CV, coefficient of variation; CIM, confidence interval of the mean.

the blank). Therefore, we concluded that the closed system as used in the MCBTS contained sufficient oxygen for complete biodegradation of organic substances under standard conditions.

Biodegradation of aniline in the MCBTS. The biodegradation results for aniline in the MCBTS are summarized in Table 3. It is obvious that the elimination of DOC is the fastest process, followed by the BOD and the production of CO₂. The lag periods for DOC elimination were the shortest and the degree of biodegradation was highest, followed by the values for BOD and CO₂ evolution (Fig. 4).

Biodegradation of sodium benzoate in different test systems. Sodium benzoate was a readily biodegradable organic compound and was also frequently used as a reference substance in standardized biodegradation tests. The disadvantage compared to aniline was that it was degraded more easily and therefore gave no characteristic sigmoid-shaped biodegradation curves. The degradation pattern of sodium benzoate was nevertheless intensively studied in a coulombometric respirometer (Sapromat), a respirometer with direct pressure metering (Oxitop), the online CO₂ evolution test, and the multicomponent biodegradation test system. The data obtained (Table 4) show that the lag periods and the degrees of biodegradation were in a similar range for all test systems, whereas the $\mu_{\max}$

was highest in the Oxitop and MCBTS (oxygen consumption) and lowest in the Sapromat and the CO₂ evolution test.

DISCUSSION

In recent years, several authors have reported difficulties with CO₂ detection in the CO₂ evolution test (OECD 301B [32] ISO 9439 [16]) due to the indirect method of CO₂ determination (44, 51). As a consequence, the 10-day window was not passed in some cases, whereas it was passed without any difficulties in the DOC removal test (OECD 301A [31] and ISO 7827 [15]). Therefore, several authors have described alternative methods for the detection of carbon dioxide. These include sampling of headspace gas and subsequent infrared analysis (7, 11) and the trapping of carbon dioxide in a 0.1 M KOH and measuring changes in electrical conductivity of the base solution (23). A combination of an electrolytic respirometer and carbon dioxide detection was also proposed (42). In our study, the continuous online determination of the biodegradation product CO₂ using a conductivity electrode was optimized for biodegradation tests based on the OECD 301B guideline (32) and the ISO 9439 standard (16). On account of the accuracy and easy performance of the method, the well-established CO₂ evolution test can be used with a new and better analytical technique. The CO₂ online test system is fully based on the existing OECD guideline and ISO standard and can be realized without any major technical effort.

An important practical application of an online CO₂ test system will be in the field of biodegradation of polymers, where direct analytical methods are restricted or complicated. Using the online method, reliable biodegradation studies may also be performed in complex matrices, such as soil, waste, or compost. The suitability of carbon dioxide trapping methods for biodegradation of starch-based materials (23), cellulose, polyhydroxyalkonates, poly(3-hydroxybutyrate), and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (30) has been described.

Aqueous KOH and NaOH solutions were tested as absorbing solutions. The KOH solution proved to absorb CO₂ faster than the NaOH solution. Fifty milliliters of a 0.25 M KOH solution with a theoretical CO₂ binding capacity of 275 mg was very suitable for the CO₂ online test. The conductivity electrodes showed their suitability even for long-term studies.

The experiments performed in our laboratories showed that the variation of the parallel measurements was rather low and the relevant biodegradation patterns were in the same range compared with the existing method based on DIC measurements. Only the degree of biodegradation of aniline was

FIG. 4. Degradation of aniline in an MCBTS. The number of replicate tests was six. A statistical analysis of the data is summarized in Table 3.

TABLE 4. Degradation pattern of sodium benzoate in different biodegradation test systems^a

Test system/ parameter ^b	Sapromat		Oxitop		CO ₂ evolution test		MCBTS			
							Oxygen consumption		CO ₂ production	
	Biodegradation (%)	$\mu_{\max}$ (day ⁻¹)	Biodegradation (%)	$\mu_{\max}$ (day ⁻¹)	Biodegradation (%)	$\mu_{\max}$ (day ⁻¹)	Biodegradation (%)	$\mu_{\max}$ (day ⁻¹)	Biodegradation (%)	$\mu_{\max}$ (day ⁻¹)
Mean	85.0	1.5	89.5	2.8	93.0	1.1	78.5	2.9	78.4	2.1
CV (%)	3.6	7.2	2.9	19.2	10.2	9.6	4.2	13.9	9.4	48.2
95% CIM	81.9–89.7	1.3–1.6	86.8–92.2	2.2–3.3	77.8–100	1.0–1.3	73.2–83.3	2.2–3.5	72.2–84.6	1.2–2.9

^a The test period was 28 days. The number of tests performed was 4 to 6. The lag period varied between 0.8 and 1.5 days.

^b CV, coefficient of variation; CIM, confidence interval of the mean.

slightly lower in the online system, but clearly above the usual limit values for ready biodegradability (60 and 70%, respectively). The major advantage of a continuous CO₂ measurement based on conductivity is the possibility of obtaining much better biodegradation curves. CO₂ measurement does not depend on the frequency of sampling for DIC measurement any longer and the risk of missing the important 10-day window is therefore reduced.

According to ISO standards and OECD guidelines, blank values for endogenous CO₂ formation should not exceed 70 mg liter⁻¹. Otherwise, the experimental technique has to be reviewed and the test has to be repeated with another inoculum (31–36). The data reported in this study for the CO₂ online method were about 30 mg liter⁻¹ over the usual test period of 28 days and were therefore in the allowed range. The endogenous production of CO₂ was due to aerobic biodegradation of residual carbon sources of the inoculum and self-digestion processes of the microorganisms. A CO₂ concentration of 30 to 70 mg liter⁻¹ was equivalent to about 8.2 to 19 mg of carbon liter⁻¹. The endogenous CO₂ production represented a fraction of 0.21 to 0.95 of the ThCO₂ of an added test substrate at the recommended test concentrations of 20 to 40 mg of carbon liter⁻¹ and was therefore significant. The most convenient way of reducing endogenous CO₂ production is to starve the inoculum. The drawback of this method is the possible reduction in the number of viable bacterial cells and subsequently the loss of biodegradation potential. The influence of a starvation process on the viability of degrading bacteria has been discussed controversially (50). Improved survivability has been reported (48), while another study found no significant effects on survivability (49). In our study, a short starvation period of about 18 to 24 h was sufficient to reduce endogenous CO₂ production effectively. Extended starvation periods up to 10 days cause a significant decrease in respiration and dehydrogenase activity (50) and may also result in a decrease in biodegradation potential.

The further development of the online CO₂ test and the Oxitop system for BOD determinations together with a port for taking samples for DOC analysis resulted in an MCBTS that offered the opportunity of monitoring two or even three relevant biodegradation parameters in parallel. This new system is very suitable for testing biodegradability of compounds, which are poorly water soluble, highly volatile, or have a high adsorption capacity to biomass. These properties may cause experimental difficulties in a system based on a single parameter only and may show no clear results in usual standard tests. In such a case, information obtained from three independent

analytical parameters may lead to a more reliable prediction of biodegradability.

The MCBTS has the advantage that better differentiation can be made between the oxidized part of a test compound and the part incorporated into new biomass, which can be expressed by the heterotrophic yield coefficient (24, 46). Heterotrophic yield coefficients vary between 0.40 and 0.80 and depend on the quality of the organic substrate used, the source of the microbial inoculum, the substrate/inoculum ratio, and the test conditions applied. Higher degrees of biodegradation for the same test substance in tests based on carbon removal in comparison to tests based on oxygen consumption or CO₂ formation can be explained by the heterotrophic yield. The usual limit values indicating sufficient biodegradation in BOD- and CO₂-based tests (>60% BOD of ThOD or CO₂ of ThCO₂) consider this fact, but they are still discussed controversially (31–36, 41). Test results based on DOC removal seem to be much more reliable, as values of >90% indicate good biodegradation. This discrepancy can be solved by monitoring BOD and CO₂ formation together with DOC removal. Both the MCBTS and the Oxitop rely on the oxygen being available in closed test vessels. For standard test conditions, the oxygen content is sufficient. An additional oxygen supply, as in the coulombometric Sapromat, may only be necessary when working under test conditions with a higher oxygen demand (higher substrate and inoculum concentrations), extended test periods, or in the case of highly volatile test substances, when the volume of the test medium is increased so that the headspace is limited to supply enough oxygen.

For biodegradation studies, aniline is a frequently used reference compound, since it has a characteristic biodegradation curve with a lag period of 3 to 5 days. In standardized test systems, it is used to show the biodegradation capability of the inoculum used. The aerobic microbial degradation of aniline has been described by Boon et al. (6). The first steps of the aerobic degradation pathway involve oxidative deamination resulting in the formation of catechol. The catechol is further degraded by *ortho*-cleavage (1, 28) or *meta*-cleavage (27).

The biodegradation patterns or curves of standardized batch tests are described by the lag phase, the final degree of degradation in the plateau phase, and the time needed to reach the plateau phase. A modern approach for describing biodegradation kinetics is the use of mathematical models. A variety of such methods exist that can be applied (2, 41). In this study, we focused on the determination of the $\mu_{\max}$. According to Blok (3, 4), the classic theory of Monod (29) to describe bacterial growth offers suitable quantitative parameters that can be used

to describe the biodegradability of substances. Blok and Struys (5) reported calculated $\mu_{\max}$ values for different classes of chemicals (aliphatic and aromatic substances and amino- and nitro-substituted aromatic substances) based on respirometric biodegradation experiments. The $\mu_{\max}$ values reported varied between 0.4 day⁻¹ (diethyleneglycol) and 9.8 day⁻¹ (*p*-nitrophenol). The $\mu_{\max}$ value for aniline of 2.5 day⁻¹ is in accordance with data found in this study (2.81 day⁻¹ in the MCBTS, 2.77 day⁻¹ in the Oxitop, and 1.46 day⁻¹ in the Sapro-mat). Tabak et al. (47) measured $\mu_{\max}$ from the oxygen uptake curves with an electrolytic respirometer. The $\mu_{\max}$ values for 15 substances varied between 1.6 day⁻¹ and 11.5 day⁻¹. Kincannon et al. (25, 26) and Stover and Kincannon (45) gave $\mu_{\max}$ values between 1 and 4.7 day⁻¹ for 25 substances, and the values of Goldsmith et al. (12) were between 3.1 and 6.2 day⁻¹ for aromatic substances. The discrepancies between $\mu_{\max}$ values were discussed in detail by Blok and Struys (5) and are mainly due to adaptation processes and the source of the inoculum used. Based on their studies, the authors proposed a critical $\mu_{\max}$ value of 1.5 day⁻¹ to differentiate between readily and inherently biodegradable compounds.

Other methods frequently used for modeling biodegradation processes are the zero and first-order plot model, the modified logistic model, and the logarithmic model. Critical discussions of these models have been published by Simkins and Alexander (43), Battersby (2), Hales et al. (14), and Reuschenbach et al. (41). For future standards, it would be advantageous to integrate these models into standardized methods. For example, the 10-day window could be replaced with the $\mu_{\max}$ model and the first-order kinetic model and the modified logistic model could be appropriate for estimating the lag phase as well as the final degree of degradation. There are already ISO standards available for determining biodegradation kinetics (ISO 14592 parts 1 [20] and 2 [21]), indicating the positive future perspectives of biodegradation kinetics.

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