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A rationale for the appropriate amount of inoculum in ready biodegradability tests

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Abstract

Several screening methods at the so-called ready biodegradability level are suitable to test poorly soluble substances. Typical for these tests is that mineralization is evaluated from monitoring oxygen uptake or carbon dioxide production. Unfortunately, they suffer from a rather low precision in the calculated percentage of mineralization caused by subtracting a too high inoculum control measurement from the response in the test system. Criteria for blank oxygen consumption, due to the metabolic activity of the inoculum, are proposed from which maximum amounts of activated sludge or secondary effluent per litre test medium can be derived to be used as an appropriate inoculum. Both for current and future standardized tests the precision of the method can be kept within acceptable margins. Inoculum material was sampled from 40 communal biological waste water treatment plants. From endogenous respiration rates it was derived that the concentration of secondary effluent in the Closed Bottle Test can be increased up to 50 mL/L but that in respirometry tests inoculated with activated sludge the appropriate concentration is 10 mg/L dry matter or below, depending of the design of the test system.

Introduction

As microbial degradation is the most dominant elimination mechanism of organic chemicals from the environment, a reliable method for estimating the biodegradability of chemicals is essential for environmental exposure and risk assessment. Standardized methods for the very diverse group of 'new chemicals' have been developed at several evaluative levels of biodegradability (OECD 1981). These methods, later adopted by the EC (1984), have been submitted to a correlation study on 44 compounds by Gerike & Fischer (1979, 1981). They found a too high variety in stringency at the level of 'ready biodegradability' tests (RBTs), with the Closed Bottle Test at the highest end of the stringency scale. This has been attributed to the high variety in inoculum concentrations, spanning a range of more than four orders of magnitude.

A recent harmonization of RBTs (EC 1992; OECD 1993) has reduced differences in mineral medium and – more important – in the inoculum strength, so enhancing the inter-comparability. The upper limit of the inoculum concentration in RBTs is still 30 mg/L dry solids of activated sludge. It was anticipated that if higher amounts are applied, those chemicals may pass RBTs also which are too slowly degradable in biological waste water treatment. Relatively fast elimination from the aqueous phase due to ultimate biodegradation both in natural aerobic environments and biological treatment systems, regardless of the physicochemical properties of the compound, is considered fundamental to the concept of 'ready biodegradability'.

The harmonization has generally resulted in a shift of inoculum strength to the higher limit, leaving a difference of only two orders of magnitude between the 'high' inoculum tests and the Closed Bottle Test. The low inoculum concentration (and of the tested compound) in the latter test is determined by its design. The total amount of oxygen that is available for mineralization of the tested compound is a limiting factor in this test because the sealed vessel *without a gas phase* lacks a gaseous oxygen reservoir. Therefore, endogenous respiration per vessel volume should be kept at a relatively low level in order to avoid subtracting a too

high inoculum control measurement from the response in the test system.

The inoculum concentration of the recently revised Closed Bottle Test has been increased hundredfold. Apparently, there has never been a technical reason to apply an inoculum concentration so extremely low as half a drop of secondary effluent per litre medium as was prescribed in the version of 1981 (OECD). Setting criteria for appropriate inoculum concentrations is convenient because new methods that are candidates for testing the 'ready biodegradability' of poorly soluble compounds are now under consideration. These methods all have in common that – without the need for expensive respirometers – mineralization is monitored by either O_2 uptake or CO_2 evolution in sealed vessels (Blok 1979; Struijs & Stoltenkamp 1990; Birch & Fletcher 1991). Until now no rationale has been presented on the maximum biomass content in a sealed vessel. Therefore, we have derived criteria based on the measured respiration rate of the most common types of inoculum, i.e. activated sludge and secondary effluent. To accomplish this, the range of respiration rates due to endogenous oxygen uptake is evaluated.

Materials and methods

Deriving criteria for the appropriate inoculum concentration in sealed vessels

Depending on the physico-chemical and toxicological characteristics of the compound, the decision can be made whether to use the OECD Closed Bottle Test (CBT), which is a sealed vessel oxygen uptake test without a headspace volume, or one of the tests that accommodate both an aqueous and a gas phase. These systems include both oxygen uptake methods such as the Two-Phase Closed Bottle Test (TPCBT), investigated by the International Organization for Standardization (ISO), and recently reported carbon dioxide evolution tests (Struijs & Stoltenkamp 1990; Birch & Fletcher 1991). In two-phase systems an appropriate gas/liquid ratio in the sealed bottles has to be chosen. The following steps are required to calculate the inoculum concentration in sealed vessels, thus avoiding too high blanks. First, the oxygen capacity (O_c) in mg oxygen per litre vessel volume is calculated as follows:

$$O_c = V_a \cdot \rho_a + V_w \cdot \rho_s \qquad (1)$$

where V_a and V_w are the volumes of air and water per litre vessel volume, respectively; ρ_a is the concentration of oxygen in air at 20° C and 101.5 kPa (= 280 mg/L) and ρ_s the corresponding saturation concentration in the aqueous phase (9.1 mg/L). Second, an appropriate concentration of the test compound, C (mg/L), always fulfills the condition:

$$C \cdot ThOD \cdot V_w \leq O_c \qquad (2)$$

where ThOD is the theoretical amount of oxygen required to convert 1 mg of test compound into carbon dioxide and water.

It is a matter of experience, gained in round-robin tests, that in 28 d RBTs the major part of the total blank respiration is observed during the first week of the test and is due to oxidation of 'reserve' substances and adsorbed organic nutrients by the active cells. The remaining blank respiration, being a minor share (about 30%), is the result of starvation of cells and subsequent mineralization of part of the biomass.

Complete mineralization usually corresponds to an oxygen uptake between 50 and 80% of ThOD. In principle this portion of O_c is available if the blank oxygen uptake after one week does not exceed 0.1 O_c.

As a consequence, also the concentration of inoculum, C_{as} (mg dry solids of activated sludge per L) or C_{ef} (ml secondary effluent per L), in the test medium has a maximum value:

$$C_{as/ef} \cdot Ox_{as/ef} \cdot V_w \leq 0.1 \cdot O_c \qquad (3)$$

where Ox_{as} is the oxygen consumption after one week in mg O_2 per mg dry weight activated sludge (as) and Ox_{ef} per mL secondary effluent (ef), respectively. Calculating the upper limit of the inoculum concentration that will not cause too high blank responses in sealed vessels, with a specific V_a/V_w rations, requires knowledge of Ox_{as} and Ox_{ef}. As most often an inoculum is applied that has been derived from conventional biological waste water treatment systems, the aim of the experimental part of this study is to find the range and confidence limits for $Ox_{as/ef}$.

Sampling and handling activated sludge and secondary effluent

During the period December 1992 – June 1993, samples were taken from 40 municipal waste water treatment plants in the center of the Netherlands. All plants are of the activated sludge type and 22 are equipped with a primary sedimentation tank. Some of the instal-

Table 1. Sampled municipal waste water treatment plants.

No	Sampling date	BOD load (kg $O_2/m^3 \cdot d$)	Population equivalents	% Industrial	Dry weight sludge (kg/m^3)	SLR (kg O_2/kg·d)
1	07-12-1992	0.53	68,450	?	2.19	0.24
	07-06-1993				nd	
2	07-12-1992	0.70	400,000	?	2.94	0.24
	07-06-1993				nd	
3	07-12-1992	0.48	56,600	?	6.85	0.07
	07-06-1993				nd	
4	07-12-1992	0.36	75,000	?	2.74	0.13
	07-06-1993				nd	
5	11-01-1993	0.52	16,000	?	1.85	0.28
	07-06-1993				nd	
6	11-01-1993	0.14	46,300	?	2.44	0.06
	07-06-1993				nd	
7	11-01-1993	0.95	30,000	?	2.73	0.35
	07-06-1993				nd	
8	11-01-1993	0.78	54,300	?	5.20	0.15
	06-06-1993				nd	
9	25-01-1993	0.62	62,000	30	4.77	0.13
10	25-01-1993	0.24	27,000	30	3.83	0.06
11	25-01-1993	0.14	10,000	10	3.50	0.04
12	25-01-1993	0.34	120,000	10	3.02	0.11
13	08-02-1993	0.30	41,900	?	6.37	0.05
14	08-02-1993	0.30	50,000	?	2.70	0.11
15	08-02-1993	0.22	35,000	?	3.09	0.07
16	08-02-1993	0.50	25,000	12	3.31	0.15
17	22-02-1993	0.26	30,000	0	4.05	0.06
18	22-02-1993	0.15	45,000	< 50	2.00	0.08
19	22-02-1993	0.54	60,000	33	6.85	0.08
20	22-02-1993	0.75	90,000	50	4.57	0.16
21	08-03-1993	0.23	12,000	?	3.79	0.06
22	08-03-1993	0.11	5,000	?	1.61	0.07
23	08-03-1993	0.30	14,000	50	1.90	0.16
24	08-03-1993	0.17	17,500	0	2.79	0.06
25	22-03-1993	0.39	285,000	?	3.44	0.11
26	22-03-1993	0.41	35,000	0	1.71	0.24
27	22-03-1993	0.50	110,000	?	3.72	0.14
28	22-03-1993	0.19	30,000	< 50	4.54	0.04
29	05-04-1993	0.21	38,000	50	3.93	0.05
30	05-04-1993	0.54	6,000	?	3.03	0.18
31	05-04-1993	0.54	6,000	?	3.64	0.15
32	05-04-1993	0.17	6,000	5	3.26	0.05
33	19-04-1993	0.16	160,000	< 50	4.38	0.04
34	19-04-1993	0.70	80,000	< 50	1.42	0.49
35	19-04-1993	0.50	100,000	< 50	1.56	0.32
36	19-04-1993	0.22	7,000	?	3.54	0.06
37	03-05-1993	0.45	160,000	27	4.25	0.11
38	03-05-1993	0.44	750,000	0	3.03	0.14
39	03-05-1993	0.61	160,000	?	7.60	0.08
40	03-05-1993	0.43	390,000	0	2.76	0.15

lations have two reactors in series in which case the second aeration tank was sampled. In Table 1 sampling dates, size of the installation and the mode of operation as BOD load in kg $O_2/m^3 \cdot d$ are summarized. The dry weight content of activated sludge, C_{ss} (g/L), was determined within 24 hours after sampling. The BOD load/C_{ss} ratio, known as the sludge loading rate (SLR), had an average value of 0.133 kg O_2/kg dry weight·d. The size of the plants varied from 5,000 to 750,000 population equivalents; the portion of industrial waste

Table 2. Oxygen consumption by the inoculum in the Two Phase Closed Bottle Test (activated sludge) and the Closed Bottle Test (secondary effluent) after 7 d.

No	mg O_2/mg Sludge (dry weight)	mg O_2/50 mL Effluent
1	1.31	nd
2	1.00	nd
3	0.71	nd
4	0.94	nd
5	1.11	nd
6	0.94	nd
7	0.58	nd
8	0.58	nd
9	0.89	0.210
10	0.66	0.040
11	0.59	0.004
12	0.65	0.080
13	0.48	0.290
14	0.78	0.140
15	0.57	0.150
16	0.59	0.150
17	0.61	0.190
18	0.90	0.940
19	0.53	0.930
20	0.63	0.200
21	0.87	0.510
22	0.90	0.530
23	0.90	0.290
24	0.82	0.270
25	0.83	0.160
26	1.24	0.370
27	0.65	0.060
28	0.51	0.210
29	0.63	0.150
30	0.83	0.220
31	0.81	0.730
32	0.69	0.350
33	0.51	0.270
34	1.38	0.540
35	1.18	1.240
36	0.56	0.020
37	0.65	0.500
38	0.69	0.080
39	0.63	0.110
40	0.44	0.100
1*	nd	0.280
2*	nd	0.500
3*	nd	0.150
4*	nd	0.180
5*	nd	0.460
6*	nd	0.130
7*	nd	0.200
8*	nd	0.490

Table 3. Oxygen consumption by the mineral medium in the Closed Bottle Test (not inoculated) after 7 d.

No (Table 1)	mg O_2/L
9–12	0.12
13–16	0.21
17–20	0.14
21–24	0.21
25–28	0.22
29–32	0.21
33–36	0.32
37–40	0.17
1– 8	0.08

water was in between 0 and 50%. The weighted average SLR was 0.14 kg O_2/kg dry weight·d. Secondary effluent of plants 1–8 was sampled a second time as results obtained from the first sampling were discarded for reasons given hereafter.

Activated sludge in quantities of approximately 0.25 L was stored in 2 L and 0.75 L secondary effluent in 1 L capped polyethylene bottles during a transportation period not longer than 6 hours. Aerobic conditions were maintained for one or two hours in the laboratory by aeration before the samples were used as inoculum.

Determination of Ox_{as} and Ox_{ef}

Experiments were performed immediately after each sampling tour (total 10) was completed. In the Two Phase Closed Bottle Test according to a Dutch Standard (NEN 6515, 1992) 10 mL of activated sludge per litre mineral medium was added. The respiration rate was measured after one week. The air/liquid ratio in the test bottles, V_a/V_w, was 1/2 and the concentration of dry weight was in the range between 25 and 35 mg per L test medium.

The oxygen uptake in mg per litre flask volume, Ox_{flask}, was calculated from:

$$Ox_{flask} = O_c(\rho_s - DO_{7d})/\rho_s \qquad (4)$$

where DO_{7d} is the concentration of oxygen in the aqueous phase after 7 days. Ox_{flask} was divided by the amount of activated sludge per litre flask volume to obtain Ox_{as}.

Secondary effluent was filtered through a coarse filter before transferring it to the test medium of the

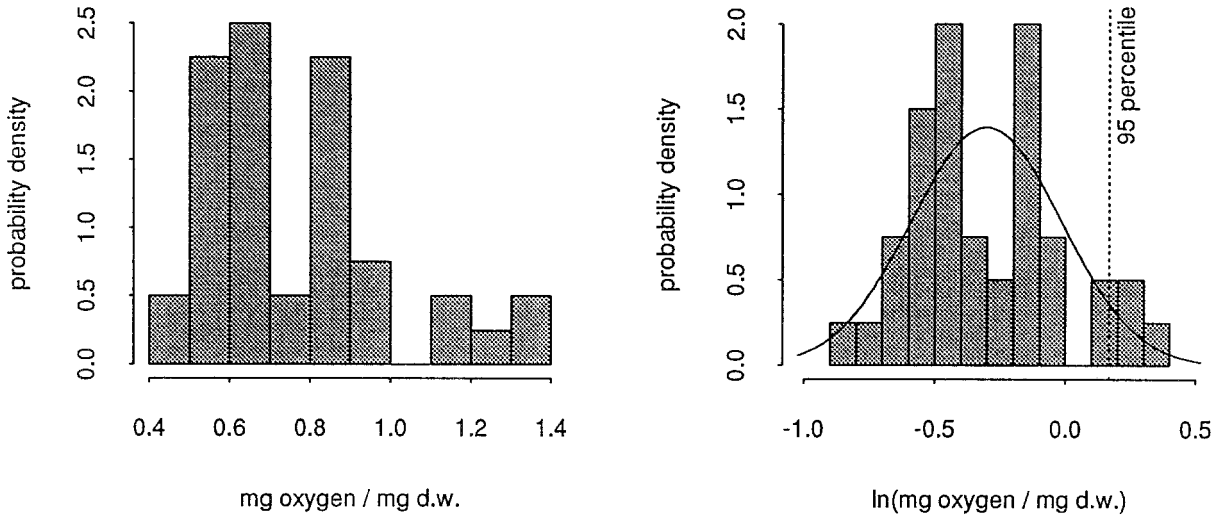

Fig. 1. Histograms of O_2 uptake (after 7 d) by activated sludge (a) and the natural logarithm of O_2 uptake (b).

Closed Bottle Test. With the exception of the addition of 50 mL secondary effluent per litre mineral medium in order to measure a significant oxygen uptake at day 7, the experiment was in accordance to the revised protocols of the CBT (OECD 1993; EC 1992). Initially, 5 mL effluent/L was applied, however, it appeared necessary to repeat the first series (plants to 1–8). The inoculum concentration in the following series was increased tenfold in order to enhance the response/background ratio. Triplicate zero inoculum control bottles were measured for oxygen uptake by the mineral medium in the CBT in parallel with each of the series.

All (triplicate) incubations were carried out in the dark on a rotary shaker (120 rpm) at 20.0 ± 1.5° C. The concentration of O_2 in the test medium was measured with a WTW OXI 530 oxygen measurement apparatus. Oxygen concentrations at t = 0 differed less than 2% from saturation values corrected for the actual air pressure and temperature.

Results

Table 2 contains blank oxygen uptake values per mg activated sludge (Ox_{as}) and per 50 mL secondary effluent (Ox_{ef}[50]). Corrections were made for oxygen uptake in non-inoculated bottles run in parallel with each series. Although these oxygen uptakes are low, typically in the range of 0.1 and 0.3 mg O_2/L (see Table 3), compared to secondary effluent in mineral medium these values are significant. An explanation for this oxygen uptake may be the mineralization of trace amounts of organic impurities present in the mineral medium, probably from the phosphate buffer (analytical grade) or from the deionized water, by microorganisms which are also present in deionized water. However, the population density is very low as well as the concentration of organic micro-impurities.

Activated sludge, Ox_{as}

The empirical frequency distribution of Ox_{as} is shown in Fig. 1a, suggesting that the population does not display a normal distribution. Figure 1b indicates that the distribution is rather log normal and that outliers are absent in the population. The Shapiro Wilk statistic (Madansky 1988) for this sample equals 0.959. The 95% point for 40 observations equals 0.940, hence the hypothesis of normality for the log transformed data is not rejected.

The sludge loading rate of the activated sludge reactor (SLR) is expected to correlate positively with Ox_{as}. The results given in Fig. 2 indicate that the relation In (Ox_{as}) = a + bSLR might be significant. This is confirmed by the least squares estimates of a and b, which turn out to be equal to - 0.56 and 1.70 respectively, with p-values smaller than 0.0001 and a multiple R^2 = 0.334. Omitting two outliers pertaining to low oxygen consumption at SLR equal to 0.15 and 0.35 but not the

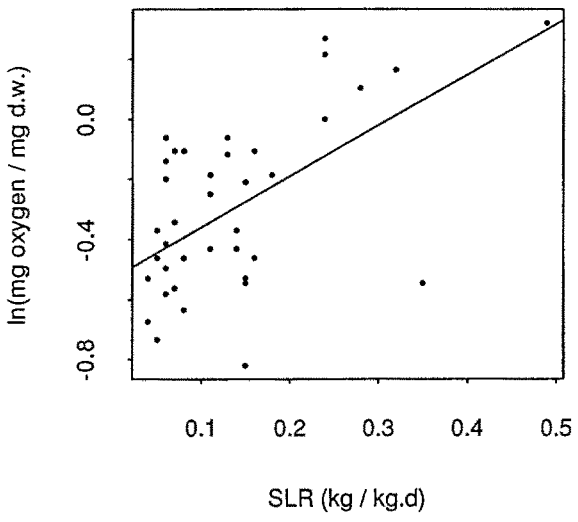

Fig. 2. Log transformed O_2 uptake (t = 7 d) vs the sludge loading rate (SLR).

one at SLR close to 0.5, would significantly increase R^2 (0.525) with least squares estimates for a and b equal to respectively - 0.56 and 2.16.

The average value of ln Ox_{as} of the 40 observations equals 0.304, which is equivalent to an oxygen uptake equal to 0.74 mg O_2/mg activated sludge, whereas the 95 percentile is equivalent to

$$Ox_{as}(95\%) = 1.18\ mgO_2/mg\ dry\ weight$$

Taking into account the SLR dependence and anticipating that more than 95% of the activated sludge reactors are operated according to SLR < 0.3 kg/kg·d if the treatment percentage of communal waste water is higher than 90%, an upper limit is derived:

$$Ox_{as}(SLR < 0.3) < 0.96\ mgO_2/mg\ dry\ weight$$

In those areas where SLR < 0.5 kg/kg·d cover the majority of the treatment plants a higher endogenous O_2 uptake is to be anticipated:

$$Ox_{as}(SLR < 0.5) < 1.38\ mgO_2/mg\ dry\ weight$$

Secondary effluent, Ox_{ef}

Initial determinations of Ox_{ef} per 5 mL ($Ox_{ef}[5]$), conducted on effluent from the first sampling tour, were too inaccurate due to a very low response. Therefore, from treatment plant 9 to 40 $Ox_{ef}[50]$ was determined and installations 1 to 8 were sampled again after nr 40. Both Fig. 3a and 3b indicate that the distribution of $Ox_{ef}[50]$ is not Gaussian. One sample station (nr 11) seems to have produced an outlier for effluent but not for activated sludge. Identification of effluent of nr 11 as an outlier is confirmed by a plot of the distribution of log transformed results (Fig. 3b). Elimination of the outlier is justified as there is evidence that the distribution is log normal.

Log $Ox_{ef}[50]$ correlated neither with the season nor with the sludge loading rate (N = 39). The estimated 95% percentile of the data (with omission of the outlier) equals

$$Ox_{ef}[50](95\%) = 0.958\ mgO_2/50\ mL$$

Discussion

According to eqn (3) the maximum activated sludge concentration in the Two Phase Closed Bottle Test depends on the V_w/V_a ratio as is shown in Fig. 4 for three different values of Ox_{as}. The version in which $V_w/V_a = 2:1$ is prescribed has recently been examined in an ISO round robin test. Also in the CO_2 headspace method (Struijs & Stoltenkamp 1991) this ratio is applied. In both tests 30 mg/L dry weight activated sludge is used for inoculation. This study indicates that 10 mg/L would be a more appropriate inoculum concentration to avoid too high blank responses when $V_w/V_a = 2:1$. In the Dutch standard (NEN 1992) higher V_w/V_a ratios are suggested in order to enhance its applicability to volatile compounds. Based on this study, the activated sludge concentrations, as prescribed in NEN 6515 for ratios 5 : 1, 9 : 1 and 19 : 1 should be reduced by approximately a factor 2 to avoid too high blank oxygen uptake rates.

Also in manometric respirometry tests where the amount of available oxygen is in principle unlimited, round robin tests have indicated that high blank O_2 uptake had an adverse effect on the accuracy of the method (Painter & King 1985; CEC 1985). In calculating the degree of mineralization, the measured inoculum blank is subtracted from the response in the system where the tested chemical is being metabolized. In manometric respirometry methods a blank oxygen consumption would preferably not exceed 10% of the ThOD. A prescribed chemical concentration corresponding to 100 mg/L ThOD in manometric respirometry tests would then allow a maximum content of

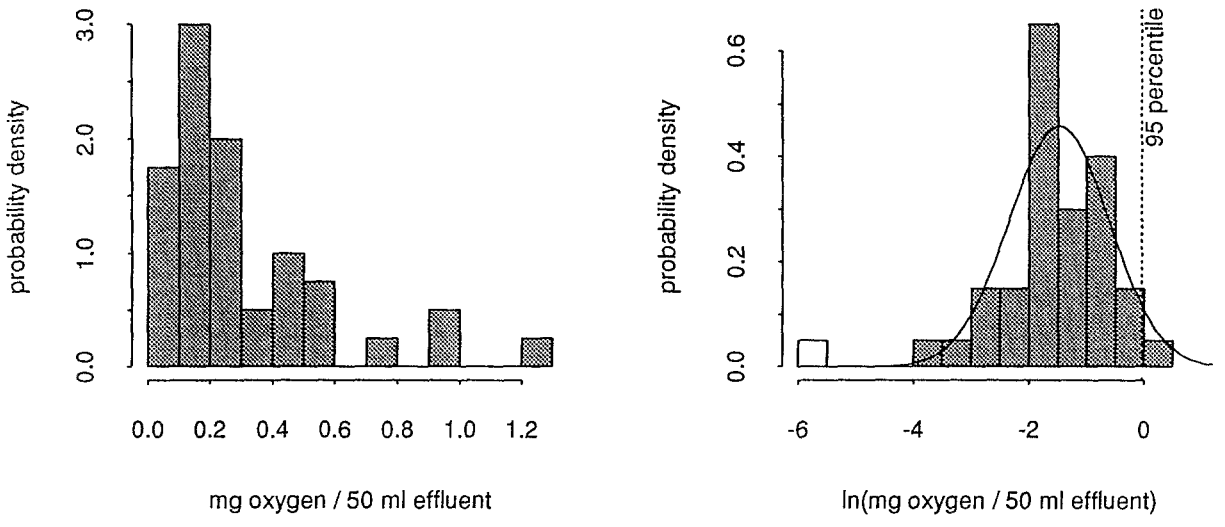

Fig. 3. Histograms of O_2 uptake (after 7 d) for effluent (a) and the log transformed O_2 uptake (b). The open box indicates the omitted observation.

Table 4. Inoculum concentrations, expressed as CFU/mL (King 1981), and normalized oxygen uptake by activated sludge and secondary effluent in mineral medium after 7 d.

parameter	value
geometric mean of Ox_{as}	0.74mg O_2/mg dry weight
geometric mean of Ox_{ef}	$4.127 \cdot 10^{-3}$ mg O_2/mL effluent
CFU per mg activated sludge (dry weight)	$(0.67\text{–}3.3)10^4$
CFU per mL secondary effluent	$(1\text{–}5)10^2$
Ox_{as} per CFU	$(0.2\text{–}1.1) \cdot 10^{-5}$ mg O_2/CFU
Ox_{ef} per CFU	$(0.1\text{–}0.4) \cdot 10^{-5}$ mg O_2/CFU

activated sludge equal to 10 rather than 30 mg/L dry matter.

At first glance it is surprising that 50 mL secondary effluent per litre test medium applied in the Closed Bottle Test would hardly violate the 10% O_c criterion. The 95 percentile of oxygen consumption per 50 mL secondary effluent is approximately equal to 10% of the oxygen saturation concentration which entirely determines O_c in the Closed Bottle Test. If we combine, however, King's data (1981) on colony forming units (CFU) per mL secondary effluent and per mg dry weight activated sludge with the geometric mean values for Ox_{as} and Ox_{ef} of this study, a rather high consistency is apparent: the oxygen uptake after 7 days *per CFU* is approximately equal for both types of inoculum (Table 4).

A quantitative statement on the appropriate amount of inoculum in all kinds of respirometry methods (sealed vessels and manometric methods) requires the knowledge of the blank oxygen uptake or carbon dioxide evolution due to the endogenous activity of the inoculum. As most often the inoculum is derived from a biological waste water treatment plant, the blank response due to endogenous respiration of activated sludge and secondary effluent in RBTs has to be characterized in order to be able to improve test methods. In this study 40 of such plants were sampled to obtain this information quantitatively.

It can be argued that not only in the TPCBT the inoculum concentration should have an higher limit equal to 10 mg/L dry weight if $V_w/V_a = 2/1$, but also in the CO_2 headspace method. The $0.1 \cdot O_c$ criterion is generally applicable if we assume that for both min-

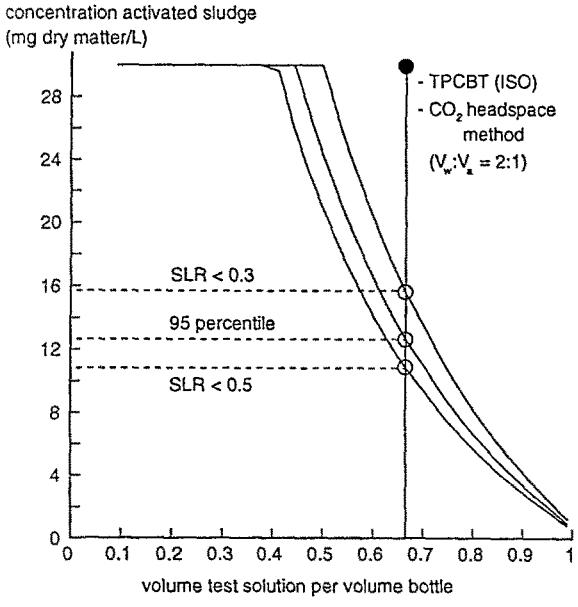

Fig. 4. Maximum inoculum concentrations in two phase closed bottle tests as a function of $V_w/(V_a + V_w)$. Lines are according to the proposed criteria if Ox_{as} is 1.18, 0.96 and 1.38 mg O_2/mg dry weight for 95 percentile, SLR < 0.3 and SLR < 0.5, respectively. • represents the inoculum concentration in two sealed vessel tests reported previously, ○ recommended in this study.

eralization and endogenous respiration the production of inorganic carbon is proportional to oxygen uptake. It is recommended that also in respirometric manometry tests the inoculum concentration is reduced by a factor three if the concentration of the test chemical is less than 100 mg ThOD/L. These methods are all considered 'high inoculum' methods whereas the Closed Bottle Test is a 'low inoculum' method for Ready Biodegradability. Our results indicate that the inoculum concentration in the latter may be *increased* by a factor of ten. This is beneficial in view of future harmonization work which again may be aimed at a further reduction of differences in biodegradation potencies of these methods. Sealed vessel tests other than the Closed Bottle Test are expected to be adopted as standardized tests in the near future as there is a growing interest in simple and inexpensive methods for mineralization of poorly soluble compounds.

List of abbreviations

BOD	biological oxygen demand
CBT	Closed Bottle Test
C_{as}	inoculum concentration in mg dry solids of activated sludge per litre test medium
C_{ef}	inoculum concentration in ml secondary effluent per litre test medium
C_{ss}	dry weight content of activated sludge (g/L)
CFU	colony forming units
DO_{7d}	dissolved oxygen concentration (mg/L) after 7 days
ISO	International Organization for Standardization
NEN	Dutch Organization for Standardization
O_c	oxygen capacity in mg oxygen per litre vessel volume
OECD	Organisation for Economic Co-operation and Development
Ox_{as}	oxygen consumption after one week in mg oxygen per mg dry weight activated sludge
Ox_{ef}	oxygen consumption after one week in mg oxygen per mL secondary effluent
$Ox_{ef}[n]$	oxygen consumption after one week in mg oxygen per n mL secondary effluent
Ox_{flask}	oxygen uptake in mg per litre flask volume
RBT	Ready Biodegradability Test
SLR	sludge loading rate in kg O_2/kg dry weight·d
ThOD	theoretical oxygen demand
TPCBT	Two Phase Closed Bottle Test
V_a, V_w	volumes of air and water per litre vessel volume, respectively
ρ_a	concentration of oxygen in air at 20° C and 101.5 kPa
ρ_s	saturation oxygen concentration in te aqueous phase

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