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A new method to determine the anaerobic degradability of surfactants: the AnBUSDiC test

Thomas Bendt[1] and Andreas Willing[2*]

Abstract

Background: Surfactants are chemicals with a high production volume and a wide dispersive use, i.e. surfactants have a high environmental impact. Most commercial surfactants are aerobically biodegradable. Only a small fraction of the surfactants is not aerobically broken down during the usual hydraulic retention times of modern WWTPs. This fraction, due to the predominantly hydrophobic nature of surfactants, adheres to the sludge. The sludge is usually collected and further treated under anaerobic conditions in digester tanks. Therefore, the knowledge about anaerobic biodegradability under digester tank conditions is important to gain an understanding about the environmental fate of surfactants.

Results: A new test method suited for the assessment of the anaerobic biodegradability of surfactant under sewage plant simulation conditions is proposed. The test method foresees that an accurately known amount of the test substance is added to the sludge inoculum, and that the test substance is added in two sequential steps to overcome possible interferences from unspecific digester gas formation caused by the surface-activity of the surfactant test substance. By measuring the difference in the gas volumes produced in the sludge inoculum plus test substance and the corresponding control (sludge inoculum only) and converting the gas volumes to the percentage degree of biodegradation, this test allows the quantification of the anaerobic biodegradability of the test substance.

Conclusions: Tests with commercial surfactants indicate that the newly developed test method allows for a quantification of the degradation of surfactants under conditions encountered in the anaerobic digester tank of municipal waste water treatment plants. The described test is particularly suitable for the testing of surfactants, because the two-step design overcomes any problems related to unspecific digester gas formation caused by the surface-activity of the test substances, therefore avoiding false positive results.

Keywords: Surfactants, Anaerobic biodegradation, Environmental fate, Digester tank simulation test

Background

Surfactants are chemicals with a high production volume and a wide dispersive use, i.e. surfactants have a high environmental impact. The question of the persistence of a particular substance is one of the most important aspects for the assessment of the risks associated with chemical substances (EU Technical Guidance Document, 2003). Therefore, strict legal requirements for the aerobic biodegradability of surfactants have been established on national and international level, e.g. the EU Detergent Regulation (EC/648/2004). The most common fate for surfactants is that they are discharged via waste water. Surfactant containing domestic and industrial waste water is collected and purified in professionally operated waste water treatment plants. Taking into account the strict legal requirements assuring the use of biodegradable surfactants, the vast majority of surfactant molecules entering a waste water treatment plant (WWTP) are readily broken down to CO_2 and H_2O. Only a very small fraction of the surfactants is not aerobically broken down during the usual hydraulic retention times of modern WWTPs. This fraction, due to the predominantly hydrophobic nature of surfactants, adheres to the sludge. The sludge is usually collected and further treated under anaerobic conditions in digester tanks.

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Therefore, the knowledge about anaerobic biodegradability under digester tank conditions is important to gain an understanding about the environmental fate of surfactants. Unfortunately, no standard method is available to test surfactants under these conditions.

The development of a new test method suited for the assessment of the anaerobic biodegradability of surfactant under sewage plant simulation conditions is based on an analysis of the existing methods, which are summarized in the Fraunhofer Report on the "Anaerobic Degradation of Detergent Surfactants" to the EU Commission (2002). As the most promising basis, the test method according to DIN 38414, part 8 was chosen. The test method was modified in that way that an accurately known amount of the test substance is added to the sludge inoculum, and that the test substance is added in two sequential steps to overcome possible interferences from unspecific digester gas formation caused by the surface-activity of the surfactant test substance. By measuring the difference in the gas volumes produced (after the second addition of the test substance) in the sludge inoculum plus test substance and the corresponding control (sludge inoculum only) and converting the gas volumes to the percentage degree of biodegradation, this test allows the quantification of the anaerobic biodegradability of the test substance.

The newly developed test method, i.e. the modified DIN 38414, part 8 test, allows for a quantification of the degradation of surfactants under conditions encountered in the anaerobic digester tank of municipal waste water treatment plants. Therefore, we propose to name this test the Anaerobic Biodegradation Under Sludge Digester Conditions test (=AnBUSDiC test). The AnBUSDiC test is relevant for the assessment of the environmental fate of surfactants, as surfactants usually end up in the anaerobic digester tank of WWTPs. The AnBUSDiC test is particularly suitable for the testing of surfactants, because the two-step design overcomes any problems related to unspecific digester gas formation caused by the surface-activity of the test substances, therefore avoiding false positive results.

Results and discussion

As the first step to develop an anaerobic degradation test, which is especially suited for surfactants a literature review has been conducted. The recent review article on Anaerobic degradation of Detergent Surfactants prepared by the Fraunhofer Institut UMSICHT at Oberhausen (Germany) by request of the EU Commission [1] has been most helpful in this regard.

The published test methods were evaluated for their suitability to determine the anaerobic biodegradability of surfactants under use-relevant, i.e. waste water treatment plant conditions. The standard test methods can be divided into two classes (see Table 1): composting tests run at 52°C and submerged tests run at 35°C. The composting tests are run under almost dry conditions as they are designed to mimic the conditions in a composting heap. Their main use is the assessment of the compostability of bio-based polymers. Therefore, they are not primarily relevant for surfactants. Analysis of the test run under submerged conditions reveals that the tests ASTM D 5210, ISO 11734, ISO 14853, OECD 311 and the ECETOC test are characterized by a low microbial density (a small inoculum) and the fact that the test substance is the only carbon source, i.e. the test conditions are stringent, but not necessary realistic with regard to the fate of chemicals like surfactants. In addition, there are indications that the reproducibility of the anaerobic screening tests is poor [2].

As a potentially promising test system the test according to DIN 38414, part 8 has been identified, because this test uses more practice-related mass concentrations. This test is set up to determine possible inhibitory effects of (unknown) waste water components for the anaerobic stage of biological waste water treatment plants. The method is based on a comparison of the degradation rates obtained in presence of the (unknown) test effluent with the untreated control (reference sludge alone). The test design was evaluated with regard to its suitability for a quantitative determination of surface-active substances (see Figure 1). Another potentially interesting simulation test for anaerobic biodegradability is the OECD 314, Part C, which has been standardized just recently at OECD level. OECD 314, part C assesses the biodegradability of organic substances by anaerobic digester sludge. This test, however, requires [^{14}C] labeled test substances, which clearly limits the scope of the test.

The digester gas formation from sludge with and without three well-known surfactants is shown in Figure 1. In the presence of anaerobically biodegradable surfactants, i.e. #35 and # 46 of the DID list [3], 900 ml resp. 1500 ml of extra digester gas is produced, whereas in the presence of a (under conditions of the ECETOC test) non-biodegradable surfactant, i.e. #1 of the DID list), no extra gas is formed, but rather an inhibition of the gas formation is observed. It is also interesting to notice that the test was run with rather high surfactant concentrations compared to the ECETOC test, which uses only 20-50 mg organic C/ml corresponding to 40-100 mg surfactant. In case of the two anaerobically biodegradable surfactants, the test substance concentration was 20-30 times higher as in the ECECOC test without causing inhibition.

In the next experiment, the influence of the test substance concentration on the degradation rate was investigated.

Table 1 Standard methods for testing of anaerobic biodegradation (taken from [4], but up-dated)

	Standards				**Draft Standards**			**Standards**	
	ASTM D 5210:1992 (1)	**ASTM D 5511: 1994 (2)**	**DIN 38414, TL 8: 1985-06 (3)**	**ISO 11734: 1995**	**ISO/DIS 14853: 1999 (4)**	**ISO/DIS 15985: 1999 (5)**	**CEN/Draft: 1995 (6)**	**OECD 311: 2001 (7)**	**ECETOC: 1988 (89)**
Degradation parameter	Biogas, CO_2 and CH_4, soluble organic carbon, residual polymer	Biogas, CO_2 and CH_4 in gas phase	Biogas in gas phase	Biogas in gas phase and soluble inorganic carbon (IC) in liquid phase	Biogas, CO_2 and CH_4, DOC, TIC resp. DIC	Biogas, disintegration of test substance, optional CO_2 and CH_4 in gas phase	Biogas and disintegration	Biogas, DIC in liquid phase	Biogas, DIC in liquid phase
Test substance	polymer	polymer		soluble organic substance	Non-soluble (polymeric) substances	Non-soluble (polymeric) substances	packaging material	div. Material	div. Material
Medium	definite mineral salt medium	digested substance	sewage sludge	definite mineral salt medium	definite mineral salt medium	digested substance	digested substance	definite mineral salt medium	definite mineral salt medium
Test volume	100 mL	ca. 1 L in 2 L Erlenmeyer-flasks	500 mL	100 – 1000 mL	250 mL	ca. 1000 mL	ca. 1000 mL	100 – 1000 mL	100 – 1000 mL
Test duration		up to 70% degradation rete in reference substance	20 – 40 d	60 d	30 – 60 d	15 d (or longer)	15 d (or longer)	60 d	8 weeks
Temperature	35 ± 2°C	52 ± 2°C	35 ± 1°C	35 ± 2°C	35 ± 2°C	52 ± 2°C	52 ± 2°C	35 ± 2°C	35 ± 2°C
Method	volumetric or manometric	volumetric	volumetric	manometric	volumetric or manometric	volumetric (as example)	Volumetric (as example)	manometric	manometric
Concentration test substance		15 – 100 g dry substance/L		100 mg/L organic carbon	100 mg/L organic carbon	20 g DS with 8 g TOC/L	20 g DS with 8 g TOC/L	20 – 100 mg/L organic carbon	20 – 50 mg/L organic carbon
Dry substance	1 – 2 g/L	300 g/L		1 – 3 g/L	1 – 3 g/L	> 200 g/L	> 200 g/L	1 – 3 g/L	1 – 5 g/L

(1): Standard test method for determining the anaerobic biodegradation of plastic materials in the presence of municipal sewage sludge.
(2): Standard test method for determining anaerobic biodegradation of plastic materials under high-solids anaerobic-digestion conditions.
(3): Evaluation of digester behavior.
(4): Evaluation of ultimate anaerobic biodegradation of plastic materials in an aqueous system – method by analysis of carbon conversion to carbon dioxide and methane.
(5): Plastic – evaluation of the ultimate anaerobic biodegradability and disintegration under high-solids anaerobic-digestion conditions – method by analysis of released biogas.
(6): Evaluation of ultimate biodegradation and disintegration of packaging materials under high-solids anaerobic digestion conditions – method by analysis of released biogas.
(7): Ready anaerobic biodegradability; gas production from diluted anaerobic sewage sludge; OECD guidelines for testing of chemicals. Organization for Economic cooperation and development (2006).
(8): ECETOC Technical Report No. 28: "Evaluation of anaerobic biodegradation; Guideline for screening of chemicals for anaerobic biodegradability" (ECETOC – European.
Chemical Industry Ecology and Toxicology Centre).

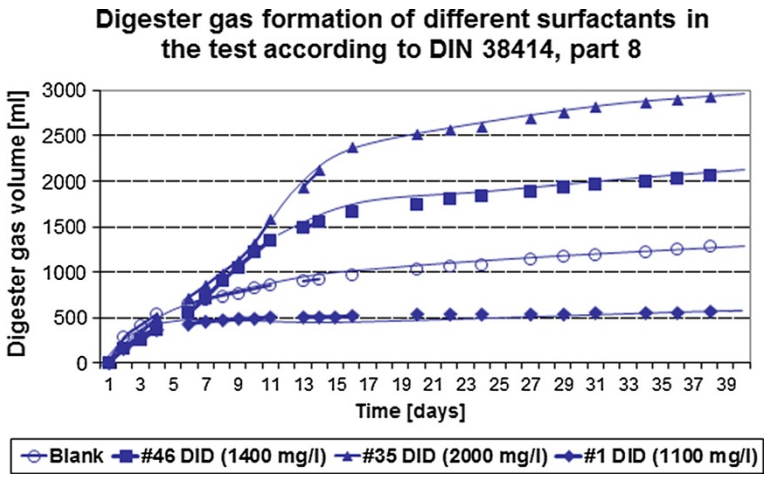

Figure 1 Gas evolution curves of well-known surfactants in the test according to DIN 38414, part 8 (conditions: 475 ml sludge from the digester tank + 25 ml raw (aerobic) sludge).

As can be seen from Figure 2, the surfactant has a rather short lag phase of about 5 days at a concentration of 500 mg/l. After about 21 days, the degradation curve of the test substance and the control become parallel, i. e. the degradation has reached the plateau phase. At the higher concentration of 1,000 mg/l the test substance seems to be initially inhibitory. After about 21 days, the inhibitory effects are overcome, probably due to primary degradation of the toxic surface-active parent structure). At the end of the test, at day 35, roughly twice as much surplus digester gas is formed at the test substance concentration of 1,000 mg/l compared to the test substance added at 500 mg/l.

The next step in method development was to quantify the degradation rate of the test substances. For this, the carbon content of the test substance needs to be determined. We have used elementary analysis for C-determination (see Table 2).

Based on the net gas volume, the C-content, the amount added to the test system according to the equations as given under material and methods, the degradation rate of the test substance as % of the complete degradation can be calculated based on the net digester gas formation determined in the modified DIN 38414, part 8 test.

Using the test according to DIN 38414, part 8, modified for degradation rate quantification, we have investigated the degradation rated of surfactants with known anaerobic degradation properties, i.e. linear alcohol ethoxylates (#35 DID) and linear alkyl benzene sulphonates (#1 DID) (see Figure 3).

The result shown in Figure 4 is interesting, as it indicates a degradation rate > 100% (120% after 28 days). As the reference TOC-value has been based on elementary analysis, we believe that the result in excess of 100% is not related to a underestimation of the reference value.

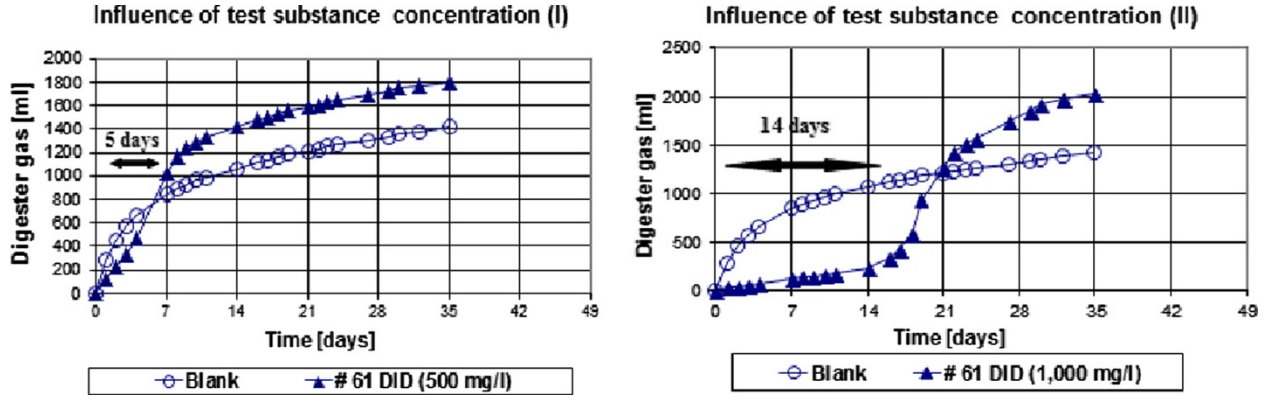

Figure 2 Biodegradation curves of known anaerobically biodegradable surfactant (#61 DID) at 500 mg/l and at 1,000 mg/l test substance concentration.

Table 2 Carbon content of test substance Betaine determined by elementary analysis

Test	Surfactant type	Chemical structure	Batch	Date of analysis	C-content	N-content
1	amphoteric	Betaine	090063	06.11.2009	20.8%	2.3%
2	amphoteric	Betaine	110013	25.02.2011	20.7%	2.5%

Even more surprisingly is the result obtained with DID-No. 1 (LAS), shown in Figure 5. It is well established (see DID list) that LAS is not anaerobically biodegradable under the conditions of OECD 311 (ECETOC test) [4], although recently an anaerobic degradation pathway has been discovered for LAS [5]. However, a very high degradation rate was observed for LAS, comparable to the degradation rate of the linear alcohol ethoxylate. As such high degradation rates are reproducible in the DIN 38414, part 8 test (results not shown), they are not considered to be artefacts (e.g. resulting from leaking seals). There are two possible explanations: (i) LAS is indeed anaerobically biodegradable under the conditions of the DIN 38414, part 8 test, (ii) or the observed surplus digester gas production is not related to the degradation of the LAS itself, but due to an enhanced gas formation of other organic components contained in the digester sludge. Our working hypothesis at this point was - that depending on the degree of surface-activity of the added surfactant - surfactants can either increase the bioavailability of nutrients (e.g. P) and/or liberate easy to degrade organic sludge components.

To distinguish between these two possibilities we have modified the testing scheme of the DIN 38414, part 8 in regard to the test substance addition. Assuming that any unspecific digester gas formation caused by the surface activity of the added surfactant takes place immediately after the addition of the surfactant, we have introduced a second test substance addition into the testing scheme. The second addition of the test substance should ideally be done after the gas formation has reached the plateau phase.

The modified method, with two-step addition of the test substance, yields the expected results. Unspecific digester gas formation is observed in phase 1, i.e. after the first addition of test substance, whereas in phase-2 the test substance specific biodegradability is observed (see Figure 6). As the modified DIN 38414, part 8 test allows for a quantification of the degradation of surfactants under conditions encountered in the anaerobic digester tank of municipal waste water treatment plants, it is named the Anaerobic Biodegradation under Sludge Digester Conditions test (=AnBUSDiC test).

However, the data shown in Figures 6, 7, 8, are – although reproducible (data not shown) - only initial test results and more data are needed to prove that the AnBUSDiC test provides reliable results on the anaerobic biodegradability of surfactants. Therefore, in another test series we have extended the test scheme and have included even a third test substance addition step. The result for the linear alcohol ethoxylate (#35 DID) is shown in Figure 9.

A detailed analysis of the results show that unspecific digester gas formation (i.e. which is not related to degradation of the test substance) takes place mainly after the first addition of the surface-active substance. The second addition of the surfactant does only lead to a little additional unspecific gas formation. In addition, we have undertaken several experiments with subsequent addition of different test substances, including surfactants as well as non surface active substances, e.g. isosorbit (Figure 10).

Taken together, the results obtained with the AnBUSDiC test demonstrate that the problems with unspecific

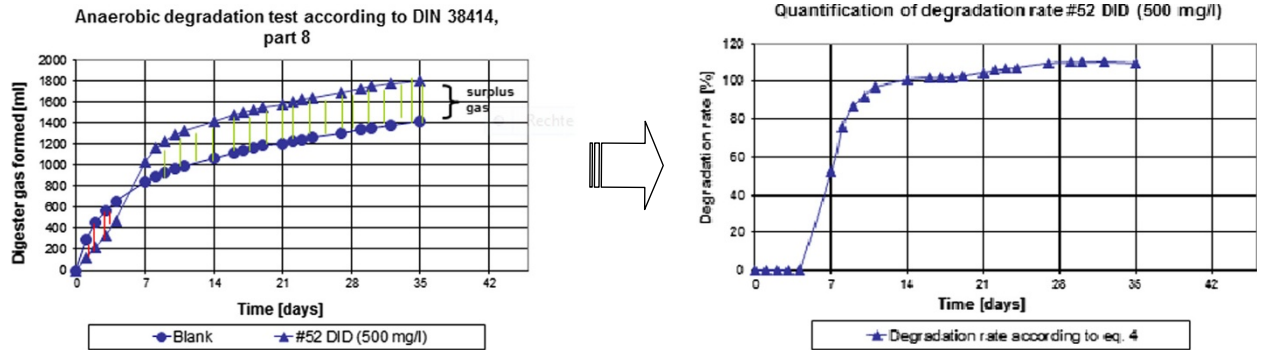

Figure 3 Quantification of degradation rates: from net gas volume the degradation rate of # 52 DID as [%] is calculated base on the added amount of test substance (as TOC) by applying equation 6.

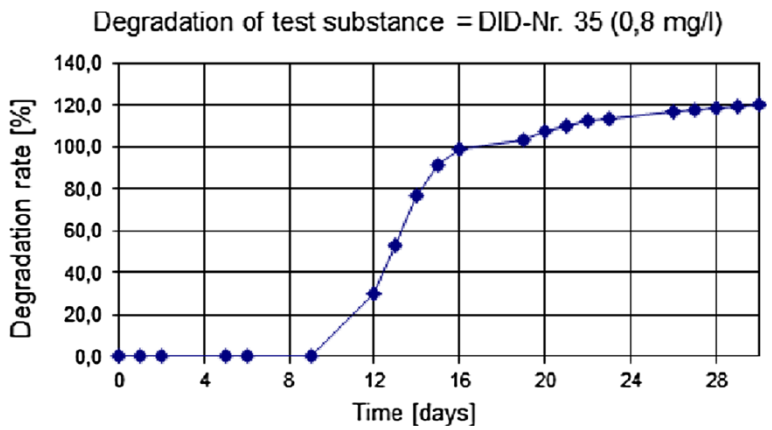

Figure 4 Degradation rates of test substance # 35 DID, calculated according to (equation 6) from the net gas evolution curves, and the TOC and the amount added to the test.

digester gas formation can be overcome using a two-step test substance addition.

Discussion

Biodegradation is the most important mechanism for the detoxification and ultimately the removal of chemicals from the environment. This is particularly true of surfactants, which are per se surface-active (i.e. toxic) and which are discharged down the drain. For the evaluation of the aerobic degradability several standardized screening tests (e.g. OECD 301 A-F for ultimate degradability, respectively OECD 302 A-C for inherent degradability) as well as some simulation tests (e.g. OECD 303 A-C and OECD 314) are available. In contrast, for the evaluation of the ultimate degradability under anaerobic conditions, the number of test methods is much lower. Two screening tests are available (OECD 311, ISO 11734), which are characterized by a low microbial density (a small inoculum) and the fact that the test substance is the only carbon source, i.e. the test conditions are stringent but not necessary realistic with regard to the fate of chemicals like surfactants. In addition, there are indications that the reproducibility of the anaerobic screening tests is poor [2]. The only simulation test, which has been standardized just recently at OECD level, is the OECD 314, part C [6]. OECD 314, part C assesses the biodegradability of organic substances by anaerobic digester sludge. This test, however, requires [^{14}C] labeled test substances, which clearly limits the scope of the test.

Taking into account that anaerobic biodegradation has or may become included as a pass/fail criterium in surfactant-specific regulations (e.g. EU Detergent Regulation, EU Ecolabel) there is a need for a test which reliably determines the anaerobic biodegradation of surfactants under relevant (use-related) conditions. Various methods for determining the anaerobic degradability of organic substances have been developed based on the ultimate aerobic biodegradation screening tests which are summarized in the Fraunhofer Report on the "Anaerobic

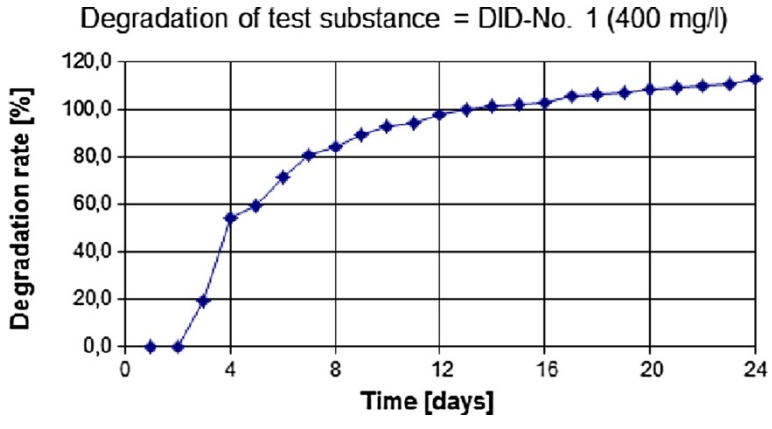

Figure 5 Degradation rates of test substance # 1 DID, calculated according to (equation 6) from the net gas evolution curves, and the TOC and the amount added to the test.

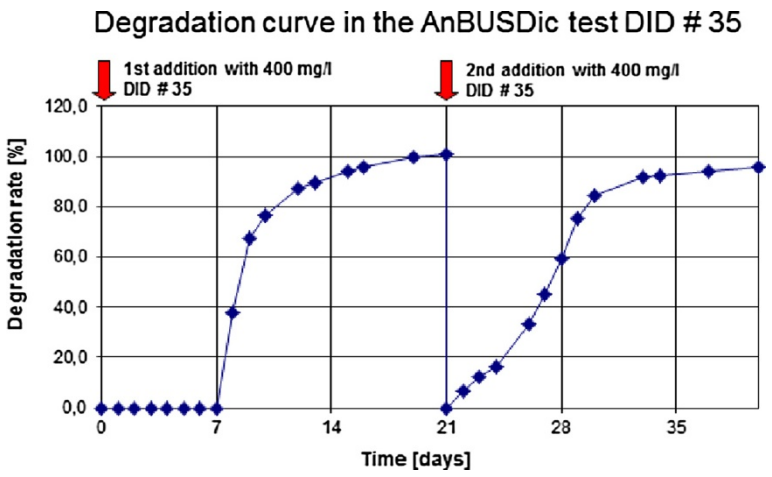

Figure 6 Degradation rates of test substance # 1 DID in the modified (two-step) testing scheme.

Degradation of Detergent Surfactants" to the EU Commission (2002) [1]. The methods can be divided in two groups, the composting test running at 52°C (which are not relevant for waste water effluents), and the tests in aqueous media running at 35°C (e.g. ISO 11734 and OECD 311). These test methods use sludge from the digestion tower of municipal sewage plants as the inoculum. A comparison of the prevailing biodegradation conditions in these tests (e.g. OECD 311) with those encountered in practice, i.e. the conditions that are found in the digestion tower of a municipal sewage plant, shows that sewage plants work with considerably higher mass concentrations both of sludge and of organic pollutants. The above-mentioned tests for determining the ultimate biodegradation under anaerobic conditions are therefore only suitable to a limited extent for making practice-related statements about the anaerobic biodegradation of substances. Much more practice-related mass concentrations are used in simulation tests, like OECD 314 [6] or in the test according to DIN 38414, part 8 [7]. When we started the project, we had also another aspect in mind, namely cost and time efficiency of the test system, so that ideally the test could be used for serial screening of research substances. Based on these considerations, we have chosen the method DIN 38414, part 8 as the starting point for the development of a new method for determining the ultimate anaerobic biodegradation of organic substances under sewage plant simulation conditions. The main difference between this test and that of the method given in DIN 38414, part 8 is that an accurately known amount of the test substance is added to the sludge inoculum. By measuring the difference in the gas volumes produced in the sludge inoculum plus test substance and the corresponding control (sludge inoculum only) and converting the gas volumes to the percentage degree of biodegradation, this test ultimately allows the quantification of the anaerobic biodegradability of the test substance. This

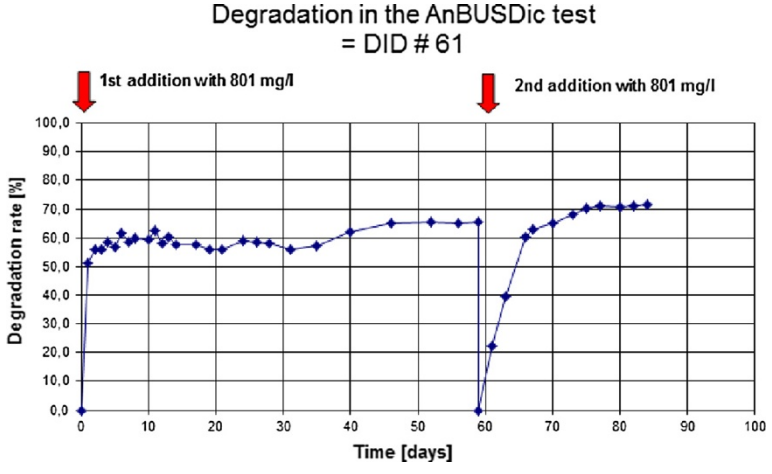

Figure 7 Degradation of test substance # 35 DID in the modified (two-step) testing scheme.

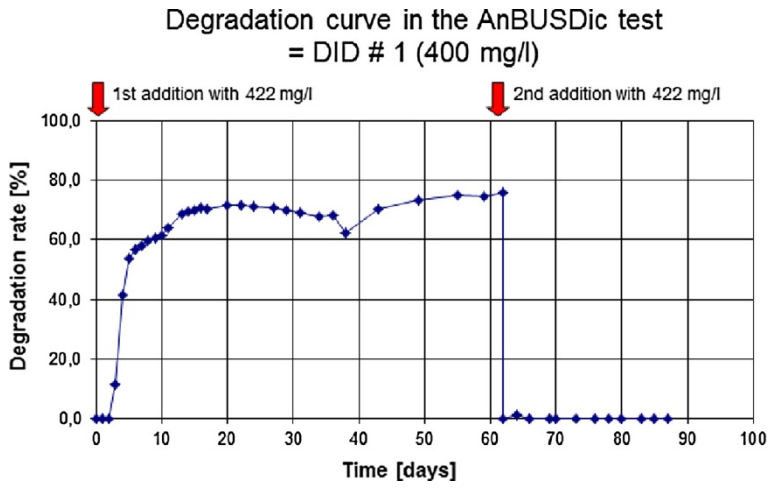

Figure 8 Degradation of test substance # 61 DID in the modified (two-step) testing scheme.

anaerobic biodegradation under sewage digester conditions test (AnBUSDiC test) is particularly suitable for testing chemicals used in consumer products which, if used in accordance with the relevant instructions, are found practically quantitatively in the wastewater and therefore also in the anaerobic treatment stage of municipal sewage plants. In order to be able to assess the potential risks involved in applying clarified sludge to the soil the ultimate anaerobic biodegradability under the conditions of a sewage plant simulation test is the method of choice. This test is carried out in order to investigate the degradation behavior of the test substance under conditions such as prevail in the anaerobic sludge treatment in municipal sewage plants. The result may also be used to demonstrate that the substance complies with the criteria for good anaerobic biodegradability demanded by surfactant-relevant environmental labels (particularly the EU Marguerite and the Nordic Swan).

The development of a new test method suited for the assessment of the anaerobic biodegradability of surfactant under sewage plant simulation condition has been based on an analysis of the existing methods, which are summarized in the Fraunhofer Report on "Anaerobic Degradation of Detergent Surfactants" to the EU Commission (2002) [1]. As the most promising basis the test method according to DIN 38414, part 8, was chosen. Although the AnBUSDiC test is more tolerant to high surfactant concentrations, the substance concentration has to be a compromise between the limit of detection (sufficient discrimination between the test sample and the blank sample) and toxicity inhibition (It is well known from the ECETOC test, that higher concentrations of surfactants

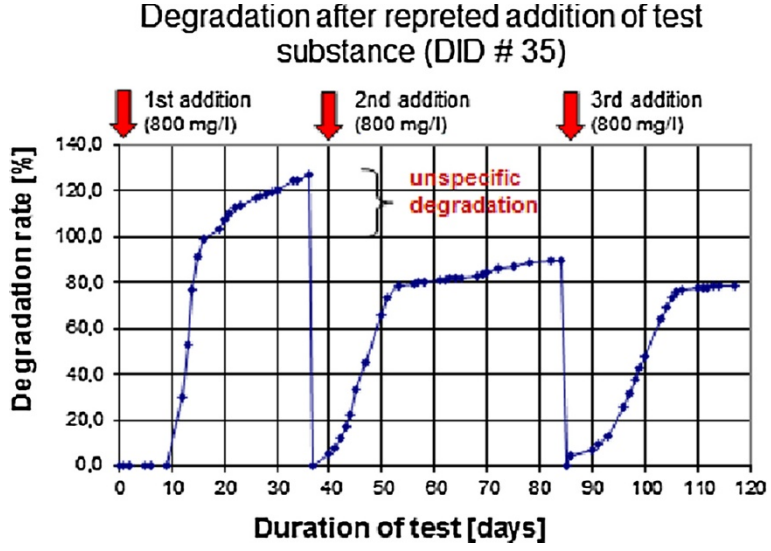

Figure 9 Degradation curve of test substance 3 (# 35 DID) from repeated additions.

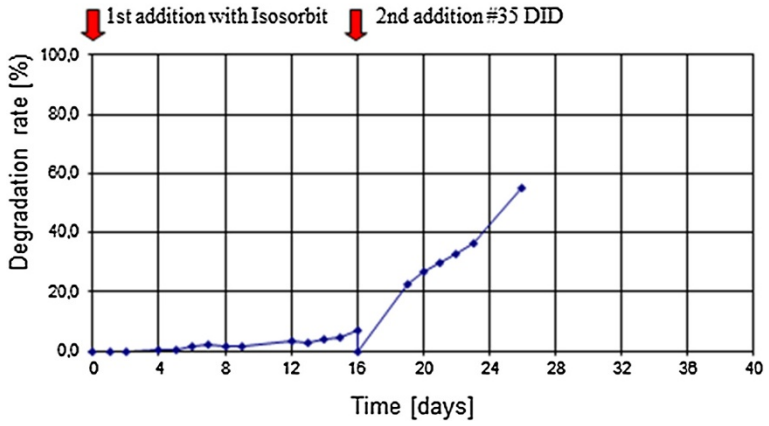

Figure 10 Subsequent addition of a non-degradable substance (isosorbit) followed by an easily degradable surfactant (# 35 DID).

can be toxic for the sludge microorganisms [8]). However, the higher inoculum concentration, named as dry residue, in the AnBUSDiC test seem to "buffer" to some extent the toxicity, so that compared to the conditions in the ECE-TOC test, generally higher surfactant concentrations are tolerated. For surfactants a test substance concentration of 200 – 800 mg active substance per liter seems to be a good choice for the 1st addition (i.e. 150 - 500 mg TOC/ per liter). For non toxic compounds, even higher test substance concentrations may be appropriate.

The original DIN 38414, part 8, test has been set up to determine the inhibition of fouling, which is of importance for assessing the suitability of organic waste streams for renewable energy production. In this context, digester gas production is the most relevant parameter and a quantification of degradation rates is not within the scope of this test method.

However, for our purpose, i.e. for the assessment of the anaerobic biodegradation of surfactants under sludge digester conditions, the quantification of the degradation rates are required. Another problem encountered with the original DIN 38414, part 8, test method is the possible unspecific digester gas formation in presence of surface-active test substances compared to the blank. On the basis of this analysis the test design has been modified with regard to two aspects

i. a well characterized amount of surfactant is added to the test system instead of the unknown test effluent to allow for the determination of an exact degradation rate
ii. a two-step addition of the test substance is introduced to avoid false positive results due to unspecific (excess) gas formation caused by the surface-activity of the test substance.

This subsequent addition of test substance allows the detection of any false positive effect caused e.g. by the increased bioavailability of nutrients or easily biodegradable organic components from the sludge due to the solubilizing action of the surfactant (for known bacteriotoxic substances (e.g. many surfactants) it may be necessary to reduce the substance concentration so far that toxic effects no longer occur). The inclusion of a toxicity control, e.g. glucose of acetate, can help to distinguish between non-degradability cased by persistence or by toxic inhibition. However, depending on the biological activity of the sludge inoculum used (gas production rate of the control), the substance concentration can only be reduced to a limited extent as the signal/noise ratio has a limiting effect from approx. 120 mg TOC/L.

The potential unspecific digester gas formation in phase 1 together with the subsequent addition of test substance (with or without a toxicity control in the second step) makes the evaluation of the test, i.e. the calculation of the degradation rates, complex, because the reference value changes with the second addition. In principle there are two options for the evaluation of the degradation rates for the second phase: either the non-degraded portion of test substance at the end of phase 1 is added to the newly added test substance (equation 1) or only the newly added test substance is taken into account (equation 2):

$$\text{TOC Reference value for phase 2} = \text{TOC } 1^1 + \text{TOC } 2^2 \quad (1)$$

$$\text{TOC Reference value for phase 2} = \text{TOC } 2 \quad (2)$$

There are good reasons to base the calculation of the degradation rate in phase 2 on equation 2, i.e. not to include the remaining TOC from phase 1 in the evaluation

of phase 2. Let us assume that the first degradation reaction has reached the plateau phase at the start of phase 2. In this case, any remaining TOC is not further degradable. If this non-degradable amount of TOC is added to TOC 2, this will lead to an underestimation of the biodegradation rate of the test substance in phase 2. Therefore, under these frame conditions (plateau phase is reached) it makes sense to evaluate phase 2 independently of phase 1 and use TOC 2 as the 100% degradation reference value.

For practical considerations the AnBUSDiC test has a fixed time span of 21 days for phase 1. It could be that in some cases 21 days are too short to reach the plateau phase (see for example Figure 4, there a linear increase in the degradation curve is observed between days 21 - 28). In this case application of equation 2, i.e. neglecting of TOC 1, would result in a potential overestimation of the degradation rate in phase 2. The magnitude of this potential overestimation can be estimated by comparing the degradation rate deduced from the test substance degradation curve at the end of phase 1 and the extrapolated degradation rate when the plateau phase would be reached: the potential overestimation is given by the difference of these two degradation rates. The degree of potential overestimation can be reduced by prolonging the time before the second addition of test substance. However, we believe that 21 days is a good compromise.

Based on the results obtained so far we believe that it is more appropriate to assess the degradation rate of phase 2 independently. Therefore, the AnBUSDiC test method foresees to evaluate the degradation rate of the test substance according to equation 2. Nevertheless, if there are good reasons that an evaluation of the test according to equation 1 may be possible, too. In such cases, it should be clearly documented in the test report that the evaluation deviates from the standard method.

Conclusion

This AnBUSDiC test allows the quantification of the anaerobic biodegradability of the test substance and overcomes possible interferences from unspecific digester gas formation caused by the surface-activity of the surfactant test substance. Further, initial results obtained with surfactants and non-surfactants of well known anaerobic degradability indicated that the modified test system is able to distinguish between degradable and non degradable substances and inhibitory surfactants. Although more work has to be done to standardize the test and to get regulatory acceptance (e.g. a ring test involving different laboratories), we believe that the AnBUSDiC test has several advantages compared to the common screening tests: it is a simulation rather than a screening test (i.e. it determines the ultimate degradability under conditions that are relevant for detergent ingredients), it shows if the surfactant under consideration is degradable, non-degradable or inhibitory at the tested concentration, and it is readily available at many municipal waste water treatment plants.

Methods

For the evaluation of the aerobic degradability several standardized screening tests (e.g. OECD 301 A-F for ultimate degradability, respectively OECD 302 A-C for inherent degradability) as well as some simulation tests (e.g. OECD 303 A-C and OECD 314) are available. In contrast, for the evaluation of the ultimate degradability under anaerobic conditions, the number of test methods is much lower. Two screening tests are available (OECD 311, ISO 11734), which are characterized by a low microbial density (a small inoculum) and the fact that the test substance is the only carbon source, i.e. the test conditions are stringent but not necessary realistic with regard to the fate of chemicals like surfactants. In addition, there are indications that the reproducibility of the anaerobic screening tests is poor [2]. The only simulation test, which has been standardized just recently at OECD level, is the OECD 314, part C [6]. OECD 314, part C assesses the biodegradability of organic substances by anaerobic digester sludge. This test, however, requires [^{14}C] labeled test substances, which clearly limits the scope of the test. Thus, there is the need for an easy-to-carry out test method suited for the assessment of the anaerobic biodegradability of surfactant under sewage plant simulation conditions.

As the first step of the project, a literature review was conducted to evaluate the published test methods for their suitability to determine the anaerobic biodegradability of surfactants under use-relevant, i.e. waste water treatment plant conditions. As a potentially promising test system the test according to DIN 38414, part 8 was identified. The test design was modified to overcome the limitations of the original test. The modified test system is described below in detail.

Basis of the method

A mixture of anaerobic fermenting and methanogenic bacteria is involved in the anaerobic decomposition process such as is usually encountered in the digestion tower of municipal sewage plants. The different microorganisms decompose the organic C-compounds via various intermediate stages to form the ultimate degradation products methane (CH_4) and carbon dioxide (CO_2). The community of all these microorganisms forms the digested sludge in the anaerobic sludge treatment stage in the sewage works.

In order to determine the anaerobic degradation, an exactly defined amount of the substance is incubated together with sludge inoculum at 35 ±1°C. The sludge

inoculum consists of digested sludge to which a certain amount of raw sludge (raw sludge is usually a mixture of 40% sludge from the pre-sedimentation and 60% sludge from the final sedimentation in WWTPs) has been added in order to "vitalize" it, i.e. to obtain a basic sludge gas development. The addition of raw sludge is part of the original DIN 38414, part 8, and when we started to evaluate the suitability of this method for determining the biodegradability of surfactants, we kept this step, as a basic sludge gas development allows to observe possible inhibitory effects caused by the test substances.

The sludge gas production is measured by using a eudiometer. The net gas production of the test batch is obtained by subtracting the volume of gas produced by the control batch. The gas law is then used to convert the net gas volume into the molar gas amount, taking the pressure and temperature into account. The degree of degradation of the test substance is then determined by the rule of three from the measured molar gas amount and the organic carbon provided by the test substance and used in the test.

Information about the test substance

The test substance should be unambiguously described by the sponsor in accordance with the quality assurance requirements (see Item 1). If doubtful results are obtained, the substance identity can be verified by using the retention sample. In addition, the relevant physico-chemical data of the test substance (solubility in water, volatility, adsorptivity) that could possibly influence the meaningfulness of the test result should be known, as well as the purity of the test substance. Last but not least, the active substance content (resp. the content of water) is important, as this is relevant for the choice of the appropriate test substance concentration, which is a compromise between the limit of detection and toxicity inhibition (see 6.3).

The organic carbon content of the test substance must also be known, as it is required as reference point for the evaluation of the test results. There are three options:

1. Use the empirical formula of the test substance together with its concentration in the sample, to calculate the theoretical carbon content (ThOC) of the test sample applied to the test.
2. Carry out an elemental analysis.
3. Determine the organic carbon content (TOC = total organic carbon).

Test procedure

Apparatus and reagents

The apparatus (see Additional file 1: Annex 2 pictures 1-2) consists of an eudiometer (e.g. from Behr Labor-Technik, Düsseldorf, Germany, Behrotest Eudiometer Unit FH 10 as per DIN 38414 p8) with a volume of 400 ml, graduated from top to bottom at intervals of 5 ml. This is attached to the incubation bottle (500 ml capacity) by a ground glass joint. A connection tube is led through the base of the eudiometer tube to allow the sludge gas produced in the incubation bottle to enter the eudiometer tube and in this way displace the barrier liquid into the leveling bottle (750 ml) through a tube. At the upper end of the eudiometer is a stopcock to allow gas samples to be removed and also to adjust the zero point of the barrier liquid. The barrier liquid consists of 30 ml sulfuric acid (density: 1.84 g/ml), 200 g sodium sulfate decahydrate and a few drops of 0.1% methyl orange solution per liter deionized water. The incubation bottles are placed in a water bath kept at 35 ± 1°C by a controlled water thermostat (e.g. Behrotest Apparatus FH 6 from Behr Labor-Technik, Düsseldorf). The water temperature is read off and recorded each day.

Special attention should be devoted to the tightness of the apparatus. A critical point is the seal between the eudiometer and the bottle (picture 3) and the valve at the top of the eudiometer (picture 4). To assure the tightness of the system, the seals should always be carefully greased. It is advisable to check the tightness or the equipment before the start of the degradation test. To do so it is recommended to let the assembled apparatus (without sludge) stand for 24 hour with the collection bottle at a significant lower height than the meniscus of the liquid in the eudiometer. This set up applies a slight negative pressure (vacuum) to the system, which allows one to detect any leaks before the test is started. The risk of leaks can be further minimized, if the bottles containing the excess eudiometer solution are places at about the same height as the meniscus of the liquid inside the eudiometer during the test to minimize the vacuum effect (see picture 5).

The sludge gas analyses (methane/carbon dioxide ratio of the sludge gas formed) are carried out using a method developed in this laboratory in which the carbon dioxide and oxygen can be absorbed in absorption solutions. The gas sample is passed through a potassium hydroxide solution (294 g KOH/L) and then through a sodium dithionite solution (200 g $Na_2S_2O_4$ and 120 g dissolved KOH) until no further reduction of the gas volume by absorption is observed. With sludge gas from the sludge treatment stage the residual gas is methane. This working method is used to check the sludge gas in the technical process. Experience has shown that the sensitive methane bacteria are not damaged with a methane volume fraction >60%.

Source, preparation and quantification of the inoculum

Source and preparation

Sludge, which has been digested to a great extent and then kept biologically active by the addition of a small

amount of raw sludge, is used as the inoculum for the test (sludge inoculum). To standardize the degradation power of the inoculum, the inoculum should be adjusted to 2.5% dry weight (± 0.5%), by using an appropriate amount of the anaerobic sludge. The sludge inoculum is prepared by taking 95% (v/v) digested sludge from the digester tower of the sewage plant (approx. 27 days old and adjusted to about 2.5% dry weight) and mixing it with 5% (v/v) thickened raw sludge taken from the aerobic stage of the sewage plant. In practice it has proved to be advisable to prepare a larger volume of sludge inoculum (approx. 10 l) in order to be able to carry out a large number of tests at the same time.

Quantification

Quantification of the final inoculum is carried out using the dry residue and ignition loss parameters. The ignition loss is determined according to EN 12879, part 3a. The ignition loss is a measure of the organic substance content of the sludge which, in a first approximation, is proportional to the microorganism content of the sludge. In addition to the ignition loss, the dry residue is determined according to EN 12880 part 2a. The pH is determined according to EN 12176, part 5. Depending on the requirements, the content of fixed organic carbon (TOC) as well as the organic carbon dissolved in the sludge (DOC) may be measured. Before the start of the test, the pH is adjusted to pH 7 ± 0.2 by the addition of inorganic buffer substances such as sodium hydrogen carbonate (according to EN 12176 part 5).

The dry residue (target value 2.5% ± 0.5%) and the ignition loss must be documented. An aliquot of 500 ml of the prepared sludge inoculum is used for each test batch.

Preparing the sample

As surfactants are a quite heterogeneous group of chemicals with regard to structure, charge, molecular weight, they have different physicochemical properties. Some a solid, others are liquid at room temperature, and some are even pasty and therefore difficult to handle. To assure that the test substance sample is homogeneous it may be necessary to gently warm up the sample under shaking before an aliquot is removed for C-analysis or addition to the degradation test. The organic carbon content of the test substance is determined as the TOC (g/g) (according to DIN EN 13137 in a double determination) and a known amount of the test substance is used in the test. A test substance concentration of 200 – 800 mg active substance per liter is used for the 1st addition (i.e. 150 - 500 mg TOC/per liter). After the net gas evolution in the test sample has come to an end (i.e. the degradation kinetic of the test sample and the control are more or less identical), a 2nd addition of another 200 – 800 mg active substance per liter is made to the test sample. Direct addition to the test system is appropriate.

Carrying out the test

The determination of the biodegradability of the test substance is made by determining the difference in the amounts of digester gas produced by the test batch and the control batch. The test and control batches are treated with identical aliquots of the sludge inoculum.

In general the DIN 38414, part 8, test is more tolerant to higher test substance concentrations than the ECE-TOC test. As a rule of thumb 150 – 500 mg TOC of the test substance per liter are a good starting concentration for most surfactants, but for some surfactants even 1500 mg TOC/liter are not inhibitory.

The test solutions (500 ml sludge inoculum and sludge inoculum with test substance) are placed in the incubation bottles of the eudiometers as described above. These are then sealed with a ground joint stopper and gently homogenized without any air entrainment. The incubation bottles are then fitted with the eudiometer attachments and treated in parallel by placing them in a water bath held at 35 ± 1°C. The eudiometers are filled to the zero mark with the barrier liquid (see 6.1) with the stopcock open. All tests and controls are carried out at least as double determinations. Each water bath contains one control batch and two test batches as a double determination.

It is assumed that any unspecific digester gas evolution caused by the surface activity of the test substance takes place within the first three weeks. Therefore, after the initial degradation reaction has reached the plateau phase (usually at about day 21), another aliquot of the test substance (with or without the toxicity control) is added. To avoid oxygen entering the system, this is done e.g. under a nitrogen atmosphere. Verification of the anaerobic status of the test system at the end of the test using an oxygen indicator like resazurin – as in the OECD 311 - is not easily possible, as the high sludge concentration does not allow per se to determine the color of the redox dye. It may be possible to remove the sludge, e.g. by filtration under nitrogen, but this will require further work.

Further it should be kept in mind that the correct TOC reference value defining 100% degradation of the added test substance(s) is used for the calculation of the degradation rate of the second phase (see evaluation).

The batches are swirled around once per day

The gas volumes are initially read off and recorded on a daily basis; subsequently the reading intervals can be lengthened to several days as is necessary. For reading the evolved digester gas volume, the meniscus of the liquid collection bottle and the meniscus of the liquid

inside the eudiometer should be aligned (see picture 5). All readings are noted in the test protocol. After each reading the barrier liquid in the eudiometer is readjusted to the zero mark. In addition, the water bath and room temperatures are measured and recorded on a daily basis (these are important for the conversion of the gas volumes to molar amounts).

The daily determined gas amounts are added together for each batch, but separately for phase 1 and phase 2 (both starting at zero), and presented as gas production curves and, after appropriate conversion, also as degradation curves.

The tests are continued until the net gas production has reached the plateau phase or the degradation has reached more than 80%.

Accompanying analyses

Depending on the requirements, the carbon dioxide/methane ratio in the sludge gas can also be determined. This is done by taking an analytical sample from each batch and analyzing it. The composition of the sludge gas can provide information about any interference to the anaerobic degradation process.

Evaluation

As the general test principal, the difference between the gas production from the bottles containing the test substance and the bottles without test substance (blank) is taken as the evaluation criterion for assessing the anaerobic degradation of the test substance. Determination of the amount of gas that is dissolved in the liquid medium is neglected to keep the method as simple as possible (it is expected that neglecting to IC does cause only a relatively small error, because the ration between the volume of the liquid medium and the amount of test substance added is much lower in the AnBUSDic test compared to e.g. OECD 311).

The gas amounts are initially read off daily and recorded in tabular form; as the test proceeds and the amounts of gas produced diminish the reading intervals can be extended to several days. For reading the evolved digester gas volume, the meniscus of the liquid collection bottle and the meniscus of the liquid inside the eudiometer should be aligned (see picture 5). For each batch the amounts of gas are converted to the standard temperature and then added together, but separately for phase 1 and phase 2. A mean value is obtained from the double determinations. The mean values in milliliters of the total gas amounts produced by the test and control batches are shown as gas production curves in a graph by plotting them against the test duration in days. In the graph, the degradation rates at day 0 as well as at day 21 should start at the x-axis, and they should refer as a reference value to the amount of TOC added at that time point.

In order to evaluate the degradation rate, the amount of gas produced theoretically by complete biodegradation needs to be known. The basis is the carbon content is the TOC used in a 500 ml batch; this must be previously determined e.g. by elementary analysis. It is advisable to double-check the TOC-value by comparison with the theoretical TOC as deduced from the structural formula of the test substance. It is important to determine the TOC with accuracy as the TOC is used as the reference value for the determination of the degradation rate, i.e. any inaccuracy in the TOC determination results directly in a proportionally wrong degradation result.

The theoretical amount of gas for the test batch at the standard temperature is obtained from the following equation:

$$V_{Th} = \frac{TOC \times M}{m_M} \times V_o \tag{3}$$

where: V_{Th} theoretical amount of gas at standard temperature [ml]

TOC organic carbon content of test sample [g/g]

M weight of test sample per 500 ml batch [g]

m_M molecular weight of carbon [12 g/mol]

V_o standard volume for ideal gas [22414 ml/mol at 273 K]

The amount of gas at standard temperature is calculated on the particular test day:

$$V_N = \frac{V \times T_o}{T} \tag{4}$$

where: V_N amount of gas at standard temperature [ml]

V amount of gas at room temperature [ml]

T_o standard temperature [273 K]

T room temperature [K]

Gas production at standard temperature is calculated on the particular test day:

$$V_{NT(K),i} = \sum V_{NT(K)} \tag{5}$$

where: $V_{NT(K),i}$ gas production of test batch (or control batch) on day i [ml]

$\sum V_{NT(K)}$ sum of mean value of gas of test batch (or control batch) up to day i [ml]

The degradation rate is calculated from the net gas production and the theoretical gas amount for the test batch at standard temperature for 100% degradation on day i according to the equation:

$$\text{Degradation } (\%)_i = \frac{(V_{NT,i} - V_{NK,i})}{V_{Th}} \times 100 \tag{6}$$

where: $V_{NT,i}$ gas production of test batch on day i [ml]

$V_{NK,i}$ gas production of control batch on day i [ml]

V_{Th} theoretical gas amount for test batch [ml]

The percentage degradation curve is obtained by plotting the degradation against the test duration.

Reporting of results

The incubation period, given in days, should be reported. It should be stated if an adaptation is required for the degradation of this test sample (see Additional file 2: Annex 1 for an example for datasheets of results).

The test sample TOC (g/g) should be stated. It is recommended to obtain this value by elementary analysis. The exact amount of test substance (in mg) added to the 500 ml digested sludge mixture should be reported. Further, the theoretical volume of digester gas resulting from complete degradation of the test substance added should be recorded (100% reference value). For each time point the digested gas formed should be recorded.

The sludge should be characterized based on % organic dry residue referred to the ignition loss of the dry residue.

Validity criteria

It should be taken into consideration that the actual operating conditions and the wastewater itself at the waste water plant, from which the digester sludge has been taken, does influence the sludge activity and the actual adaptation capability of the sludge. Therefore, the absolute gas production volumes cannot be used as validity criteria. To overcome this problem, a biodegradable standard reference substance should be included in each test series. The test is considered valid, if the reference substance surpasses 60% degradation at the plateau phase. As the test method is designed to assess particularly surfactants, we recommend using also a surfactant as the reference substance. Surfactants with known biodegradation behavior can be found in the EU Detergent Ingredient Database [3], for instance linear fatty alcohol ethoxylates, e.g. DID # 28 or linear alkyl polyglycosides, e.g. DID # 49.

Endnotes

[1]TOC 1 = remaining TOC at the end of phase 1

[2]TOC 2 = TOC of substances added at the start of phase 2

Additional files

Additional file 1: Annex 2. Pictures.

Additional file 2: Annex 1. Information summary.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AW initiated the project and contributed with his know-how about surfactants. AW introduced the basic idear for quantification of degradation rates and the tow-step concept to overcome the problem of unspecific digester gas evolution. Both authors read and approved the final manuscript.

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