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Water quality — Selection of tests for biodegradability

Qualité de l'eau — Sélection d'essais de biodégradabilité

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Foreword

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The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

In exceptional circumstances, when a technical committee has collected data of a different kind from that which is normally published as an International Standard ("state of the art", for example), it may decide by a simple majority vote of its participating members to publish a Technical Report. A Technical Report is entirely informative in nature and does not have to be reviewed until the data it provides are considered to be no longer valid or useful.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO/TR 15462 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

This second edition cancels and replaces the first edition (ISO/TR 15462:1997), which has been technically revised.

Introduction

The biodegradation of substances and wastewater ingredients depends not only on the molecular structures of the test material, but also on important additional factors, such as the

— aquatic or terrestrial test environments;

— aerobic or anaerobic test conditions;

— source and concentration of the microorganisms of the inoculum;

— acclimatization and adaptation of the inoculum;

— concentration of the test material;

— presence of other organic substrate;

— possible toxic effects of the test material under the test conditions;

— physical and chemical properties and bioavailability of the test material (e.g. volatility, water solubility, adsorption on surfaces);

— physical and chemical properties of the test system (e.g. volume of test mixture and test vessels, CO_2 removal and oxygen concentration, temperature);

— test conditions (e.g. mixing, shaking, mode of aeration, batch or dynamic, closed or open test vessels);

— test duration;

— analytical parameters used (sum parameters, such as DOC, BOD, CO_2 or substance specific analysis).

As so many factors can influence the test results, it is not possible to define a “true” or “reference” method. The reproducibility of the test results using different methods or conditions or even using identical test methods can be low and differing test results can be obtained. Usually, a test material, which is either easily or poorly biodegradable, will produce similar test results in replicates and on repetition. Substances, which are partly or moderately biodegradable and need special consortia of bacteria or long adaptation periods, will often produce disparate results.

The biodegradation tests listed in this Technical Report are designed to determine the biodegradability of chemical substances or wastewaters under standardized conditions. The test results are required to predict the biodegradation behaviour of the test materials in natural or technical aquatic environments, for example, in rivers, lakes, ponds, sea, wastewater treatment plants, digesters. To improve their predictive value, the test methods should simulate, to a certain degree, such environments. As the conditions in these environments are often very different, sometimes even diametrically opposed, the standard methods reflect these differences. Therefore, it is necessary to provide a sufficient number of different standardized test methods to allow the choice of the best one for a specific purpose.

Water quality — Selection of tests for biodegradability

1 Scope

This Technical Report gives an overview of biodegradation tests for the aquatic environment standardized by ISO and provides recommendations on their use. In Annex A, the biodegradation guidelines for the aquatic medium of the OECD are included, because these methods are sometimes identical to ISO standards or are useful supplements. In addition, inhibitory tests with bacteria and mixed bacterial inocula are included in this Technical Report because a possible toxicity on the inoculum is important information for the choice and performance of biodegradation tests. It is very helpful to determine bacteria toxicity in advance using the same inoculum as the planned biodegradation test before starting biodegradation testing.

2 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

2.1
activated sludge
biomass and inert matter produced in the aerobic treatment of wastewater by the growth of bacteria and other microorganisms in the presence of dissolved oxygen

2.2
biochemical oxygen demand
BOD
mass concentration of dissolved oxygen consumed under specified conditions by the aerobic biological oxidation of a chemical compound or organic matter in water

NOTE For the purpose of this Technical Report, it is expressed as milligrams of oxygen uptake per milligram or gram of test compound.

2.3
biodegradation phase
time from the end of the lag phase of a test until about 90 % of the maximum level of biodegradation has been reached

NOTE It is expressed in days.

2.4
biogas
carbon dioxide and methane produced by anaerobic bacteria

2.5
chemical oxygen demand
COD
mass concentration of oxygen equivalent to the amount of a specified oxidant consumed by a chemical compound or organic matter when a water sample is treated with that oxidant under defined conditions

NOTE It is expressed as milligrams oxygen uptake per milligram or gram test compound.

2.6
concentration of suspended solids of an activated sludge
amount of solids obtained by filtration or centrifugation at known conditions of a known volume of activated sludge and drying at about 105 °C to constant mass

NOTE Mixed liquor suspended solids is also often used.

2.7
degree of adsorption on activated sludge
percentage of a test compound eliminated by any processes but biodegradation under the conditions of a specific aqueous batch test with activated sludge, determined by comparing the concentration at the beginning with that at the end of the test

2.8
digested sludge
mixture of the settled phases of sewage and activated sludge, which have been incubated in an anaerobic digester at about 35 °C to reduce biomass and odour problems and to improve the dewaterability of the sludge, and which consists of a consortium of anaerobic fermentative and methanogenic bacteria producing carbon dioxide and methane

2.9
dissolved inorganic carbon
DIC
part of the inorganic carbon in water which cannot be removed by specified phase separation

NOTE Phase separation may be obtained, for example, by centrifugation of the water sample at 40 000 m/s^2 for 15 min or by membrane-filtration using membranes with pores of 0,45 µm diameter.

2.10
dissolved organic carbon
DOC
part of the organic carbon in a sample of water which cannot be removed by specified phase separation

NOTE Phase separation may obtained, for example, by centrifugation of the water sample at 40 000 m/s^2 for 15 min or by membrane-filtration using membranes with pores of 0,45 µm diameter.

2.11
lag phase
time from the start of a test until adaptation and/or selection of the degrading microorganisms are achieved and the biodegradation degree of a chemical compound or organic matter has increased to about 10 % of the maximum level of biodegradation

NOTE It is expressed in days.

2.12
maximum level of biodegradation
maximum biodegradation degree of a chemical compound or organic matter in a test above which no further biodegradation takes place during the test

NOTE It is expressed in percent.

2.13
mixed liquor suspended solids
MLSS
concentration of solids, expressed in a specified dried form, in the mixed liquor

[ISO 6107-3:1993, 48]

2.14
plateau phase
time from the end of the biodegradation phase to the end of the test

NOTE It is expressed in days.

2.15
pre-conditioning
pre-incubation of an inoculum under the conditions of the test in the absence of the chemical compound and/or organic matter, with the aim of improving the performance of the test by acclimatisation of the microorganisms to the test conditions

2.16
pre-exposure
pre-incubation of an inoculum in the presence of a chemical compound and/or organic matter, with the aim of enhancing the ability of this inoculum to biodegrade the test material by adaptation and selection of the microorganisms

2.17
primary anaerobic biodegradation
level of degradation achieved when a test compound undergoes any structural change, other than complete mineralization, as a result of anaerobic microbial action

2.18
primary biodegradation
structural change (transformation) of a chemical compound by microorganisms resulting in the loss of a specific property

2.19
theoretical oxygen demand
ThOD
theoretical maximum amount of oxygen required to oxidize a chemical compound completely, calculated from the molecular formula

NOTE In this case, it is expressed as milligrams oxygen uptake per milligram or gram test compound.

2.20
total organic carbon
TOC
all carbon present in organic matter which is dissolved and suspended in the water

2.21
ultimate aerobic biodegradation
breakdown of a chemical compound or of organic matter by microorganisms in the presence of oxygen to carbon dioxide, water and mineral salts of any other elements present (mineralization) and the production of new biomass

2.22
total inorganic carbon
TIC
all that inorganic carbon in the water deriving from carbon dioxide and carbonate

2.23
theoretical amount of formed carbon dioxide
$ThCO_2$
maximum amount of carbon dioxide formed after oxidizing a chemical compound completely, calculated from the molecular formula

NOTE It is expressed in milligrams of carbon dioxide per milligram or gram of test compound.

2.24
theoretical amount of inorganic carbon
ThIC
maximum amount of inorganic carbon formed after oxidizing a chemical compound completely, calculated stoichiometrically from the molecular formula

NOTE It is expressed in milligrams of carbon per milligram or gram of test compound.

2.25
ultimate anaerobic biodegradation
level of degradation achieved when a test compound is utilized by anaerobic microorganisms resulting in the production of carbon dioxide, methane, mineral salts, and new microbial cellular constituents (biomass)

3 Evaluations and recommendations

3.1 Biodegradation test methods

The test methods for aerobic biodegradability are not of equal potential, largely because of the different microbial densities, the concentrations of the test substances and the test durations used. ISO 7827 (DOC removal test), ISO 9439 (CO_2 evolution test), ISO 9408 (oxygen consumption test), ISO 10708 (BOD demand in a two-phased closed bottle test) and ISO 14593 (CO_2 headspace test) are of roughly equal potential. These methods are widely used standards for biodegradation studies in the aquatic environment and correspond in principle to the OECD tests for ready biodegradability. The test duration is 28 d. As inoculum, activated sludge with a concentration of not more than 30 mg/l of dry substance is often used. The test flasks of ISO 10707 (closed bottle test) have a low inoculation, are not stirred or aerated and have therefore a lower degradation potential, but they are especially useful and applicable to volatile and inhibitory test compounds. This test corresponds in principle to the known BOD tests (ISO 5815-1 and ISO 5815-2), which are, however, not recommended for substances because the conditions are very stringent, and the test time (5 d) is very short. Many substances would be classified as not biodegradable and, therefore, discriminated. The BOD_5 is the oldest aquatic biodegradation test and has shown its suitability for wastewaters since many years.

ISO 9887 (SCAS test) and ISO 9888 (Zahn-Wellens test) are tests with a high inoculum concentration. ISO 9887 uses an additional substrate (sewage) and may be extended further than the usual 28 d test duration. Hence, these tests have a high degradation potential and may be used to determine the intrinsic biodegradability of chemicals, which is, in the OECD philosophy, called “inherent biodegradability”. As these tests are open systems based on DOC determination, they cannot be applied directly to volatile or water-insoluble substances. ISO 11733 (activated sludge simulation test) is a continuous dynamic test simulating wastewater treatment plants including nitrification and denitrification techniques. ISO 14592 (shake flask test/river simulation test) is a special test for biodegradation of substances at low environmentally realistic concentrations and is suitable to determine biodegradation kinetics in the aquatic environment. ISO 14592-1 is a batch test simulating standing water bodies like lakes or ponds, ISO 14592-2 is a dynamic system and simulates flowing waters like rivers. ISO 11734 (biogas production measurement) is the only standardized aquatic test for anaerobic biodegradability and is applied independently on tests for aerobic biodegradability. Priority for application should be given to those chemicals which are not sufficiently degraded aerobically and preferentially adsorb onto activated sludge, and which enter in this way anaerobic digesters in wastewater treatment plants. ISO 16221 is a standard for marine biodegradation testing and includes five different tests with different analytical parameters which are based on established fresh water tests adapted for marine conditions.

The kinetics and the degree of degradation can be variable in different environmental compartments; therefore, results from different test methods can vary for the same test material. It is clear that differences are expected between aerobic and anaerobic biodegradation tests as well as between marine and fresh water test systems. It is also obvious that the potential for degradation increases if the test conditions are favourable. Tests with high inoculum concentrations and which allow even pre-exposure of the inoculum and permit the extension of the test duration until the plateau phase is reached, will more often show biodegradation than tests with less favourable conditions. An important parameter is an optimal test concentration which is neither too high thus avoiding the risk of inhibition nor too low thus making it difficult or impossible to determine the DOC removal, oxygen uptake or carbon dioxide production accurately and precisely. In the extreme, at very low concentrations (e.g. much less than 1 mg/l), biodegradation may not occur at all because the threshold value for a successful degradation is too low.

Chemicals which do not degrade in the rather stringent tests on ready biodegradability may, however, degrade in the powerful inherent tests. Nevertheless, they may fail to degrade in the continuously performed activated sludge simulation test. Tests on ready biodegradability may deliver contradictory results, which cannot be explained just by the different degradation power, as in the case of the rather weak, closed bottle test (ISO 10707). Experience also shows that tests, which are supposed to have the same degradation potential (such as ISO 7827, ISO 9408 and ISO 9439), and which are prepared, stirred and aerated identically and use the same inoculum concentration, may give different results. The reason is the different analytical techniques used. Consumption and hence the measurement of oxygen differs from the production and measurement of carbon dioxide which is the last step of aerobic biodegradation processes. Furthermore, a part of the carbon dioxide will be left dissolved in the test water and its determination lags behind its biological production. The full amount of carbon dioxide is measured only after acidification at the end of the test. This fault of the CO_2 production test is almost completely eliminated when the CO_2 headspace method (ISO 14593) is used. Another improvement is a new analytical development which determines the produced CO_2 by continuous conductivity measurement instead of DIC. The equivalent degrees of biodegradation will take longer than with DOC removal or oxygen uptake and could influence the fulfilling of the 10-days-window (3.5).

Even within one test method, different results may be obtained in parallel vessels if, e.g. the lag-phases vary and the test is finished before the plateau phase is reached. Experience has shown that easily degradable chemicals usually give comparable results as well as poorly degradable substances, which show their relative persistence in nearly all test systems. Chemicals of intermediate ability to biodegrade give more consistent results in tests with higher degradation potential or when pre-exposed inocula are used. Poorly biodegradable substances may, nevertheless, be well eliminated in wastewater treatment plants if they have, for example, a high adsorption potential onto activated sludge. Such adsorption processes do not always take place in parallel to the biodegradation processes. For the determination of this special elimination, ISO 18749 (batch test) is suitable.

3.2 Analytical parameters and expression of test results

In most of the standardized tests, information on ultimate biodegradability is requested, i.e. the complete breakdown of an organic substance to the inorganic catabolites CO_2 and water. To determine the complete aerobic degradation (mineralization), the sum parameters DOC, BOD or CO_2 -evolution are used. BOD and CO_2 always clearly indicate biodegradability, whereas DOC removal may be due to biodegradation as well as to abiotic elimination, such as adsorption onto activated sludge or evaporation. In the case of substances with low water solubility, the DOC cannot be determined because the test substance would be removed from solution by filtration or centrifugation, and therefore cannot be used. In anaerobic tests, the production of biogas (methane and carbon dioxide), measured by changes in pressure, is the usual analytical parameter.

For analytical reasons, sum parameters require rather high concentrations of test substance. If the test concentration should be as close as possible to natural concentrations, e.g. for kinetic reasons, they should be very low in the test. In this case, substance-specific analytical techniques are used to investigate biological transformations of a chemical, the so-called primary biodegradability. If even lower concentrations are required, radio-labelled substances are necessary. There may be also other analytical techniques which are suitable to follow biodegradation, but only the methods mentioned here are used in standardized tests.

In the DOC measurement, the initial concentration is compared with the final concentration. When using BOD and CO_2, the measurements during the test are accumulated and compared at the end with the respective theoretical values ThOD or $ThCO_2$ which are calculated from the molecular formula of the test substance. From the measured values, the percentages of biodegradation are calculated and plotted against the period of incubation to show a degradation curve. Biodegradation curves frequently have

— a marked lag phase, in which the microorganisms of the inoculum adapt to the test substance, followed by

— the actual degradation phase, in which the conversion of the test substance takes place, DOC is removed, oxygen used and CO_2 produced, and

— a plateau phase, in which biodegradation is completed and no further significant biodegradation is measured.

The test result is usually the degree of degradation in percent, determined as a mean value in the plateau phase. If this is not possible, because there are only a few data points available or no clear plateau phase is obtained, the highest value in the plateau phase or the value measured on the last day of the test is used instead.

3.3 Pass levels for biodegradation

The percentage biodegradation as expressed by DOC removal for ultimate biodegradability or test substance removed for primary biodegradability can reach 100 %. It should be verified in a test that abiotic elimination processes, such as physical adsorption are negligibly small or they should be considered in the calculation. Under the limiting batch conditions in ready biodegradation tests (3.5) it is assumed that 100 % cannot be expected in a 10 days window, or even in a total test time of 28 d, and therefore values of > 70 % DOC and > 80 % test substance removal determined with a substance-specific analytical method have been adopted as pass levels ultimate or primary biodegradation. Experience has shown that substances which fulfil these criteria usually reach higher values in activated sludge simulation tests and in the real environment. In fact, in many cases well over 70 % and 80 % respectively are often found in the “ready” batch tests.

The production of CO_2 and oxygen uptake in batch tests expressed as a percentage of the respective theoretical values $ThCO_2$ and ThOD is always less than the percentage determined by DOC removal (see References [2] and [3]). This can especially be seen in cases where simultaneous measurements are made, for example, using the CO_2/DOC combination method described in Annex D of ISO 9439:1999. The reason for this is that in bacterial metabolism some of the organic carbon of the test substance is biochemically oxidized and thus converted to CO_2, whilst other fractions are synthesized into new cellular material or into organic metabolic products. These fractions are not oxidized and do not contribute to the CO_2 production, but are, nevertheless, part of the ultimate biodegradation. The proportion of organic carbon converted to cell material depends on factors, such as the nature of the test substance and the collection of bacterial species present. There is, however, not a single value, which can be adopted as a “general pass” level in these cases. Data from interlaboratory tests performed during the standardization and establishment of these methods and the experience of many laboratories led to the presently used and accepted pass level of > 60 % ThOD or $ThCO_2$ in ready biodegradation tests. Examples and a discussion of the OECD-based pass levels are given in Reference [3].

3.4 Toxicity to bacteria

It has been observed that chemicals, which are inhibitory towards bacteria and do not degrade at the usual test concentrations, may degrade at lower concentrations of the test compound or by using an inoculum from another source or after pre-adaptation. So, additional information is required to prevent the classification of a chemical as poorly biodegradable. However, in reality, it is not degraded because it is toxic under the test conditions. One way to avoid this is to add an inhibition control during the test. Most of the standard tests include additional vessels containing the test substance and the reference substance together. The biodegradation degree of this mixture is a good assessment of any inhibition. But, it may be better to have this information before an expensive and time-consuming biodegradation test is started and to know the effective concentration EC_{20} of the test substance in advance to choose the right test concentration. Therefore, it is recommended to perform bacteria-toxicity tests using the same mixed inoculum as in the degradation tests, which is often municipal activated sludge. Suitable methods for aerobic conditions are the test for inhibition of oxygen consumption by activated sludge (ISO 8192) and the test on the growth of sludge microorganisms (ISO 15522). For anaerobic biodegradation testing, such an inhibition control is not possible due to the high concentration of organic material in the test. However, two toxicity tests with different conditions, ISO 13641-1 and ISO 13641-2, are available for different concentrations of the digested sludge and test durations. The results of these toxicity investigations may not only be used to choose the best degradation tests and the correct test concentrations, but also to predict the toxic behaviour of a test substance in wastewater treatment plants or digesters. In this context, the nitrification inhibition test (ISO 9509) should also be mentioned; this allows the prediction of toxic effects on the very sensible microbial nitrification process.

Also, standardized bacteria toxicity tests exist using pure bacteria cultures. For example, ISO 10712 uses *Pseudomonas putida* and ISO 11348 (all parts) uses the luminescent bacterium *Vibrio fischeri*. It is not recommended to use these methods because the effective concentrations obtained may be far beyond what happens in biodegradation tests or in the real environment. The reason may be that the test substance could be toxic to the used test species, but not to the more tolerant microbial community of activated sludge bacteria.

There is the risk that, for many substances, a high inhibition is predicted which, in reality, will not take place and that, therefore, low test concentrations are used which are not favourable for the usual analytical methods.

3.5 Test strategies

As there are differences between the complexity, duration, quality of results and costs of the various tests, these should be considered when choosing which test or test strategy to use when commencing a study. The choice of a test normally depends on the purpose of testing or on legal requirements, which should be fulfilled, but it may also depend on the physical and chemical properties of the test chemical. For example, volatile test compounds can be tested only in closed systems, such as ISO 10707, ISO 10708 or ISO 11593. Insoluble and poorly soluble chemicals cannot be assessed by any methods based on DOC determination. Guidance in preparing such difficult chemicals for testing is given in ISO 10634.

Usually, biodegradation testing will start with a method using low-cell density to answer the question - is the test compound biodegradable in any environmental compartments? A low or zero value for biodegradation obtained in such a simple batch test may be sufficient in some cases. Otherwise, a test with a higher cell density, a longer test period or with an inoculum pre-exposed to the chemical could be applied. When a test substance is expected to be only poorly biodegradable, it would make sense to start with a method using such improved conditions and to find out whether a biodegradation or elimination potential is available at all. Pre-adapted inocula could be obtained in one of the high-density tests ISO 9887 or ISO 9888. Because of their complexity, tests like the activated sludge simulation test (ISO 11733) or the river model (ISO 14592-2) will not usually be applied until after other simpler tests have first been carried out. Biodegradation tests under anaerobic or marine conditions are performed on special request if this information is needed.

For the routine testing of chemicals, the test strategy of the OECD Chemicals Testing Programme is very important. The applied OECD Guidelines for aquatic biodegradation tests are listed and compared with ISO International Standards in Annex A. The procedures in these guidelines are generally the same as in the ISO International Standards, but the latter are more precise and clearer than the OECD guidelines.

The OECD strategy is to apply a simple low-cell density method at the beginning of testing, i.e. a so-called test for “ready biodegradability” which requires that

a) an accepted standardized test method is used, such as one of the OECD guidelines 301A-F or a comparable ISO test of the same quality,

b) the inoculum is not pre-exposed to the test substance,

c) the test is terminated after about 28 d, and

d) one of the sum parameters DOC, BOD or CO_2 is used.

A given pass level is reached within 10 d after the beginning of the degradation phase, which is defined as the time when the extent of mineralization exceeds 10 % and is identical to the end of the lag-phase. The pass levels which should be reached within this so called 10-days-window are > 70 % of DOC removal compared to the measured start concentration, $\geqslant$ 60 % of BOD of the calculated ThOD or $\geqslant$ 60 % CO_2 evolution compared to the calculated ThCO_2.

If under these conditions the “10-days-window” is met, it is assumed that complete mineralization will be achieved either within the usual 28 d test period, or within a reasonable time in the real environment. This can be verified either when in the remaining test period a high plateau phase is reached, i.e. the stage at which degradation is complete, or by comparison of test data from more stringent tests on inherent biodegradability or of measured data from the real environment. A substance gets the label “readily biodegradable” if these criteria are fulfilled, and it is not necessary to measure a full and complete degradation under the rather stringent test conditions, and within the short test period of 28 d of the tests on ready biodegradability. This assumption is, however, only reasonable for pure chemicals, but not necessarily for chemical mixtures or chemicals containing significant proportions of impurities. For this reason, chemicals should be tested in the purest available form. On the other hand, the testing of the environmental behaviour of chemicals according to laws or directives requires often the use of real production charges with all impurities.

The next step for substances, which are not readily biodegradable, is endeavouring to demonstrate an inherent biodegradability. A substance is considered to have inherent biodegradability if in an appropriate test, ultimate degradation (mineralization) was achieved. Such substances are not persistent and can be assumed to be degraded in the aquatic environment in the medium or long term, for example, in wastewater treatment plants or in other environmental compartments. The two main tests for inherent biodegradability, OECD Guidelines 302 A and B, correspond to ISO 9887 and ISO 9888. They allow only water-soluble chemicals to be tested directly, since DOC is used as an analytical parameter. They may, however, be used to pre-expose an inoculum for use in other tests, e.g. ISO 9408, ISO 9439 or ISO 14593.

The last test category in the OECD philosophy consists of simulation tests, which reproduce, in the laboratory, defined environmental conditions as well as possible, and thus give more extensive and better information on the degradation behaviour of substances under simulated environmental conditions than simple batch tests. There are, however, only a few standardized methods available. The best known is the activated sludge simulation test (ISO 11733), which corresponds to OECD 303.

Other tests of this category are the biofilm test (OECD 303 B) and the tests at low concentrations (ISO 14592-1 and ISO 14592-2), which simulate surface water conditions.

The label “readily biodegradable” is a very important criterion for many purposes in the field of product testing. A readily biodegradable substance is regarded to be rapidly and completely degraded in any aerobic environmental compartment, especially in wastewater treatment plants. Further testing on biodegradation is therefore unnecessary and the need of other ecological parameters, such as bioaccumulation or ecotoxicity may be reduced. Experience gained with many substances within the last decades showed, however, that the existing criteria for ready biodegradability are not indisputable. Studies with detergents, made for a European Regulation for testing of detergents, and with general chemicals showed that products which failed the criteria “ready biodegradability” were, nevertheless, found to be adequately removed in simulation tests and in the real environment. The OECD criteria for “ready biodegradability” seem to be too stringent; therefore, the most recent European Regulation for testing of detergents no longer stipulate the 10-days-window. A detergent may considered as being biodegradable if, in a standard test based on BOD or CO_2, the limit values of $\geqslant$ 60 % are reached within 28 d. Additionally, it should be taken into account that detergents are not pure chemical substances, but mixtures of different chemical compounds. Therefore, the biodegradability of detergents should be considered in a different way than the biodegradability of pure chemical compounds.

An expert group compared test results from ready biodegradability tests with results from inherent and simulation tests and with data from the real environment (see Reference [1]). Substances which reached the given pass levels of 70 % DOC and $\geqslant$ 60 % BOD or CO_2 in a “ready biodegradability” test were checked against achieving a very high mineralization level in tests (> 90 % DOC removal or > 75 % BOD or CO_2 after 28 d) or in the environment. The statistical investigation of over 800 test results with chemicals showed that the fulfilling of the 10-days-window allowed, in about one third of all cases, no reliable prediction of complete mineralization because either false-positive or false-negative interpretations were possible. With DOC removal as a parameter, the ready biodegradation criterion > 70 % in 10 d is usually reached much more easily than with oxygen uptake or CO_2 production, because these analytical parameters are based on an end product and not just on a substance removal. To achieve a comparable level of stringency as in DOC tests, a time window of 15 d would be needed with tests based on BOD or CO_2 and a pass level of $\geqslant$ 60 %.

New analytical techniques allow the continuous measurement of BOD and CO_2 and result in a sufficient number of data points well distributed over the test period. With suitable curve-fitting programmes for the biodegradation curves, characteristic descriptors can be extracted. One is the A-value, which describes the extent of biodegradation in the plateau phase and is expressed as biodegradation degree in percent. The other is the k-value, which describes the biodegradation rate after the beginning of the degradation phase and is expressed as a figure per time unit. A combination of A-values and k-values give reliable information for both extent and rate of biodegradation of a substance. Suitable A-values and k-values which correspond to the existing criteria would be for tests based on DOC removal > 80 % and > 0,3 per hour, and for tests based on BOD or CO_2 > 75 % and > 0,1 per hour. It could be shown by the mentioned statistical investigations that substances fulfilling these criteria are removed in an activated sludge simulation test, or in real wastewater treatment plants by > 95 %. This would give the possibility for a reliable alternative for the existing 10-days-window criterion and needs to be discussed in detail among scientists, regulators and in the industry.

Table 1 — Overview of ISO methods

ISO No	Title	Principle	Scope	Comments
5815-1	*Water quality — Determination of biochemical oxygen demand after* n *days* (BOD_n) *— Part 1: Dilution and seeding method with allylthiourea addition*	Dilution of a sample of water with varying amounts of a specified dilution water rich in dissolved oxygen and containing a seed of aerobic microorganisms and allylthiourea to prevent nitrification. Incubation at 20 °C for usually 5 d in the dark. Determination of the dissolved oxygen concentration before and after incubation. Calculation of the mass of oxygen consumed per litre of sample.	ISO 5185-1 is applicable to all waters with BOD values > 3 mg/l and not exceeding 6 000 mg/l. The results obtained provide an indication from which the quality of waters and, if compared with the COD, the biodegradability of its ingredients can be estimated.	Only for waters and wastewaters, not for substances.
5815-2	*Water quality — Determination of biochemical oxygen demand after* n *days* (BOD_n) *— Part 2: Method for undiluted samples*	The same technique as in ISO 5815-1, but optimized test conditions for the determination of low BOD values.	ISO 5185-2 is applicable to all waters with BOD values > 0,5 mg/l and not exceeding 6 mg/l.	Only for waters, not for substances.
7827	*Water quality — Evaluation in an aqueous medium of the "ultimate" aerobic biodegradability of organic compounds — Method by analysis of dissolved organic carbon (DOC)*	Batch aquatic test system using organic test compounds as the sole source of carbon and energy for an inoculum of aerobic mixed microorganisms, usually activated sludge at 30 mg/l dry substance. Regular measurement of the removal of DOC to determine the ultimate biodegradability within 28 d. Evaluation of the test results by comparing the DOC concentration at the beginning and the end of the test. Additionally, specific analysis can be used to determine the primary biodegradability.	ISO 7827 is applicable to organic compounds which are: — water-soluble at the test concentration (10 mg/l to 40 mg/l DOC); — non-volatile, or having a negligible vapour pressure; — not significantly adsorbable on glass and activated sludge; — not inhibitory to the test microorganisms at the test concentration.	Only for water-soluble test compounds. DOC elimination is not always a clear indicator for biodegradation.

Table 1 *(continued)*

ISO No	Title	Principle	Scope	Comments
8192	*Water quality — Test for inhibition of oxygen consumption by activated sludge (carbon and ammonium oxidation)*	ISO 8192 specifies a method for assessing the inhibitory or stimulatory effect of a test material on the oxygen consumption of activated sludge microorganisms. Activated sludge in the presence of an easily biodegradable substrate will consume oxygen rapidly at a rate depending on the concentration of microorganisms. Addition of toxic concentrations of a test material can result in a decrease in the oxygen consumption rate. The rates are measured after a short exposure (30 min or 180 min) to the test material using an oxygen electrode and the percentage inhibition is calculated by comparison with that of a control mixture containing only the substrate. The inhibitory effect may include heterotrophic or carbonaceous respiration and nitrification.	The method is applicable to any test material. Special care should be taken with low water soluble or volatile materials and those which consume or produce oxygen abiotically. Two examples of the application are given, one better represents the conditions in surface waters, the other represents the conditions in biological wastewater treatment plants.	Simple and short-time test for substances and wastewaters. Indication for the toxicity effect in biodegradation difficult.
9408	*Water quality — Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium by determination of oxygen demand in a closed respirometer*	Determination of the biodegradation of organic compounds and wastewaters by aerobic microorganisms using a batch aqueous test system. The test mixture contains an inorganic medium, the organic compound as the sole source of carbon and energy at a concentration of normally 100 mg/l or 100 mg/l ThOD and as inoculum, usually activated sludge at 30 mg/l dry weight. The mixture is agitated over 28 d in the closed test vessel of a respirometer. The evolved CO_2 is absorbed and the resulting change in pressure is used to determine the BOD. The BOD is compared with the ThOD. Biodegradation is expressed as a percentage.	ISO 9408 is applicable to any organic compound. Special care is necessary for poorly water-soluble and volatile compounds. The test substance should not react and react with the CO_2 absorbent and should not be inhibitory to the test microorganisms at the test concentration.	Method for all kinds of test materials. Requires a respirometer which produces continuous biodegradation curves. BOD is a clear indicator for biodegradation. N-containing test substances may be nitrified, this should be considered with the test result.

Table 1 *(continued)*

ISO No	Title	Principle	Scope	Comments
9439	*Water quality — Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium — Carbon dioxide evolution test*	Determination of the biodegradation of organic compounds and wastewaters by aerobic microorganisms using a batch aqueous test system. The test mixture contains an inorganic medium, the organic compound as the sole source of carbon and energy at a concentration of normally 10 mg/l to 40 mg/l organic carbon and as inoculum, usually activated sludge at 30 mg/l dry weight. The mixture is agitated over 28 d. The evolved CO_2 is trapped in external vessels and determined by an appropriate analytical method. The biogenic CO_2 is compared with the theoretical amount ($ThCO_2$). Biodegradation is expressed as a percentage. A test modification is given as Annex D. As additional information the removal of DOC is determined regularly.	ISO 9439 is applicable to any organic compound. Special care is necessary for poorly water-soluble and volatile compounds. The test substance should not be inhibitory to the test microorganisms at the test concentration. For water-soluble compounds, DOC removal may also be determined (see Annex D, ISO 9439:1999).	Test method for all kinds of test material. CO_2 evolution is a clear indicator for biodegradation. With the additional DOC measurement, a good differentiation is possible between biodegradation and abiotic elimination, e.g. adsorption.
9509	*Water quality — Toxicity test for assessing the inhibition of nitrification of activated sludge microorganisms*	ISO 9509 specifies a method for assessing the short-term inhibitory effects of test substances on nitrifying bacteria in activated sludge. The inhibitory effect is estimated over an exposure period of 4 h in parallel vessels in the presence and absence of a test material. The concentration of nitrite and nitrate produced by the oxidation of ammonium is assessed and the effective concentrations (EC-values) are determined.	The method requires a nitrifying activated sludge and is applicable to non-volatile, water-soluble substances and wastewaters.	Special test for nitrification without predictive value for biodegradation tests.
9887	*Water quality — Evaluation of the aerobic biodegradability of organic compounds in an aqueous medium — Semi-continuous activated sludge method (SCAS)*	Semi-static, aquatic test system using organic test compounds and easily biodegradable organic medium (sewage) as the source of carbon and energy for a high-density inoculum of activated sludge. Daily fill-and-draw of the test vessels with sewage and test compound and measurement of the removal of DOC in the test and blank control vessels to determine the ultimate biodegradability within the test time of up to 26 weeks. Evaluation of the test results by comparing the DOC concentration before and after the fill-and-draw procedure.	ISO 9887 is applicable to organic compounds which are: — water-soluble at the test concentration (20 mg/l to about 50 mg/l DOC); — non-volatile, or with a negligible vapour pressure; — not lost by foaming from the test solution; — not significantly adsorbable on glass and activated sludge; — not inhibitory to the test microorganisms at the test concentration.	Test with high biodegradation potential especially for compounds not easily degradable and for wastewater, including co-metabolic degradation. Useful method for attempting the pre-adaptation of an inoculum.

Table 1 *(continued)*

ISO No	Title	Principle	Scope	Comments
9888	*Water quality — Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium — Static test (Zahn-Wellens method)*	Batch aquatic test system using organic test compounds as the sole source of carbon and energy for a high-density inoculum of aerobic mixed microorganisms (activated sludge). Measurement of the removal of DOC or COD to determine the ultimate biodegradability or elimination from water within 28 d. Evaluation of the test results by comparing the DOC concentration at the beginning and the end of the test.	ISO 9888 is applicable to organic compounds which are: — water-soluble at the test concentration (50 mg/l to 400 mg/l DOC); — non-volatile, or with a negligible vapour pressure; — not lost by foaming from the test solution; — not inhibitory to the test microorganisms at the test concentration.	Batch method for the evaluation of elimination and biodegradation of test compounds and wastewater. Predictive value for wastewater treatment plants.
10634	*Water quality — Guidance for the preparation and treatment of poorly water-soluble organic compounds for the subsequent evaluation of their biodegradability in an aqueous medium*	Description of techniques for preparing poorly water-soluble organic compounds and introducing them into test vessels for a subsequent test for biodegradability in an aqueous medium.	ISO 10634 is applicable to poorly water-soluble test compounds	No biodegradation test method.
10707	*Water quality — Evaluation in an aqueous medium of the "ultimate" aerobic biodegradability of organic compounds — Method by analysis of biochemical oxygen demand (closed bottle test)*	Batch aquatic test system using organic test compounds as the sole source of carbon and energy for a low concentrated inoculum of aerobic mixed microorganisms. Measurement of the BOD in completely filled closed bottles to determine the ultimate biodegradability within 28 d. Evaluation of the test results by comparing the BOD with the ThOD.	ISO 10707 is applicable to organic compounds which: — are water-soluble at the test concentration (2 mg/l to 10 mg/l); — are water-insoluble at the test conditions, provided a suitable dosing technique is used; — are volatile, provided a suitable dosing technique is used; — are not inhibitory to the test microorganisms down to the low test concentrations used.	Test method for low test concentrations especially recommended for volatile and toxic test compounds. The test conditions cause a rather low biodegradation potential.

Table 1 *(continued)*

ISO No	Title	Principle	Scope	Comments
10708	*Water quality — Evaluation in an aqueous medium of the ultimate aerobic biodegradability of organic compounds — Determination of biochemical oxygen demand in a two-phase closed bottle test*	Batch aquatic test system using organic test compounds as the sole source of carbon and energy for an inoculum of aerobic mixed microorganisms. Measurement of the BOD in closed bottles with head-space to determine the ultimate biodegradability within 28 d. Evaluation of the test results by comparing the BOD including the uptake of oxygen from the headspace with the ThOD.	ISO 10708 is applicable to organic compounds which: — are water-soluble at the test concentration (100 mg/l ThOD); — are water-insoluble at the test conditions, provided a suitable dosing technique is used; — do not significantly adsorb on or react with the oxygen electrode; — are not inhibitory to the test microorganisms at the low test concentrations used.	Test method especially for poorly water-soluble test compounds using simple equipment.
10712	*Water quality —* Pseudomonas putida *growth inhibition test (*Pseudomonas *cell multiplication inhibition test)*	ISO 10712 specifies a method for assessing the inhibitory effect of a sample on *Pseudomonas putida* by measurement of cell growth under the influence of varying dilutions of the test sample, compared to the cell growth of a culture obtained under the same conditions, but without the test sample. Determination of the cell concentration as optical density after a test period of 16 h. Calculation of the EC_{10} and EC_{50}.	ISO 10712 is applicable to wastewater and chemical substances. It is not suitable for highly coloured samples, for samples with undissolved or volatile materials or those which react with the nutrient solution or undergo changes during the test.	The test is not recommended for the prediction of bacteria toxicity in biodegradation tests or wastewater treatment plants.
11348-1 11348-2 11348-3	*Water quality — Determination of the inhibitory effect of water samples on the light emission of* Vibrio fischeri *(Luminescent bacteria test)* *Part 1: Method using freshly prepared bacteria* *Part 2: Method using liquid-dried bacteria* *Part 3: Method using freeze-dried bacteria*	Determination of the light emission by cultures of *Vibrio fisheri*. The test criterion is the decrease of the luminescence, measured after a contact of 15 min or 30 min in a luminometer. Calculation of the EC_{20} and EC_{50}.	ISO 11348-1, ISO 11348-2 and ISO 11348-3 are applicable to wastewater, aqueous extracts and leachates, fresh water, marine and brackish water as well as pore water.	The test is not recommended for the prediction of bacteria toxicity in biodegradation tests or wastewater treatment plants.

Table 1 *(continued)*

ISO No	Title	Principle	Scope	Comments
11733	*Water quality — Evaluation of the elimination and the biodegradability of organic compounds in an aqueous medium — Activated sludge simulation test*	Continuously operated, aquatic test system using organic test compounds and easily biodegradable organic medium (sewage) as the source of carbon and energy for an inoculum of aerobic mixed microorganisms (activated sludge). Continuous addition of sewage and test compound to the test vessels and measurement of the DOC and COD in the influent and effluent of test and control units to determine the ultimate biodegradability within the test time of up to 12 weeks. Evaluation of the test results by calculating the DOC removal. Additionally, specific analysis can be used to determine the primary biodegradability of a test compound.	ISO 11733 is applicable to organic compounds which are: — water-soluble or satisfactorily dispersible at the test concentration (10 mg/l to 20 mg/l DOC); — non-volatile, or having a negligible vapour pressure; — not inhibitory to the test microorganisms at the test concentration.	Test used if detailed information in wastewater treatment plants is required.
11734	*Water quality — Evaluation of the "ultimate" anaerobic biodegradability of organic compounds in digested sludge — Method by measurement of the biogas production*	Batch aquatic test system using organic test compounds as the sole added source of carbon and energy for an inoculum of anaerobic mixed microorganisms (diluted digested sludge). Measurement of the biogenous produced gas (carbon dioxide and methane) by measurement of pressure and dissolved inorganic carbon to determine the ultimate biodegradability within 60 d. Evaluation of the test results by comparing the yield of biogas with the theoretical amount. Additionally, specific analysis may be used to determine the primary biodegradability of a test compound.	ISO 11734 is applicable to organic compounds which are: — water-soluble at the test concentration (20 mg/l to 100 mg/l organic carbon); — are water-insoluble under the test conditions, provided a suitable dosing technique is used; — are volatile, provided a suitable dosing technique is used (case by case decision); — not inhibitory to the test microorganisms at the test concentration.	Test used to evaluate anaerobic biodegrad-ability.

Table 1 *(continued)*

ISO No	Title	Principle	Scope	Comments
13641-1	*Water quality — Determination of inhibition of gas production of anaerobic bacteria — Part 1: General test*	Screening method for assessing the potential anaerobic toxicity. Aliquots of a mixture of undiluted anaerobically digesting sludge and a degradable substrate are incubated alone (control) and simultaneously with a range of concentrations of the test material in sealed bottles for 2 d to 3 d at 35 °C. The growth rate of anaerobic bacteria is much lower compared with that of aerobic microorganisms. For this reason, the test periods in anaerobic methods are longer than in those with aerobic bacteria. The amount of biogas produced is measured by the increase in pressure in the test and control bottles. The percentage inhibition and the effective concentrations, such as EC_{50} are calculated.	ISO 13641-1 is applicable to substances which are soluble or insoluble in water including volatile chemicals, mixtures, wastewaters, effluents, sludges or other environmental samples to the production of biogas from the anaerobic digestion of sewage sludge over periods of up to 3 d. Information obtained by this method is a guide to the likely effect of a test material on biogas production in anaerobic digesters and may also be helpful in choosing suitable initial concentrations for anaerobic biodegradability tests.	This method is recommended to predict toxic effects in anaerobic digesters.

Table 1 *(continued)*

ISO No	Title	Principle	Scope	Comments
13641-2	*Water quality — Determination of inhibition of gas production of anaerobic bacteria — Part 2: Test for low biomass concentrations*	Screening method for assessing the potential anaerobic toxicity. Aliquots of mixtures of diluted digesting sludge or other sources of anaerobes, and a degradable substrate are incubated alone and simultaneously with a range of concentrations of the test material in sealed bottles for several days at 35 °C. The growth rate of anaerobic bacteria is much lower compared with that of aerobic microorganisms. For this reason, the test periods in anaerobic methods are longer than in those with aerobic bacteria. The amount of biogas produced is measured in the test and control bottles by the increase in pressure before and after addition of acid to release carbon dioxide from carbonates. The percentage inhibition of biogas production is calculated as well as effective concentrations, such as the EC_{50} . It is possible to use this technique for special investigations, for example, with sediments from anaerobic sites in nature. In this case, the incubation temperature in the test bottles can be that of the natural sediments. Anaerobic sediments may contain a high amount of inorganic matter and the cell number and hence the bacterial activity may be very low in such cases, so that the incubation period should be extended.	ISO 13641-2 is applicable to substances which are soluble or insoluble in water including volatile chemicals, mixtures, surface-, ground- and wastewaters, effluents, sludges, or other environmental samples to the production of biogas from anaerobic environments with low biomass concentration. The growth rate of anaerobic bacteria is much lower, compared with that of aerobic microorganisms. The inoculum may be collected from anaerobic sediments or from large, or laboratory scale, anaerobic digesters. Information obtained by this method may be helpful prior to anaerobic biodegradability testing with low inoculum concentrations and for estimations of the potential effects of chemicals and wastewater to anaerobic processes in habitats characterized by a relatively low anaerobic biomass, for example, natural sediments and soils.	This method is recommended to predict toxic effects in anaerobic natural environments and as a pre-test for anaerobic biodegradation tests.
14592-1	*Water quality — Evaluation of the aerobic biodegradability of organic compounds at low concentrations — Part 1: Shake-flask batch test with surface water or surface water/sediment suspensions*	Test system simulating the conditions of a batch aerobic surface water system. Determination of the primary biodegradability of a water soluble test substance under realistic environmental conditions especially at low concentrations. Ultimate biodegradability can be determined if the test substance is radio-labelled in the appropriate position(s).	ISO 14592-1 is applicable to organic compounds which are: — known to be in principle biodegradable; — water-soluble at the test concentration; — non-volatile and for which a substance specific analytical technique or radio-labelled substances are available.	Relatively simple method to determine biodegradation kinetics under environmentally realistic conditions in a batch system.