Review of Standard Testing methods and specifications for measuring biodegradation of bio-based materials in fresh water

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Abstract

Biodegradation in fresh water of materials such as plastics, lubricants, and other chemicals, which might end up in the environment after use, is of great importance. Therefore, certification of bio-based materials that may end up as waste in fresh water, with respect to their biodegradation behavior, is necessary. Standard testing methods for measuring biodegradation in fresh water are described in OECD guidelines, ASTM standards, ISO standards, etc. The most widely used testing methods for evaluating biodegradation of chemicals in aerobic aqueous medium are OECD 301 and OECD 310 for ready, and OECD 302 for inherent biodegradability, respectively. Similarly, the International standards ISO 7827, ISO 9408, ISO 9439, ISO 10707, ISO 10708, and ISO 14593 determine the biodegradability of organic compounds in an aerobic aqueous environment and are equivalent to OECD 301 and OECD 310. Test methods comparable to OECD 302 (inherent biodegradability) were also developed at ISO level (ISO 9887, ISO 9888). The American Standards ASTM D 5271 (plastics) and ASTM D 5864, ASTM D 6139 and ASTM D 6731 (lubricants) address the biodegradability of final products which are soluble, poorly soluble, or insoluble in water. Despite the availability of various testing methods for determining biodegradability in fresh water, further research is necessary for identifying the key factors affecting the accuracy and reproducibility of the results. Moreover, the corresponding specifications to characterize the biodegradability of bio-based complex materials in fresh water need to be further investigated.

Keywords: biodegradation, bio-based materials, standard testing methods, fresh water pollution

1 Introduction

Biodegradation can be defined as the breakdown or mineralisation of an organic material due to microbial activity. The availability of oxygen determines to which molecules the organic carbon is converted. If complete biodegradation takes place under aerobic conditions the organic carbon of the material will be converted to carbon dioxide and water (Reaction 1), while under anaerobic conditions biogas, which is a mixture of carbon dioxide and methane, will be formed (Reaction 2). The organic carbon of the material is in both cases also partly converted to new biomass \( (C_{\text{biomass}}) \) and it is possible that some carbon is not converted or remains present under the form of metabolites \( (C_{\text{residual}}) \) (De Wilde, 2013).
\[ C_{\text{sample}} + O_2 \rightarrow CO_2 + H_2O + C_{\text{biomass}} + C_{\text{residual}} \]  
\[ C_{\text{sample}} \rightarrow CO_2 + CH_4 + C_{\text{biomass}} + C_{\text{residual}} \]

The biodegradation rate is strongly affected by the environment in which the biodegradation takes place. An environment is determined by the moisture content, the temperature, the type of micro-organisms, the inorganic nutrient availability, etc. Due to the large differences between environments, a biodegradability claim corresponds to a specific well-defined environment. Therefore, test methods, which determine the biodegradability of a material, always refer to a specific environment (fresh water, marine water, soil or compost) and conditions (aerobic or anaerobic conditions).

Biodegradation tests have their roots in the development of degradable synthetic surfactants in the mid-seventies. The first guidelines were developed by the Organization for Economic Cooperation and Development (OECD) in 1981. CEN (European committee for standardisation), ISO (International Organization for Standardization) and ASTM (American Society for Testing and Materials) also developed biodegradability testing methods for organic chemicals and also for more complex materials (plastics, etc.). The major part of these standards is based on the principles of the OECD guidelines. Currently a trend towards internationalisation is observed: European standards are automatically transformed into national standards and the Vienna treaty guarantees that an agreement exists between ISO and CEN standards (De Wilde, 2005).

Aerobic aquatic freshwater biodegradation tests form a prediction method for the fate of chemicals in an aerobic aqueous environment, especially in the aerobic stages of wastewater treatment as the used inoculum is often activated sludge from a wastewater-treatment plant. Different parameters can be monitored during a test in order to determine the biodegradability of organic products:

- Dissolved Organic Carbon (DOC)
- Dissolved Oxygen (DO)
- Respirometry: carbon dioxide production
- Respirometry: oxygen consumption (BOD)
- Inorganic Carbon (IC)

The choice of the method to be used largely depends on the physical properties of the considered chemical (solubility, volatility and adsorptivity). DOC removal can be influenced by abiotic elimination, such as adsorption onto activated sludge or evaporation.

The biodegradation percentages based on CO\(_2\) production and oxygen consumption are always lower when compared to biodegradation percentages based on DOC removal because of the conversion of carbon to biomass. The percentage carbon used for biomass production varies between species of bacteria and between chemicals. Consequently biodegradation estimates based on CO\(_2\) production and oxygen consumption will vary from test to test and from chemical to chemical (Painter, 1995).

The majority of the standards for testing biodegradation in fresh water were developed for “chemicals” or “organic compounds”, while a few standards are especially developed for plastics and lubricants. No specific standards for solvents are currently available. The suitability of the measurement techniques for determining the biodegradability of lubricants, which are often poorly soluble in water and consist of complex mixtures of chemicals, and solvents, which are often volatile, differs.

Especially methods based on DOC measurements are not suitable for non-soluble and volatile materials. Moreover tests which measure CO\(_2\) production are frequently done in systems that are regularly aerated to maintain aerobic conditions. Consequently, materials with a high vapour pressure, which are in the air space of the test medium will be removed (ASTM D 6006). Therefore, volatile test compounds can only be tested in closed systems.
2 Definitions

2.1 Ultimate aerobic biodegradation

Ultimate biodegradation is the breakdown of an organic compound by microorganisms into carbon dioxide, water, and mineral salts of any other elements present (mineralization) plus new biomass.

2.2 Ready biodegradability

The term \textit{“ready biodegradability”} refers to an arbitrary classification of chemicals, which have passed certain specified screening tests for ultimate biodegradability. It is assumed that such substances will rapidly and completely biodegrade in aquatic environments under aerobic conditions. Ready biodegradability tests are no simulation tests, but tests for potential to biodegrade. Consequently using data from them to assess the performance of a substance within a particular route to the environment would be a misuse of the data and unwarranted extrapolation. The data of such tests state that chemicals passing the test do not offer a serious challenge to the metabolic capability of aerobic aquatic environments (given the presence of bacteria, nutrients, etc.) and that they would be readily degraded in the real environment (Painter, 1995).

2.3 Inherent biodegradability

The term \textit{“inherent biodegradability”} is less strict when compared to ready biodegradability. The test procedures offer a higher chance of detecting biodegradation compared to tests for ready biodegradability. Inherent biodegradability refers to a classification of chemicals for which there is unequivocal evidence of biodegradation (primary or ultimate) in any test of biodegradability. Such substances are considered not to be persistent and can be assumed to degrade in the aquatic environment in medium to long time periods (in wastewater treatment plants or in other environmental compartments). If an inherent test is negative, this could indicate the potential for persistence in the environment. In these tests the ratio biomass to food is shifted in favour of the biomass and the potential for adaptation is increased significantly.

3 Standard Testing Methods

3.1 OECD guidelines

OECD has developed several guidelines for testing of chemicals on biodegradation in an aerobic aqueous medium. As these guidelines are developed for chemicals (a form of matter that has a constant chemical composition and which cannot be separated into components by physical separation methods (without breaking chemical bonds)), these test methods are not always suitable in order to determine biodegradation of complex materials.

OECD guidelines do not provide explicit guidance with regard to biodegradability testing of poorly water-soluble substances (substances with a water solubility < 100 mg/l). The only critical guidance provided is the applicability of a restricted range of analytical methods (OECD 301 B, C, D (±) and F and OECD 310) and the requirement of additional control vessels where emulsifiers, solvents and carriers are used. Whilst advocating the use of emulsifiers, solvents and carriers, none are specifically identified and no guidance is provided regarding the acceptable level. Consequently, many approaches of introducing the test substance can be applied, which make it difficult to identify a set of core acceptable or workable solutions (ECHA (2012)). For volatile substances analytical methods OECD 301 C (±), D and F (±) and OECD 310 are suitable.
OECD 301 and 310 are both designed in order to determine ready biodegradability, while OECD 302 determines inherent biodegradability. OECD 303 and 309 are both guidelines for simulation biodegradation tests in specific freshwater aquatic environments. The duration of these tests is always 28 days, pre-exposure of the inoculum to the chemical is not allowed, the test substance is provided in a rather high concentration (2 to 100 mg/l) as the sole source of carbon for energy and growth while the amount of DOC in the test solution due to the inoculum should be kept as low as possible compared to the amount of DOC due to the test substance. The endogenous activity of the inoculum is corrected by running parallel blank tests with inoculum but without test substance.

The pass levels for ready biodegradability for chemicals as prescribed by OECD 301 and OECD 310 are:

- 70 % removal of DOC
- Biodegradation > 60 % Theoretical Oxygen Demand (ThOD)
- Biodegradation > 60 % Theoretical carbon dioxide (ThCO₂)
- Biodegradation > 60 % Theoretical inorganic carbon (ThIC)

The pass level is higher for methods based on the measurement of the residual sample (DOC), while the pass level is lower for methods, which are based on respirometric measurements. This is caused by the fact that the biodegradation percentage based on CO₂ production and oxygen consumption is always less than the percentage determined by DOC removal due to the bacterial metabolism. Some of the organic carbon of the test substance is biochemically oxidized and converted to CO₂, while other fractions are synthesized into new cellular material or into organic metabolic products. These fractions are not oxidised and do not contribute to the CO₂ production. The biomass formation is related to different factors (nature of test substance, bacterial species, etc.).

The pass levels need to be reached within a 10-d window, which starts when the degree of biodegradation has reached 10 % DOC, ThOD, ThCO₂ or ThIC and must end before day 28 of the test. If chemicals are classified as readily biodegradable, the need for other ecological parameters (e.g. bioaccumulation and ecotoxicity) may be reduced. As no complete biodegradation needs to be demonstrated, these pass levels (60 % or 70 %) for ready biodegradability are only reasonable for pure chemicals, but not necessarily for chemical mixtures or for chemicals containing significant proportions of impurities.

OECD 302 A prescribes that test chemicals giving a result > 20 % loss of DOC may be regarded as inherently biodegradable, whereas a result > 70 % loss of DOC is evidence of ultimate biodegradability. No recommendations with regard to the interpretation of the results are given in guidelines OECD 302 B and OECD 302 C. The definitions mentioned in Annex B of ISO 7827 (2010) also mention that removal of 20 % DOC, consumption of 20 % ThOD or evolution of 20 % ThCO₂ in any degradation test within 28 days, with or without pre-exposure of the inoculum, indicates that the test substance in question is inherently biodegradable and non-persistent.

### 3.2 European standards

For testing the aerobic biodegradability of organic compounds and plastic materials in fresh water CEN refers to existing ISO standards.

### 3.3 International standards

ISO proposed a series of methods for evaluating the aerobic biodegradability in fresh water. First the preparation method of the sample is selected based on the physico-chemical properties of the test substance (ECHA, 2012). These preparation methods are described in ISO 10634 (1995) “Water quality – Guidance for the preparation and treatment of poorly watersoluble organic compounds for the subsequent evaluation of their biodegradability in an aqueous medium”. Four preparation techniques are described in this standard:
- Direct addition
  a. Test component is weighted and direct addition
  b. Using an inert support (for example: microscope slides)
  c. Using a volatile solvent, which is removed prior to testing
- Ultrasonic dispersion
- Adsorption on an inert support (for example: silica gel or glass fibre filters)
- Dispersions or emulsions with an emulsifying agent

After the pre-treatment, the biodegradation in an aerobic aqueous medium can be tested using the standard methods, but methods based on DOC measurements are normally not suitable. ISO has developed various test methods in order to determine the biodegradability of organic compounds and plastics in an aerobic aqueous environment. These standards are mainly based on the principles of the OECD guidelines, but the international standards are often more precise and clearer. Most of these international standards concern the determination of the biodegradability of organic compounds in aqueous media (ISO 7827, ISO 9408, ISO 9439, ISO 9887, ISO 9888, ISO 10707, ISO 10708, ISO 11733, ISO 14592, ISO 14593). Only two standards are related to the biodegradability of plastic materials (ISO 14851 and ISO 14852), based on the same principles as ISO 9408 and ISO 9439, respectively.

The various ISO biodegradability standards differ with respect to microbial density, test item concentration, and test duration. Consequently these standards may exhibit different biodegradation potential for the same material. Test method ISO 14592 is characterised by low microbial densities and very low test item concentrations (< 200 µg/l) as it is developed in order to evaluate the biodegradation of substances at low environmentally realistic concentrations in the aquatic environment. ISO 14592-1 is a batch test simulating standing water bodies (lakes or ponds), while ISO 14592-2 is a dynamic test simulating flowing waters (rivers). ISO 10707 is performed in closed bottle without stirring or aeration and is also characterised by a rather low microbial density and a low test item concentration (2 mg/l). This method of low biodegradation potential is especially developed for volatile and inhibitory test compounds.

Test methods ISO 7827 (DOC), ISO 9408 (oxygen consumption), ISO 9439 (CO₂ production), ISO 10708 (two-phase closed bottle) and ISO 14593 (CO₂ headspace test) are all characterised by a higher biodegradation potential when compared to ISO 14592 and ISO 10707 as the microbial density and the test item concentration are considerably higher. These standards are comparable to the OECD guidelines for ready biodegradability (OECD 301 and OECD 310). In spite of the similar biodegradation potential of these methods different results may be obtained due to differences in measurement techniques.

Test methods ISO 9887 (SCAS test) and ISO 9888 (Zahn-Wellens test) have a high inoculum concentration and may be extended beyond the usual duration of 28 days. The conditions as prescribed in these methods are optimal in order to allow the maximum biodegradation value of a test item. These methods can be used to determine the intrinsic biodegradation of chemicals, which corresponds to the OECD guidelines for inherent biodegradability. ISO 11733 (activated sludge simulation test) is characterised by the highest inoculum concentration, as this test simulates the conditions of a wastewater treatment plant.

Different inoculum sources are prescribed by the various international standards for testing aerobic aqueous biodegradation of organic compounds. ISO prescribes that normally no pre-exposed inoculum should be used, especially in the case of standard tests simulating biodegradation behaviour in natural environments. Depending on the purpose of a test, a pre-exposed inoculum may be used, provided this is clearly stated in the test report and the method of pre-exposure needs to be clarified.

The standards ISO 14851 and ISO 14852 are testing methods concerning final products (plastics). The main difference with the testing methods for organic compounds (ISO 9408 and ISO 9439) is: 1) the higher concentration of the sample; 2) the longer maximum duration; and 3) the possibility to use compost extract as inoculum. Both standards recommend that the test mixture should preferably contain about 10⁵ - 10⁶ CFU/ml. The tests are normally executed at a constant temperature between 20°C and 25°C, but it is mentioned that higher
temperatures might be appropriate when compost is used as inoculum. Higher test item concentrations (up to 2000 mg/l of organic carbon) can also be tested in both methods on condition that an optimised test medium is used. An optimized medium is highly buffered and contains more inorganic nutrients. ISO 14851 and ISO 14852 acknowledge that both the determination of the BOD (after taking into account nitrification) and the determination of the evolved CO₂ are in some cases not enough to characterise the biodegradability of test materials with complex compositions. During a biodegradation test with a long duration the carbon in the test material is partly transformed to biomass but not biochemically oxidized. In this case, the measurements will not reach 100% of the theoretical values even in case of complete biodegradation of the test material. In order to confirm complete biodegradability, the carbon balance can be determined. The total carbon sum is based on five measurements: 1) carbon evolved as CO₂, 2) carbon produced as biomass, 3) carbon transformed into water-soluble organic metabolites, 4) carbon determined as DOC and 5) carbon remaining in the non-degraded polymer material.

3.4 American standards

ASTM has developed three standard test methods for the determination of the aerobic aquatic biodegradation of lubricants (ASTM D 5864, ASTM D 6139, and ASTM D 6731), and one standard test method for predicting the biodegradability of liquid-based lubricants using a bio-kinetic model (ASTM D 7373). Standard test methods ASTM D 5864 and ASTM D 6139 are not designed for volatile lubricants or lubricants components, while standard test method ASTM D 6731 is suitable for evaluating the biodegradation of non-volatile as well as volatile lubricants. Two other ASTM testing methods can be used to determine biodegradation of plastics in aqueous environment (ASTM D 5271 and ASTM D 6340).

Different inoculum options are prescribed such as activated sludge from wastewater-treatment plant, secondary effluent, surface water, soil extract, or mixture of sources. A criterion with regard to the quantity of micro-organisms in the inoculum is given.

4 Toxicity

The OECD guidelines are the most commonly used tests for assessing ecotoxicity of materials in aquatic environment. They comprise internationally agreed testing methods for environmental effects. Tests following the OECD guidelines are useful for both risk assessment and classification purposes. These guidelines describe test methods designed to evaluate the aquatic toxicity of chemicals. The toxic effect on different types of freshwater species can be evaluated: 1) algae, 2) plants, 3) invertebrates and 4) fish. An overview of these tests is presented in Table 1.

<table>
<thead>
<tr>
<th>Guideline</th>
<th>Adopted</th>
<th>Description</th>
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<tbody>
<tr>
<td>OECD 201</td>
<td>2006</td>
<td>Freshwater Alga and Cyanobacteria, Growth Inhibition Test</td>
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<tr>
<td>OECD 202</td>
<td>2004</td>
<td><em>Daphnia</em> sp., Acute Immobilisation Test</td>
</tr>
<tr>
<td>OECD 203</td>
<td>1992</td>
<td>Fish, Acute Toxicity Test</td>
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<tr>
<td>OECD 204</td>
<td>1984</td>
<td>Fish, Prolonged Toxicity Test: 14-day Study</td>
</tr>
<tr>
<td>OECD 209</td>
<td>2010</td>
<td>Activated Sludge, Respiration Inhibition Test (Carbon &amp; Ammonium Oxidation)</td>
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<tr>
<td>OECD 210</td>
<td>1992</td>
<td>Fish, Early-life Stage Toxicity Test</td>
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<tr>
<td>OECD 211</td>
<td>2012</td>
<td><em>Daphnia magna</em> Reproduction Test</td>
</tr>
<tr>
<td>OECD 212</td>
<td>1998</td>
<td>Fish, Short-term Toxicity Test on Embryo and Sac-fry Stages</td>
</tr>
<tr>
<td>OECD 215</td>
<td>2000</td>
<td>Fish, Juvenile Growth Test</td>
</tr>
<tr>
<td>OECD 221</td>
<td>2006</td>
<td><em>Lemna sp.</em> Growth Inhibition Test</td>
</tr>
<tr>
<td>OECD 229</td>
<td>2012</td>
<td>Fish Short Term Reproduction Assay</td>
</tr>
</tbody>
</table>

There are similar international (ISO) and American (ASTM) testing methods for aquatic ecotoxicity, which can also be applied alternatively.
5 Specifications

Although no European specifications are currently available for the biodegradation of bio-lubricants and bio-solvents, CEN has recently developed a technical report with recommendations for terminology and characterisation of bio-lubricants: CEN/TR 16227 (2011) “Liquid petroleum products – Bio-lubricants – Recommendation for terminology and characterisation of bio-lubricants and bio-based lubricants”. This technical report recommends that the claim “bio” should refer to an international or at least European agreed standard. In order to avoid market hindering confusion, the following minimum requirements are recommended in this technical report for bio-lubricants:

1. Minimum content of renewable raw material: 25 % (ASTM D 6866)
2. Minimum biodegradation percentage: 60 % (oils) or 50 % (lubricating greases) (OECD 301 B, C, D or F or adequate ISO or EN standards)
3. Not labelled as “Dangerous to the environment” (Symbol N) (EC50/LC50/IC50 fully formulated product > 100 mg/l; Method: OECD 201/202/203)
4. Minimum performance criteria
5. Any lubricant according to the present criteria of the EU Ecolabel for Lubricants is per definition a bio-lubricant

The technical report also gives an overview of the biodegradation and ecotoxicity test methods which are of importance for lubricants.

Moreover, there exists an international standard, which gives specifications for environmentally acceptable hydraulic fluids (HE): ISO 15380 (2011) “Lubricants, industrial oils and related products (class L) – Family H (Hydraulic systems) – Specifications for categories HETG, HEPG, HEES and HEPR”. The specification is related towards ready biodegradability (in aerobic aqueous environment) and (aquatic) ecotoxicity towards bacteria, Daphnia and fish.

6 Conclusions

Based on the literature review of the different biodegradation test methods in an aqueous aerobic freshwater environment it can be concluded that a sufficiently broad range of measurement techniques already exists. The biodegradation rate can be determined based on the measurement of dissolved organic carbon, dissolved oxygen, CO2 production, oxygen consumption or inorganic carbon. However, several improvements are required in order to enhance the reproducibility of the results and the credibility of the testing methods.

When testing poorly water soluble test items the addition of the test item to the test system needs to be adapted when compared to the addition of water soluble test items. Therefore the addition method as prescribed in the previous mentioned OECD, ISO and EN methods need to be modified. Such modifications are already described in ISO 10634, which was especially developed towards test items that are poorly water soluble.

When testing bio-lubricants and bio-solvents, more appropriate reference materials should be chosen when compared to the currently proposed water soluble and very easily degradable reference materials which are mentioned in the guidelines. Such a modification has already been introduced in biodegradation standards for plastics (ISO 14851 and ISO 14852).

The biodegradation activity of the inoculum will be determined by various factors (geographical location of sampling, season of sampling, etc.). The majority of the standards refer to activated sludge, secondary effluent, surface water and soil as a suitable inoculum. It is expected that the diversity of the microbial flora and as such the biodegradation potential will increase if different sources are mixed in order to prepare the inoculum. A standardised inoculum could be a solution in order to increase reproducibility of test methods and this could reduce the dependence on seasonal and geographical variability. However, it still needs to be evaluated if they are diverse enough in order to degrade difficult products. The diversity of standardised inocula should be compared to the diversity of natural inocula.
No specific criteria are given in ASTM D 5864, ASTM D 6139 and ASTM D 6731. These test methods state that if a test material achieves a high degree of biodegradation, it may be assumed that the test material easily biodegrades in many aerobic aquatic environments. However, no specific pass level (“high” degree: > 60 %, > 70 %, > 90 %?) nor a maximum duration are given. This should be clearly defined in order to avoid miscommunications.

Based on the literature review of the available standards with regard to environmental safety, it can be concluded that a sufficiently broad range of testing methods towards freshwater organisms on different trophic levels (bacteria, algae, freshwater aquatic plants, crustacean and fish) already exists. For bio-lubricants and bio-solvents, additional attention is especially needed towards the addition of bio-lubricants that are poorly water soluble and volatile bio-solvents to the testing systems as this can influence the test results. There exist already documents with specific guidance toward the sample preparation and the interpretation of the results for difficult substances (OECD), poorly water soluble substances (ISO 14442) and lubricants (ASTM D 6081). These guidelines should be taken into account when testing bio-lubricants and bio-solvents.

The literature review on the specifications revealed that currently no clear specifications for biodegradation in fresh water exist on European and American level. Therefore, there is an urgent need as standardisation can play an important role in the uptake of products such as bio-based lubricants and bio-based solvents and consequently can help to increase market transparency by providing reference methods and criteria.

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8 References

