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Water quality — Preparation and treatment of poorly water-soluble organic compounds for the subsequent evaluation of their biodegradability in an aqueous medium

Qualité de l'eau — Préparation et traitement des composés organiques peu solubles dans l'eau en vue de l'évaluation de leur biodégradabilité en milieu aqueux

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document 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 10634:1995), which has been technically revised to take into account user feedback, new technologies and available reagents.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

The standardizing work carried out by ISO/TC 147/SC 5 has shown that the development of a single method for evaluating the biodegradability of organic compounds with a low solubility in water (i.e. < 100 mg/l[1][2][3]) cannot be envisaged in the immediate future. In fact, the selection of the most suitable working method to obtain a satisfactory emulsion or dispersion of these compounds in the test media depends particularly on their physicochemical properties. Consequently, the selection of the most suitable method has to be left to the judgement of laboratories responsible for the tests based on their experience and the product information supplied by the applicant. For this reason, this document describes various techniques for treating poorly water-soluble organic compounds before they are investigated for biodegradability tests. The objective is to reach a stage where, for any given technique, the same working method is used by all laboratories, thus making it easier to compare results. Specificities of the selected protocol should be kept in mind for the evaluation and interpretation of the results of the biodegradation test.

The techniques described in this document will not necessarily produce the same biodegradability results of the test compound if they are used in parallel. The use of solvents and dispersing or emulsifying techniques can be additional sources of uncertainty and can lead to test results which differ from those obtained without using these techniques. Furthermore, dispersions or emulsions can be produced that would not exist as such in nature. It is recommended to perform biodegradability tests with the direct addition of a test compound and using dispersion techniques in parallel because activity of inoculum used should be comparable. The presence of microorganisms with potential to degrade the test compound is assumed to be identical. The composition and activity might change when the tests are conducted subsequently.

According to current standards for testing biodegradability, only pure or compounds containing a low amount of impurities should be tested. Biodegradability tests are not recommended for heterogeneous mixtures or multicomponent compounds as the results of such tests are difficult to interpret, especially when the degradation is partial. Moreover, the use of solvents and dispersion techniques can lead to unrepresentative heterogeneous distributions and to misleading test results in the subsequent biodegradability tests.

Water quality — Preparation and treatment of poorly water-soluble organic compounds for the subsequent evaluation of their biodegradability in an aqueous medium

WARNING — Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices.

IMPORTANT — It is absolutely essential that tests conducted in accordance with this document be carried out by suitably qualified staff.

1 Scope

This document specifies techniques for preparing poorly water-soluble organic compounds (i.e. liquid and solid compounds) with a solubility in water of less than approximately 100 mg/l and introducing them into test vessels for a subsequent biodegradability test in an aqueous medium using standard methods.

The subsequent tests on biodegradability are primarily methods using the analysis of the released carbon dioxide described in ISO 9439 and the determination of the oxygen described in ISO 9408 and following the usual precautions for ISO 10707. Thus, one can notice that the methods measuring the removal of dissolved organic carbon (DOC) are not appropriate.

This document does not specify the biodegradation test methods. It is restricted to describing techniques for introducing the test compounds into the test medium and to keeping them in a dispersed state[4]. These techniques are implemented while observing the experimental conditions described in the standardized methods for evaluating biodegradability. ISO 9439, based on CO_2 evolution, is not suitable for testing volatile compounds.

Some of the preparation methods described in this document might not be accepted by regulators for making conclusions on the ready biodegradability of tested compounds.

Examples of biodegradability curves are given in Annex A.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 9408, *Water quality — Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium by determination of oxygen demand in a closed respirometer*

ISO 9439, *Water quality — Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium — Carbon dioxide evolution test*

ISO 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)*

3 Terms and definitions

No terms and definitions are listed in this document.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

4 Presentation of suitable preparation and analytical methods

4.1 Preparation methods

In this document, several techniques for introducing the test compounds into the test medium are described. The preparation methods are as follows:

— direct addition: this technique is recommended for poorly soluble compounds instead of the preparation of a stock solution;

— ultrasonic dispersion: this technique can be applied to non-volatile liquid and solid compounds;

— adsorption or weighing on an inert support;

— dispersion or solubilization with additive;

— combination of methods listed above.

NOTE Regarding the combination of methods, the techniques are generally run individually in parallel (i.e. simultaneously by the same method and with the same inoculum) to gain insight into whether one technique is dominant or whether both are contributing to enhance bioavailability and biodegradation.

4.2 Analytical methods

The test compound concentration shall fulfil the requirements of ISO 9439, ISO 9408 and ISO 10707.

When the test compound is introduced directly or on an inert support in the test vessel, it is not necessary to confirm the tested concentration.

When the preparation method uses a stock solution of the tested compound, it is necessary to confirm the concentration tested. For this purpose:

— a specific analytical method is required if the support or additive is an organic chemical (for example, surfactant);

— the total organic carbon (TOC) analysis is acceptable if the support or additive is an inorganic compound (for example, silica gel) or if a homogeneous dispersion is obtained by physical treatment (for example, ultrasonic treatment).

5 Direct addition and addition with inert support

5.1 General

Biodegradability tests should be performed in parallel, with the direct addition of a test compound and using dispersion techniques, because the activity of the inoculum used should be comparable. The presence of microorganisms with the potential to degrade the test compound is assumed to be identical.

The test compound is weighed and directly introduced into the test vessels or weighed onto an inert support and introduced into the test vessels, which are subjected to continuous agitation.

Solid compounds can be grinded (e.g. using a mortar and a pestle) as finely as possible before weighing them. Liquid nitrogen may be used.

NOTE Adding the tested substance adsorbed on inert support can affect the final biodegradation result. The bioavailability of the compound can be limited by the adsorption to the inert support. Therefore, the measured biodegradation result can decrease (e.g. if the adsorption to the surface of the inert material limits the access of the tested substance to the inoculum). In the case of a tested compound that is toxic to microorganisms, the limitation of the bioavailability could limit the toxic effect and increase the biodegradation result.

5.2 Reagents

5.2.1 Inert supports.

Silica gel, fibreglass filters, microscope slides or other non-biodegradable inert supports that do not release organic or inorganic carbon into the aqueous medium can be used.

It should be validated by preliminary work that the support is inert and carbon-free. To avoid or minimize surface area effects, the quantity of the support shall be minimal. The test compound should be adsorbed on the surface.

For example, the following supports are suitable:

— microscopic slide;

— polyethylene slide;

— stainless steel slide;

— silica gel used for thin-layer chromatography (15 µm particle size);

— silica gel used for column chromatography (200 µm to 500 µm particle size).

5.3 Apparatus

5.3.1 Stirrers.

Sufficient stirrers are required to agitate all the test vessels used in a given biodegradability test except for the closed bottle test (see ISO 10707).

Stirrer-rods shall be made of a material such that no ingredient of a plastic coat will contaminate the test medium and no adsorption of test compounds will occur. Heating the test vessels by stirring and raising the test temperature shall be avoided.

5.3.2 Vessels.

It is recommended to use laboratory glass or chemically inert labware for weighing and sample preparations to avoid carbon contamination and adsorption of the test compound.

5.3.3 Mechanical disperser (e.g. Ultra-turrax®[1)]).

5.4 Procedure

5.4.1 Direct addition

Test compounds shall be weighed and directly added to the test vessels.

1) Ultra-turrax® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Non-viscous liquid compounds shall be added with a high precision volumetric syringe, taking into account their relative density.

5.4.2 Addition on inert support

5.4.2.1 Solid test compound

Weigh onto the support (5.2.1) a quantity of the compound corresponding to the concentration of the organic carbon required by the test method to be used.

Introduce a support into each of the test vessels.

Introduce a support without the test compound into each of the control vessels.

5.4.2.2 Liquid test compound

Weigh the liquid, including the viscous compound, without treatment. Prepare the quantity of test compound required by the biodegradability test method to be mixed with the support.

For example, with a final test solution volume of 1 l, add 50 mg of the support (5.2.1) and the amount of the test compound needed for the test flask by direct weighting and emulsify with the mechanical disperser for 1 min. At the same time, carry out the same procedure using only the support in the control vessels. After mixing the inert support with the liquid test compound, the dilution water and inoculum are added.

6 Ultrasonic and physical treatment

6.1 General

An emulsion or dispersion of the compound to be tested is prepared using an ultrasonic probe or an ultrasonic bath and is introduced into the test vessels, which are continuously agitated (see 5.3.1 and 5.3.2).

6.2 Apparatus

6.2.1 Ultrasonic probe, capable of producing a frequency of approximately 20 kHz to 35 kHz.

6.2.2 Ultrasonic bath, capable of producing a frequency of approximately 20 kHz to 35 kHz.

6.2.3 Stirrers, in sufficient numbers to ensure that all the test vessels can be agitated (see 5.3.2).

6.3 Procedure using an ultrasonic probe

6.3.1 Preparation of the test compound

6.3.1.1 Preparation of a stock solution

Add, for example, 1 g or 1 ml of the test compound to a 500 ml beaker containing approximately 400 ml of deionized water.

The test compound shall be present in excess so that a saturated solution is obtained.

6.3.1.2 Preparation with the required quantity

Add the required quantity of the test compound into the test vessels containing mineral medium without inoculum.

6.3.2 Experimental protocol

Install the ultrasonic probe (6.2.1) in such a way that its tip is as close as possible to the interface between the mineral medium and the test compound.

Use a stirrer (6.2.3) to agitate the test vessel so that the compound is drawn down to the bottom.

Set the probe to give a frequency of about 20 kHz to 35 kHz and maintain this for about 5 min to 30 min.

Switch off the probe and leave the emulsion or dispersion to settle for 15 min to 30 min.

Some compounds are subjected to ultrasonic decomposition, possibly due to an increase in the temperature of the bulk solution. This problem can be avoided by measuring and controlling the temperature, by reducing the power of the ultrasonic probe or by intermittent sonification. It is possible to cool the test vessel to avoid overheating, e.g. by placing the test vessel in an ice bath or cold water. In some cases, problems can be encountered because of the destruction of the compounds. If this is the case, a different method should be used.

When using a stock solution, analyse an aliquot of the emulsion or dispersion obtained and determine the concentration of the test compound after decantation by using an appropriate analytical method. Introduce an appropriate volume of emulsion or dispersion into the test vessels to obtain the concentration of the organic carbon required by the test method to be used.

It can be difficult to obtain a stable emulsion or dispersion. Special care is therefore required when aliquots are distributed to the test vessels. If it proves impossible to obtain a sufficiently stable emulsion or a sufficiently high concentration to carry out the test, the test compound can be introduced directly into the test medium and can be dispersed ultrasonically in the test vessels before the inoculum is added.

6.4 Procedure using an ultrasonic bath

Prepare the test vessels with the required concentration of the test compound and the mineral medium without inoculum. Introduce the test vessels in the ultrasonic bath (6.2.2).

Set the bath to give a frequency of about 20 kHz to 35 kHz for about 5 min to 30 min. The energy input of ultrasonic treatment depends on many factors and the effect should be tested in pre-tests in order to obtain a suitable combination of electric power and treatment duration.

Switch off the bath and leave the emulsions or dispersions to settle for 15 min to 30 min (see 6.3.2).

6.5 Other methods

Except sonication, other physical bioavailability improvement methods are feasible, for example pickering emulsions, as described by Kalashnikova[5]:

> "Emulsions are stabilized by surface-active species such as surfactant molecules with an affinity for both phases. Surfactants, the conventional stabilizers, are continuously adsorbed and desorbed at the interface. This is at the origin of the phase separation phenomenon since competition between adsorption and coalescence occurs. In the past few years, solid particles have been used to replace surfactant molecules. These types of emulsions are called Pickering emulsions".

These other methods will be carried out on the test compound suspended in the mineral medium. Its use will be acceptable if the test medium remains unchanged and retains its properties. It is necessary to:

— use an appropriate specific analytical method, analyse an aliquot of the emulsion or dispersion obtained and determine the concentration of the test compound after decantation;

— prepare control vessels with mineral medium and used treatment without the test compound.

In some cases, problems can be encountered because of the destruction of the compounds. If this is the case, a different method should be used.

This method has demonstrated its ability to improve the bioavailability of the tested compound in biodegradation tests[6].

7 Adsorption on an inert support with a volatile solvent removed from the system

7.1 General

The test compound is adsorbed or weighed onto an inert support and introduced into the test vessels. It is kept dispersed in the medium by continuous agitation.

7.2 Reagents

7.2.1 Inert supports.

Silica gel, fibreglass filters, microscope slides or other non-biodegradable inert supports which do not release organic or inorganic carbon into the aqueous medium can be used (see 5.2.1).

7.2.2 Solvent.

A volatile solvent is selected for its suitability to dissolve the test compound.

It shall be non-toxic to bacteria and non-biodegradable under the conditions of the subsequent biodegradability tests and have a high purity which ensures that insignificant organic carbon is introduced with non-volatile residues. This shall be tested in advance or in the subsequent biodegradability test.

Depending on the test compound, trichloromethane (CAS RN: 67-66-3) can be suitable.

NOTE 1 The residual carbon content due to unevaporated solvent can affect the test results despite the use of a solvent control.

NOTE 2 In order to prevent volatile solvents from mobilizing adsorbed organic residues from the equipment, it can be necessary to consider an additional washing step with the solvent before using the equipment.

Non-toxic and non-biodegradable solvents may be used (e.g. 3,7,11,15-tetramethylhexadecane-1,2,3-triol, CAS RN: 74563-64-7 or di-methyl isosorbide, CAS RN: 5306-85-4). Nevertheless, their suitability for biodegradation tests should be demonstrated beforehand.

7.3 Apparatus

7.3.1 Stirrers, in sufficient numbers to ensure that all the vessels can be agitated (see 5.3.1).

7.3.2 Vessels (see 5.3.2).

7.4 Procedure

7.4.1 Prepare the quantity of test compound to be soaked into the support required by the biodegradability test method to be used. For this, two types of preparation could be performed: individual (7.4.2) or distribution (7.4.3).

7.4.2 As an example of individual preparation, mix together by agitation, for each test vessel, in a 100 ml vessel for 2 h, 50 mg of the support (7.2.1) and the quantity of the test compound required by the

test in the chosen solvent (7.2.2). At the same time, carry out the same procedure using only the support and the solvent as a control.

In both cases, recover the support and dry by totally evaporating the solvent. This can be performed using, in succession, a rotary evaporator, a ventilated oven and a vacuum oven at about 45 °C.

7.4.3 As an example of distribution preparation, mix together by agitation, for each test vessel, in a 250 ml vessel for 2 h, 30 g of the support (7.2.1) and the quantity of 150 ml of a solution of 1 g/l of the test compound in the chosen solvent (7.2.2). At the same time, carry out the same procedure using only the support and the solvent as a control.

In both cases, recover the support and dry by totally evaporating the solvent. This can be performed using, in succession, a rotary evaporator, a ventilated oven and a vacuum oven at about 45 °C.

7.4.4 For analytical verification, determine the amount of the compound soaked into the support in three samples of 1,5 g or more, using one of the following methods:

— elemental quantitative analysis of the amount of carbon originating from the compound, using a high-temperature total carbon analyser, and then deducting the values obtained for the support treated with solvent only;

— determination of the chemical oxygen demand of the compound impregnated on the inert support and then deducting the values obtained for the support treated with solvent only;

— extraction of the compound using an organic solvent and quantitative analysis using a specific analytical method.

From the amount of test compound effectively adsorbed on the support, determine the quantity of support to be introduced into the test vessels to obtain the concentration of the organic carbon of the test compound required by the test method used.

Introduce the same quantity of support treated with the solvent alone into each of the control vessels.

The solvent control shall be taken into account in the calculation of the test compound results.

NOTE The last traces of solvent can be difficult to remove. Interferences can occur if the solvent is biodegradable or inhibitory to bacteria.

8 Addition with a non-biodegradable solvent or emulsifying agent

8.1 General

A dispersion, emulsion or solubilization of the test compound is prepared using a solvent, an emulsifying agent, an oil or another chemical support and is introduced into the test vessels, which are continuously agitated.

NOTE In general, the addition of solvents or emulsifying agents or other additives not removed from the system increases the overall uncertainty of the tests. The use of an emulsifier to prepare stock or test dispersions is generally the least preferred method[7].

8.2 Reagents

8.2.1 Solvent.

A solution of the test compound is prepared in an organic solvent and is introduced into the test vessels which are subjected to continuous agitation.

A solvent, sufficiently miscible in water, is chosen for its capacity to dissolve the test compound. The chosen solvent shall not react with the test compound or with any component of the medium. The

solvent shall be used in minimal amounts (1 ml·l^{-1} or less). The solvent shall be non-toxic to bacteria and non-biodegradable under the conditions of the subsequent biodegradability test.

When the solvent is volatile, it shall be removed before the inoculum is added. It can be difficult to eliminate solvents which are highly miscible in water[8]. In this case, they should not be used.

8.2.2 Emulsifying agent.

The emulsifying agent shall be non-toxic to bacteria and non-biodegradable under the conditions of the subsequent biodegradability tests.

If the biodegradability and the inhibitory effects to bacteria of the emulsifying agent are unknown, they should be investigated in advance or in additional assays of the subsequent biodegradability test, e.g. using one vessel containing only emulsifying agent for measuring biodegradability and one vessel containing emulsifying agent and a reference compounds, for example: sodium benzoate, aniline for measuring potential inhibitory effects.

As an example, the following compounds[2)] can be used:

a) a block copolymer of ethylene oxide and propylene oxide, with a hydrophilic-lipophilic balance value of about 9;

b) a block copolymer of ethylene oxide and propylene oxide, with a hydrophilic-lipophilic balance value of about 13,5.

8.2.3 Mineral oil.

The mineral oil shall be non-toxic to bacteria and non-biodegradable under the conditions of the subsequent biodegradability tests as, for example, the 2,2,4,4,6,8,8-heptamethylnonane (CAS RN: 4390-04-9)[9].

8.2.4 Silicone oil.

Silicone oil as polydimethylsiloxane and polyphenylmethylsiloxane, for example, AR 20®[3)] (CAS RN: 63148-58-3), are often non-biodegradable and not toxic to bacteria[10][11].

8.3 Apparatus

8.3.1 Stirrers, in sufficient numbers to ensure that all the test vessels can be agitated (see 5.3.1).

8.3.2 Mechanical disperser (e.g. Ultra-turrax®[1)]).

2) Pluronic P9400® and Pluronic P10300® are examples of suitable products available commercially for compounds a) and b), respectively. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

3) AR 20® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

9 Preliminary tests

9.1 Reagents

9.1.1 Selection of emulsifying agent concentration

As an example, prepare three solutions of the test compound (x mg) in y ml of the solvent (8.2.1) or mineral medium with each of the following emulsifying agents:

a) block copolymer [8.2.2 a)] alone at 0,5 x mg;

b) block copolymer [8.2.2 b)] alone at 0,5 x mg;

c) mixture of block copolymer [8.2.2 a)] at 0,25 x mg plus block copolymer [8.2.2 b)] at 0,25 x mg.

The amount of the compound dissolved in the solvent (x mg) is calculated to obtain the required concentration of organic carbon of the test compound in the test medium to be used.

Homogenize by steady agitation (e.g. for 10 min), and then add the solution obtained drop by drop to the volume of test medium required for each test vessel specified for the biodegradability test. Remove the solvent by persisting with the agitation or by any other appropriate method.

Select by visual assessment the preparation a), b) or c) which produces the most homogeneous and stable emulsion. The achievement of homogeneous and stable emulsion can require several days.

9.1.2 Selection of mineral oil concentration

As an example, prepare two solutions of the test compound (x mg) in the test medium with each of the following mineral oil quantity:

a) 0,5 x mg;

b) 0,25 x mg.

Emulsify with the mechanical disperser for 1 min. Select by visual assessment the preparation a) or b) which produces the most homogeneous emulsion.

9.1.3 Selection of silicone oil concentration

As an example, prepare two solutions of the test compound (x mg) in the test medium with each of the following silicone oil quantity:

a) x × 50 mg;

b) x × 25 mg.

Emulsify with the mechanical disperser for 1 min. Select by visual assessment the preparation a) or b) which produces the most homogeneous emulsion.

9.2 Procedure

9.2.1 Use of solvent

Prepare a solution of the test compound in a minimum volume of the chosen organic solvent (8.2.1).

Introduce into the test vessels the quantity of solution needed to obtain the concentration of the organic carbon required by the test method used.

Introduce the same quantity of the solvent, without any test compound, into each of the control vessels.

Evaporate the solvent, if possible completely, using, for example, in succession, a rotary evaporator, a ventilated oven and a vacuum oven at about 45 °C.

The last traces of solvent can be difficult to remove. Interferences can occur if the solvent is biodegradable or inhibitory to bacteria.

The test solution can be spread over the base of the test vessels and the system is then purged with gas (e.g. air, nitrogen) and/or stirred. The last traces of solvent can be difficult to remove. Interferences can occur if the solvent is biodegradable or inhibitory to bacteria.

Prepare a mix containing only the test medium and the solvent, to provide control vessels to verify that the solvent biodegradation does not exceed 10 % compared to the test compound biodegradation.

The results of this solvent control shall be taken into account in calculating the test compound results.

9.2.2 Use of emulsifying agent

Prepare a sufficient amount of the emulsion or dispersion, combining the emulsifying agent and the test compound, in accordance with the procedure selected as a result of the preliminary tests for all test vessels needed to carry out the biodegradability test to be used.

Alternatively, an emulsion or a dispersion could be prepared for each test vessel with the emulsifying agent concentration in accordance with the procedure selected as a result of the preliminary tests.

Prepare an emulsion or dispersion containing only the test medium and the emulsifying agent. This provides control vessels to verify that the emulsifying agent biodegradation does not exceed 10 % compared to the test compound biodegradation.

The results of this emulsifier control shall be taken into account in calculating the test compound results.

9.2.3 Use of mineral oil

Prepare a sufficient amount of the emulsion or dispersion, combining the mineral oil and the test compound, in accordance with the procedure selected as a result of the preliminary tests, for all the test vessels needed to carry out the biodegradability test to be used.

Alternatively, an emulsion or a dispersion could be prepared for each test vessel with the mineral oil concentration in accordance with the procedure selected as a result of the preliminary tests.

Prepare an emulsion or dispersion containing only the test medium and the mineral oil. This provides control vessels to verify that the emulsifying agent biodegradation does not exceed 10 % compared to the test compound biodegradation.

The results of this mineral oil control shall be taken into account in calculating the test compound results[10][11].

9.2.4 Use of silicone oil

Prepare a sufficient amount of the dispersion, combining the silicon oil and the test compound, in accordance with the procedure selected as a result of the preliminary tests, for all the test vessels needed to carry out the biodegradability test to be used.

Alternatively, a dispersion could be prepared for each test vessel with the silicone oil concentration in accordance with the procedure selected as a result of the preliminary tests.

As an example, prepare, for each test vessel, a dispersion in a tube by mixing y ml of silicone oil and x mg of test compound in the mineral medium to obtain a final silicone oil concentration of 1 ml/l and the final test compound concentration needed to carry out the biodegradability test. Homogenize this dispersion by suction and discharge with a micropipette. Heat for 10 min at 40 °C in a heating

block. Heating should be omitted if it can alter the structure of the test compound. Disperse with the mechanical disperser for 1 min.

Prepare a dispersion containing the test medium and the silicone oil only. This provides control vessels to verify that the silicone oil biodegradation does not exceed 10 % compared to the test compound biodegradation.

The results of this silicone oil control shall be taken into account in calculating the test compound results[10][11].

9.3 Other additives

Other compounds or natural additives can be used as bioavailability improvement methods of test compounds such as some biosurfactants. For these other additives, it is possible to apply the selection of emulsifying agent (9.1.1) to select the additive and the emulsifying agent procedure (9.2.2) to perform the preparation.

The additive shall be non-toxic to bacteria and non-biodegradable under the conditions of the subsequent biodegradability test, especially if it cannot be removed sufficiently. It should not change the structure of the test compound and should be used in small amounts (around 10 mg/l or less) to prevent any significant change of the test medium. Therefore, compounds as readily biodegradable rhamnolipids are not suitable to perform the test compound preparation.

It is essential to prepare a dispersion containing the test medium and the additive only. This provides control vessels to verify that the additive biodegradation does not exceed 10 % compared to the test compound biodegradation.

The results of this additive control shall be taken into account when calculating the test compound results.

10 Combination of methods

It is also possible to combine the chemical methods described in Clauses 5, 7 and 8 and the physical methods described in Clause 6 (for example, the use of ultrasonic dispersion (6.3) with silicone oil (8.2.4) [4][5][6]). This process will be carried out on the test compound suspended in the mineral medium.

Its use will be acceptable if the test medium remains unchanged and retains its properties. It is necessary to:

— use an appropriate analytical method (see 4.2), analyse an aliquot of the emulsion or dispersion obtained and determine the concentration of the test compound in the decanted medium after decantation;

— prepare control vessels with mineral medium and used treatment without the test compound.

In addition, it is preferable to run the techniques individually in parallel (i.e. simultaneously by the same method and with the same inoculum) to gain insight into whether one technique is dominant or whether both are contributing to enhance bioavailability and biodegradation.

11 Test report

The test report shall contain at least the following information:

a) the test method used, together with a reference to this document, i.e. ISO 10634:2018;

b) any pretreatment of the compound before the test;

c) method of introduction of the test compound;

d) duration and intensity of treatment;