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**Water quality — Determination of  
inhibition of gas production of anaerobic  
bacteria —**

**Part 2:  
Test for low biomass concentrations**

*Qualité de l'eau — Détermination de l'inhibition de la production de gaz  
des bactéries anaérobies —*

*Partie 2: Essai à de faibles concentrations de biomasse*



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# Contents

Page

|                                                                                          |           |
|------------------------------------------------------------------------------------------|-----------|
| <b>Foreword</b> .....                                                                    | <b>iv</b> |
| <b>1 Scope</b> .....                                                                     | <b>1</b>  |
| <b>2 Normative references</b> .....                                                      | <b>1</b>  |
| <b>3 Principle</b> .....                                                                 | <b>2</b>  |
| <b>4 Reagents and media</b> .....                                                        | <b>2</b>  |
| <b>5 Apparatus</b> .....                                                                 | <b>4</b>  |
| <b>6 Test environment and interferences</b> .....                                        | <b>5</b>  |
| <b>7 Procedure</b> .....                                                                 | <b>5</b>  |
| <b>8 Calculation</b> .....                                                               | <b>9</b>  |
| <b>9 Validity criteria</b> .....                                                         | <b>9</b>  |
| <b>10 Test report</b> .....                                                              | <b>10</b> |
| <b>Annex A</b> (informative) <b>Calibration of the pressure meter</b> .....              | <b>11</b> |
| <b>Annex B</b> (informative) <b>Expression of results in tests with wastewater</b> ..... | <b>12</b> |
| <b>Bibliography</b> .....                                                                | <b>13</b> |

## 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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

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.

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 13641-2 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

ISO 13641 consists of the following parts, under the general title *Water quality — Determination of inhibition of gas production of anaerobic bacteria*:

- *Part 1: General test*
- *Part 2: Test for low biomass concentrations*

# Water quality — Determination of inhibition of gas production of anaerobic bacteria —

## Part 2: Test for low biomass concentrations

**WARNING** — Sludge samples may contain hazardous and inflammable substances. They contain pathogens and are liable to biological action. Consequently, it is recommended that samples be handled with special care. The gases that may be produced by microbiological activity are potentially inflammable and will pressurize sealed bottles. Exploding bottles are likely to result in infectious shrapnel and/or pathogenic aerosols. Glass bottles should be avoided wherever possible. Care is necessary when sampling, transporting and utilizing the sludge and when using microsyringes and pressure-meter syringe needles. National regulations should be followed with respect to microbiological hazards associated with this method. Toxic test materials and those with unknown properties should be handled with care.

### 1 Scope

This part of ISO 13641 specifies a screening method for assessing the potential toxicity of substances, mixtures, surface waters, groundwaters and wastewaters, effluents, sludges, or other environmental samples by determining the production of biogas (carbon dioxide and methane) from muds, sediments and other anaerobic environments with low biomass concentration. 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 conditions of this test (for example amount of inoculum and substrate in the test bottles) were adopted to a defined test period over several days. The inoculum can be collected from anaerobic sediments or from large, or laboratory scale, anaerobic digesters.

This method is applicable to materials, soluble or insoluble in water, including volatile chemicals (see Reference [1] in the Bibliography).

**NOTE** Special care is necessary with compounds of low water-solubility, and in these cases, see for example, ISO 10634. For general information on biotesting see ISO 5667-16<sup>[2]</sup>.

Information obtained by this method can be helpful prior to anaerobic biodegradability testing with low inoculum mass 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.

### 2 Normative references

The following referenced documents are indispensable for the application 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 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*

### 3 Principle

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 mass concentrations of the test material in sealed bottles for a defined incubation time at 35 °C. The amount of biogas (methane and carbon dioxide) produced is measured by the increase in pressure in the bottles before and after addition of acid to the release carbon dioxide from carbonates. The percentage inhibition of biogas production by the various mass concentrations of the test material is calculated from the amounts produced in the respective test and control bottles. The  $EC_{50}$  and other effective mass concentrations are calculated from the plots of percentage inhibition against the logarithm of mass concentration of the test material.

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 can contain a high amount of inorganic matter and cell numbers and hence the bacterial activity can be very low in such cases, so that the incubation period needs to be extended.

### 4 Reagents and media

#### 4.1 Reagents

##### 4.1.1 Dilution water, previously de-aerated and de-ionized.

Analytical controls of this water are not necessary, but make sure that the deionizing apparatus is regularly maintained. Prior to addition of the anaerobic inoculum to any solution or dilution of test material, make sure that these are oxygen-free. Therefore, either bubble nitrogen gas (4.1.2) through the dilution water or through the dilutions for 1 h before adding the inoculum, or alternatively heat the dilution water to boiling then cool it to room temperature in an oxygen-free atmosphere.

##### 4.1.2 Nitrogen gas, of high purity with a content of less than 5 µl/l oxygen.

##### 4.1.3 Phosphoric acid ( $H_3PO_4$ ), 85 % by mass in water.

#### 4.2 Media

##### 4.2.1 Inoculum.

Collect active digesting anaerobic sludge from a full-scale, or a laboratory, digester or anaerobic sediment from a suitable natural source. Record the source and type of inoculum in the test report. Bottles for collection should be equipped with gas-tight seals and be made of high-density polyethylene or a similar material, which can expand. Glass is not recommended since the bottle may explode. Fill the sample bottles up to 1 cm from the top, seal them tightly and place them in insulated containers (5.1) to minimize temperature shock, until being transferred to an incubator (5.10) maintained at the desired test temperature. When opening the bottles, take care to release excess gas pressure either by periodically loosening the seal or by fitting a three-way pressure release valve (5.3) to the bottle cap. It is preferable to use the inoculum within a few hours of collection, otherwise store at the test temperature (6.1) under a headspace of nitrogen for up to 3 days when little loss of activity will normally occur.

Immediately prior to use, mix the inoculum by gentle stirring and pass it through a mesh sieve (5.2) into a suitable bottle (5.4) through the headspace of which a stream of nitrogen (4.1.2) is passed. Set aside a sample for determination of the mass concentration of total dry solids (see for example ISO 11923). The mass concentration of digester sludge is usually between 20 g/l and 40 g/l total dry solids but anaerobic sediment will be more variable. Thus some sludges may require diluting using dilution water (4.1.1) and some sediments will require concentrating by centrifugation. Use a final mass concentration of  $0,20 \text{ g/l} \pm 0,05 \text{ g/l}$  of total dry solids.

Check the pH value of the inoculum and adjust if necessary to  $7 \pm 0,5$ . During centrifuging or dilution of the inoculum make sure that no oxygen penetrates into the suspension. Use, for example, for centrifugation