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Ships and marine technology — Bioassay methods for screening anti-fouling paints —

Part 2: Barnacles

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Foreword

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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).

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This document was prepared by Technical Committee ISO/TC 8, *Ships and marine technology*, Subcommittee SC 2, *Marine environment protection*, in collaboration with Technical Committee ISO/TC 35, *Paints and varnishes*, Subcommittee SC 9, *General test methods for paints and varnishes*.

A list of all parts in the ISO 21716 series can be found on the ISO website.

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

Anti-fouling paints that contain biocides are widely used to prevent fouling of ship hulls by marine organisms. Effective anti-fouling technologies are critical to maintaining the fuel consumption efficiency of ships and also for minimizing possible translocation of aquatic species through maritime trade. The evaluation of anti-fouling paints is generally undertaken by adopting a tiered approach whereby paint manufacturers use a battery of laboratory, raft, patch tests and full vessel trials. Raft, patch tests and full vessel trials are generally conducted over extended periods of time and are predominantly relied upon for the prediction of coating performance when used commercially on in-service ships.

The results of raft, patch test and full vessel trials (field testing) can be used as part of the regulatory process for pesticidal or biocidal products in certain countries in order to demonstrate the efficacy of an anti-fouling paint. Laboratory testing alone is recognized as being unable to predict in-service performance or efficacy. For example, guidance published by the European Chemicals Agency (ECHA) on the assessment and evaluation of efficacy for anti-fouling products states clearly that laboratory testing of individual anti-fouling paints is not undertaken as it is not considered to be a realistic evaluation of the product; field testing, which permits anti-fouling products to be tested under similar operating conditions and stresses as those encountered when the anti-fouling products are in service is routinely undertaken instead (see Reference [28]).

Whilst laboratory tests are unable to reliably predict in-service coating performance, they have merit in the screening of experimental coatings for further evaluation during the research and development process.

Reproducible objective data obtained by following standardized screening methods, independent of the test location or the season, can be a useful tool to support the selection of anti-fouling paints for higher tier testing, e.g. raft or ship tests. ISO 21716 provides a compilation and description of *in vitro* bioassay methods intended to aid the process of screening anti-fouling paints prior to higher tier raft or ship tests. Toxicological screening methods included in each part of ISO 21716 can be used for such purposes as early decision-making in research and product development, rapid feedback on potential toxicological concerns, or for the preliminary assessment of anti-fouling paints. For instance, ISO 21716 provides information on methods that can be used to screen anti-fouling paints in order to determine whether to continue development of an experimental paint and/or a product that contains a particular ingredient, or to determine whether to take on the cost of performing the remaining tiers within a complete tiered-testing strategy.

ISO 21716 provides screening bioassays related to certain common genera of fouling organisms, namely barnacles, mussels and algae. These screening tests are relatively simple and rapid laboratory tests that can be performed to provide an indication of the toxicity of a painted surface towards selected test organisms. The screening tests described in each part of ISO 21716 can be used as part of a tiered approach to predict the ability of an anti-fouling paint to prevent fouling on ships. Alternatively, to prevent the translocation of invasive marine species by progressively involving subsequent semi-field (e.g. raft panels) and field testing (e.g. ship trials). On their own, the screening tests described in each part of ISO 21716 do not reliably predict the ability of an anti-fouling paint to prevent fouling on ships or the translocation of invasive marine species.

ISO 21716 is not intended to provide a list of validated tests for testing the efficacy of anti-fouling paints; this can be covered in regulations. It is not intended to provide a list of validated tests for this purpose, nor for predicting the ability of a fouling control paint to prevent fouling on ships or to prevent the translocation of invasive marine species.

Barnacles are typical marine sessile organisms regarded as harmful fouling organisms because of their impact on fuel consumption and the potential for translocation of non-indigenous species if they become attached to ship hulls.

This test method utilizes cyprid juveniles to assess settling behaviour in the presence of treated panels. Cyprid larvae are considered the most relevant life stage for such evaluations as it is at this point that the barnacle settles on appropriate substrate prior to metamorphosis into the adult. More information is provided in Annexes B and C.

Ships and marine technology — Bioassay methods for screening anti-fouling paints —

Part 2: Barnacles

1 Scope

This document specifies a laboratory test method for screening anti-fouling paints in a flow-through system using barnacle cyprid larvae as the test organism. It is intended to be used in conjunction with ISO 21716-1, which specifies the general requirements. The purpose of the test is to determine if there is a difference in barnacle settlement on painted test panels compared with barnacle settlement on inert non-toxic control panels under the conditions of the test. Examples of statistical analysis to determine if the difference in barnacle settlement is statistically significant 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 21716-1:2020, *Ships and marine technology — Bioassay methods for screening anti-fouling paints — Part 1: General requirements*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 21716-1 and the following apply.

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/

3.1
culturing
growing hatched nauplius larva to cyprid stage under controlled conditions prior to the test

Note 1 to entry: Refer to Figure B.2.

3.2
rearing
growing adult barnacle to enhance larval hatching under controlled conditions prior to the *culturing* (3.1) stage

3.3
settlement
stage of the sessile phase involving *juvenile* (3.4) barnacles and cyprids metamorphosing into juveniles on the substrates

Note 1 to entry: Refer to Figure B.2.

3.4
juvenile
individual of barnacle after the metamorphosis and molting of cyprid during the test

Note 1 to entry: Refer to Annex B.

3.5
purified water
water with an electric conductivity of 2 µS/cm or less prepared by distillation and/or treatment with ion exchange resin(s)

4 Principle

The test procedure consists of the following 5 sequential steps, summarized in Figure 1:

— preparation of the test organism and the test seawater;

— preparation of the triangular prisms;

— operation of the test (cyprid viability test and bioassay);

— validation of the test; and

— data treatment and interpretation of the results.

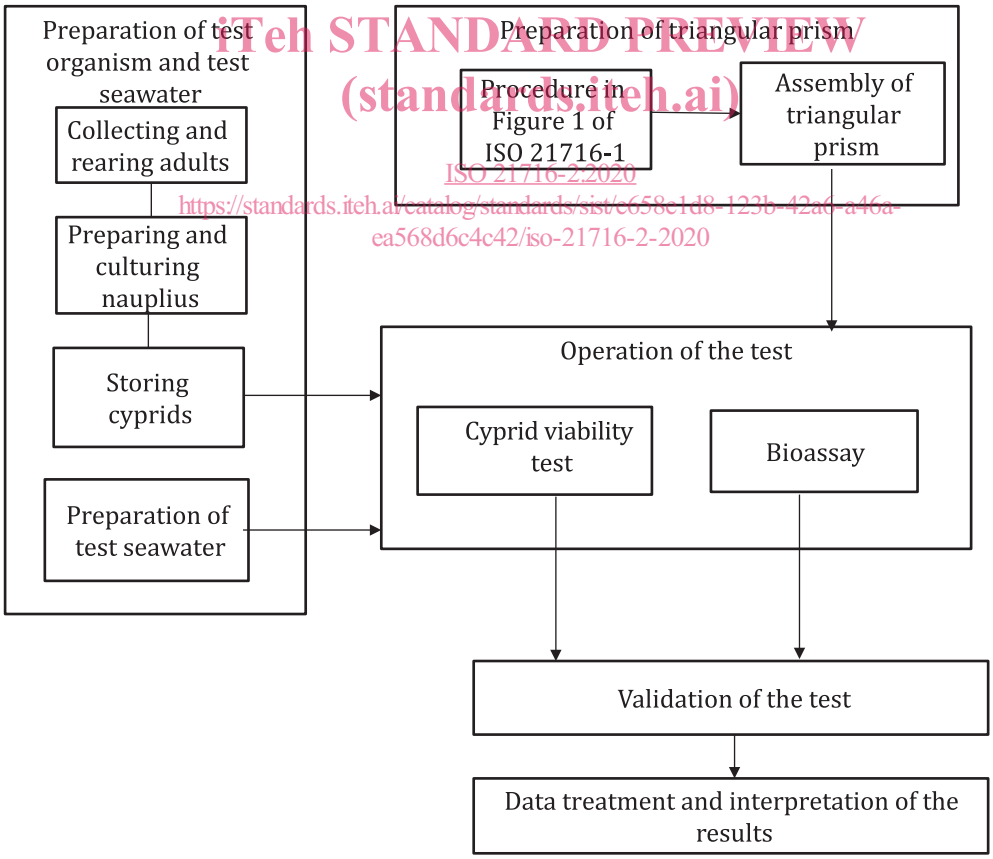


Figure 1 — Schema of the test procedure

Each bioassay shall consist of three runs as a minimum. Each run shall consist of a test group of three or more test panels, and a control group of three or more control panels. Provided that the cyprid viability and settlement on the control groups are both shown to be acceptable, then the barnacle settlement rates of the test and control groups can be compared.

5 Material and apparatus

The items listed in Tables 1 and 2 shall be used for the test. For recommended items, refer to Annex D.

Table 1 — List of material used

Material	Remarks
Adhesive tape	Used to assemble a prism. Approx. 50 mm long without any harmful effect on cyprids, e.g., double-sided carbon tape is recommended.
Abrasive media	F20 macrogrit or F20 macrogrit bonded abrasive[1]
Cultured stock of live barnacle cyprids	*Amphibalanus amphitrite* should be used with a larval density of 2-3 nauplius larvae per ml of seawater. Other barnacle species may be used if *Amphibalanus amphitrite* cyprids are not available.
Natural seawater	Defined in ISO 21716-1:2020, 3.8
Pipettes	10 ml capacity, glass or disposable [see 8.2 (i)], used for filling the microtiter plates.
Plankton net	Approx. 11 cm^2 mesh size (NXX13), 100 µm
Plastic legs	2 mm × 2 mm × 30 mm, used to support prism
Polishing agent	Used for surface treatment of control panels, sandpaper or other bonded materials with F-20 macrogrit.
Purified water	Defined in 3.5
PVC plates	Used as substrates for control panels. Black panels with same size as test/control panels are recommended.
Test panels	Specified in ISO 21716-1:2020, 4.2. 50 mm square is recommended.
White panel	White acrylic plates with same size as test/control panels should be used as they are considered as the material on which cyprids hardly settle, resulting in increased settlement on the test surface. White plates used to assemble a prism with control or test panels.
1 µm filters	Used to prepare test seawater.

Table 2 — List of apparatus used

Apparatus	Remarks
Incubator	Thermostatic chamber with a means of maintaining the ambient temperature at 25 °C
Light	White fluorescence or LED
Light intensity meter	Accuracy: ±10 lx
6-well (or 12-well) microtiter plates with lids	Made of polystyrene (may be replaced to petri dish)
pH meter	Accuracy: ±0,1
Salinometer	Accuracy: ±0,1
Stereo microscope	Magnification: 5-30x with fiber light
Thermometer	Accuracy: ±0,1 °C
Water flow-through system	As specified in ISO 21716-1:2020, 5.2, with a means of maintaining the test seawater tank at 25 °C ± 1 °C and alternately illuminating the test seawater tank with a light intensity of 3 000 lx (see 8.2 f), light conditions) and with a light intensity of <50 lx (see 8.2 f) dark conditions).

6 Preparation of the test organism and the test seawater

6.1 General

The cultured stock of live barnacle cyprids is used to perform the bioassay test in seawater.

6.2 Preparation of the test organism

Live barnacle cyprids are generally prepared by collecting and rearing an adult barnacle followed by preparing and culturing nauplius larvae of the barnacle. Guidance on this process and on storing cyprids can be found in Annex B. Information on the life cycle of barnacles can be found in Annex C, and information on the identification of adult *Amphibalanus amphitrite* barnacles can be found in Annex E.

6.3 Preparation of the test seawater

Pass natural seawater through a 1 µm filter unit and adjust to salinity 28,0 ± 0,5 using purified water.

7 Preparation of the triangular prisms

7.1 General

Each test panel and each control panel shall be used with two white panels to construct a series of triangular prisms for use in the bioassay (see Figures 2 and 3). The same test and control groups shall be used throughout the whole test.

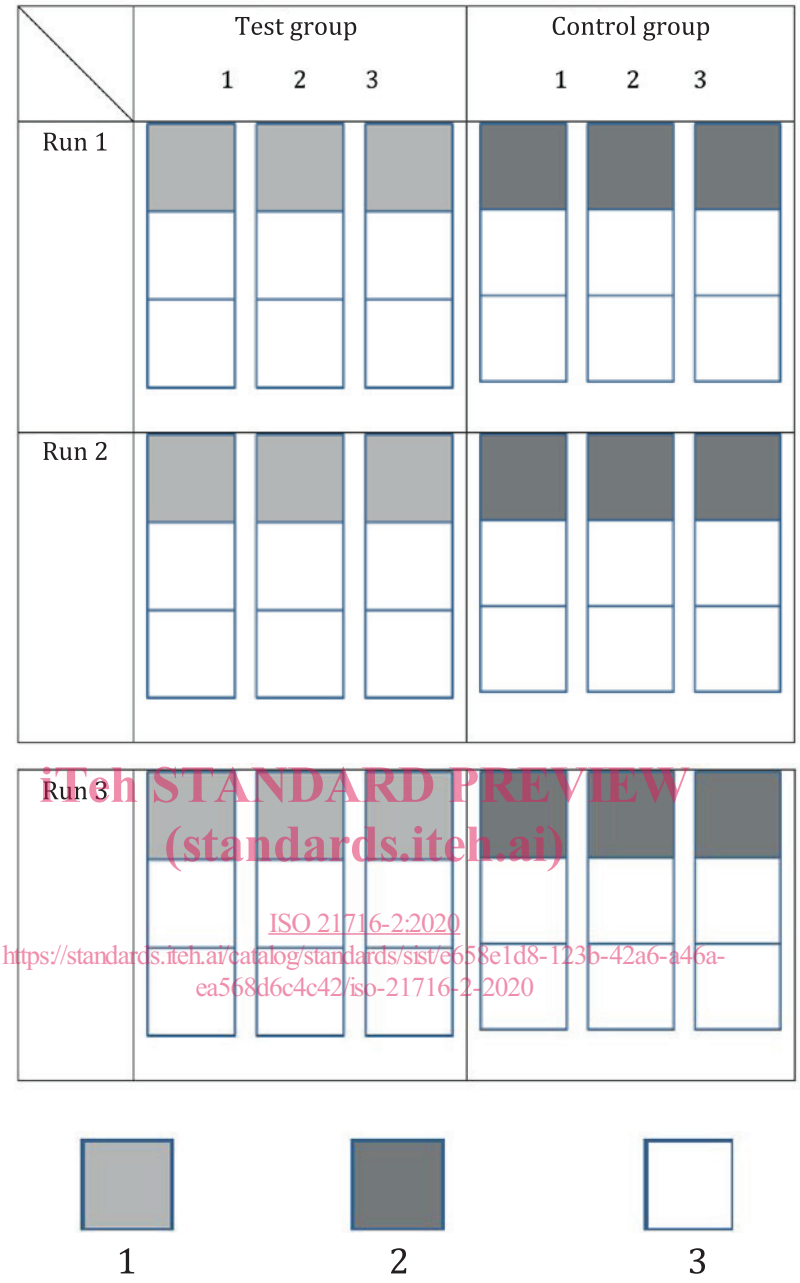
7.2 Preparation of the test panels and control panels

Test panels and control panels shall be prepared following the specifications of ISO 21716-1:2020, Clause 4.

Abrade the surface of the control panels prior to use in the test by gently blasting with F20 macrogrit or by abrading with F20 macrogrit bonded abrasive (Reference [1]).

7.3 Assembly of the triangular prisms

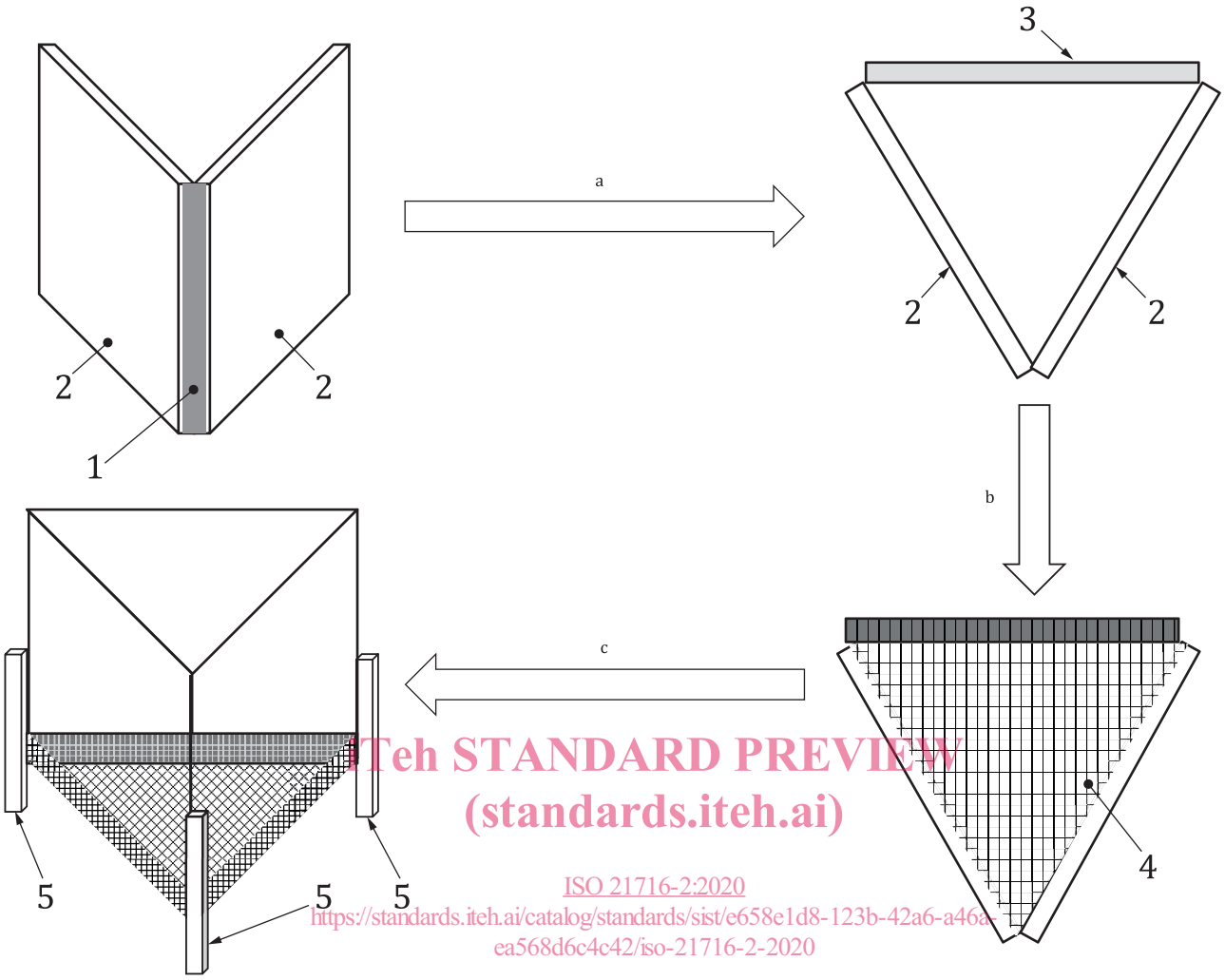
Construct the required number of prisms for the required number of replicates for each run. Test group prisms shall use one test panel and two white panels. Control group prisms shall use one control panel and two white panels. Panels shall be of the same size (see Figure 2). Prisms shall be constructed with test and control surfaces facing inwards. The bottom of the triangular prism is covered with plankton net. The panels, the plankton net and a plastic leg at each bottom corner shall be assembled using adhesive tape. Ensure that all components are tightly fixed together without any gaps between them. The triangular prism should be supported by the three legs 10 mm or more from the bottom surface of the test tank to ensure sufficient flow of test seawater through the prism. The surface of test panels shall be kept wet with test seawater during the assembly of the prisms and up until immersion in the test tank. The triangular prisms are assembled according to the process described in Figure 3.



Key

1 test panel
2 control panel
3 white panel

Figure 2 — Formation of the triangular prism for the test and control group



Key

1 adhesive tape
2 white panel
3 test/control panel
4 plankton net
5 plastic leg
a Assemble one test/control panel and two white panels to a triangular prism using adhesive tape.
b Attach plankton net to the bottom side of the triangular prism using adhesive tape.
c Attach plastic leg s to the triangular prisms; length of the legs from the bottom side of the prism ≥10 mm.

Figure 3 — Assembly of triangular prism for the test

8 Operation of the test

8.1 Cyprid viability test

The cyprid viability test is conducted in order to verify the health of cultured cyprids in the bioassay, and should be performed in parallel with the bioassay. The test shall be performed according to the following procedure.

a) Place cyprids collected from the cultured stock into a well of a microtiter plate filled with the test seawater.

