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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.

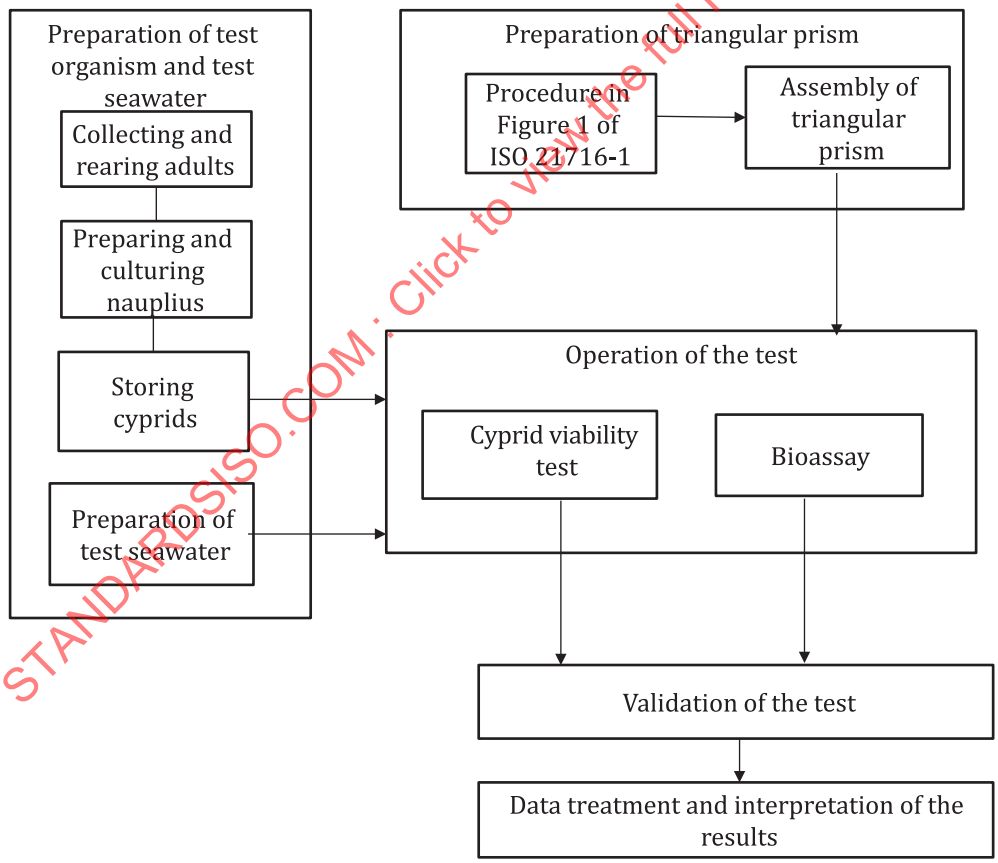


Figure 1 — Schema of the test procedure

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.

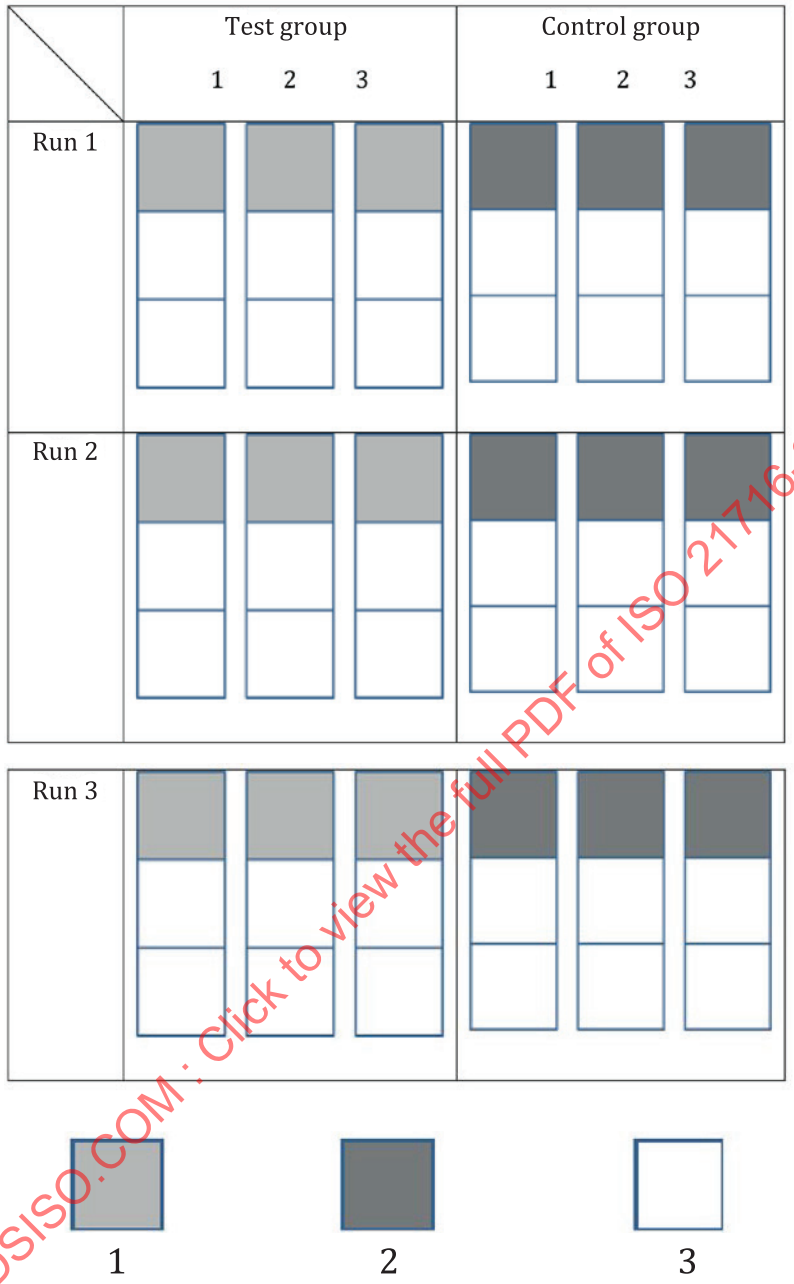
7.2 Preparation of the test panels and control panels

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

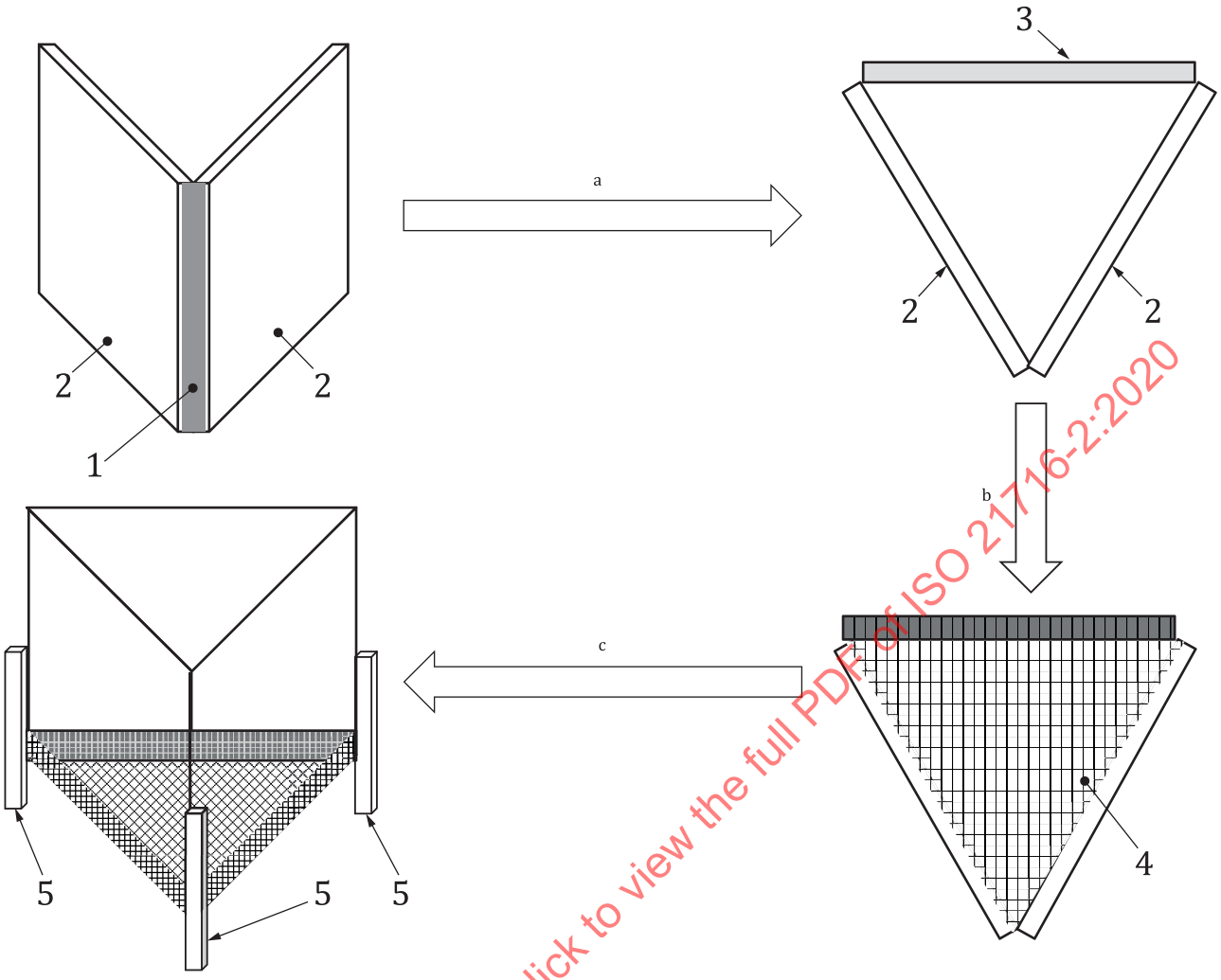
Construct the required number of prisms for the required number of replicates for each run. Test group prisms shall use one test panel and two white panels. Control group prisms shall use one control panel and two white panels. Panels shall be of the same size (see Figure 2). Prisms shall be constructed 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 legs 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.

b) Fill 5 wells of at least three 6-well (or 12-well) microtiter plates with at least 10 cyprids at a maximum density of 3 individuals/ml of test seawater. Lids should be used to prevent evaporative loss of test seawater throughout the test period.

c) Record the number of cyprids in each well prior to starting the test.

d) Conduct the test according to 8.2 f) for 48 h. Maintain water temperature at 25 °C ± 1 °C throughout the test period.

e) After completion of d) above, count the number of juvenile, cyprids and dead individuals using a stereo microscope and record using e.g. Table 3.

8.2 Bioassay

The bioassay shall be simultaneously performed on the test group and on the control group, using the triangular prisms as follows (see Figures 4 and 5).

The experimental system specified in ISO 21716-1 shall be used for the test. The system is equipped with the devices that maintain the specified water temperature and light irradiation of the test.

a) Wash each triangular prism thoroughly prior to the test with fresh running test seawater.

b) Fill the test seawater tank with the test seawater and provide a continuous flow of the test seawater from the seawater storage tank. Maintain the temperature of the test seawater tank within the range 25 °C ± 1 °C for the duration of the test. The flow rate should be set to achieve about 1 turnover per hour of the water of test seawater tank.

NOTE If the flow rate is too high, the test seawater can overflow from the prisms and dislodge cyprids from the test or control surface. If the flow rate is too low, the result can be affected by the concentration of biocide in seawater of the test seawater tank.

c) Place the prism in the test seawater tank and adjust water level to 1,0 cm ± 0,2 cm below the top of the prism to ensure the flow rate of the seawater from the bottom of the prisms as shown in Figure 5.

d) Place cyprids at the density of 2 to 3 individuals/ml inside of the prism ensuring the water does not overflow from the top of the prism. In case of using 5,0 cm square panels, 60 to 100 cyprids should be placed.

e) Measure and record the temperature, pH and salinity of the test seawater in the test seawater tanks at the initial stage of the test. Measure and record these parameters again after 24 h and 48 h from the beginning of the test.

f) Illuminate the test seawater tank with a light intensity of 3 000 lx for the initial 12 h, maintain a dark condition for 12 h, and then alternate subsequent 12 h light and dark periods for 48 h in total.

g) After 48 h from the beginning of the test, collect the remaining cyprids and dead individuals inside the prism around the test seawater surface irradiated with light, using pipettes.

NOTE Cyprids exhibit phototactic behaviour, this can be exploited to facilitate collecting nauplius larvae.

h) Carefully remove the prism from the tank and dismantle immediately after the completion of step g) above to separate the white panels and test or control panels, noting which surfaces had formed the inner surface of the prism.

i) Using a pipette, rinse the inner surface of each panel three times with 10 ml of the test seawater to remove and collect unattached cyprids.

NOTE Unsettled individuals are easily detached by rinsing from the test surface.

j) Using a stereo microscope, count the number of live and dead cyprids and juveniles as shown in Figure B.1, both on the test surface and on the other surfaces (white panels, edges of the test

plates and plankton net), the number of live and dead cyprids and juveniles collected from the prism [see (g)] and rinsing from the inner surface [see (i)], and record the results using e.g. Table 4. Metamorphosing cyprids should be counted as cyprids and not as juveniles.

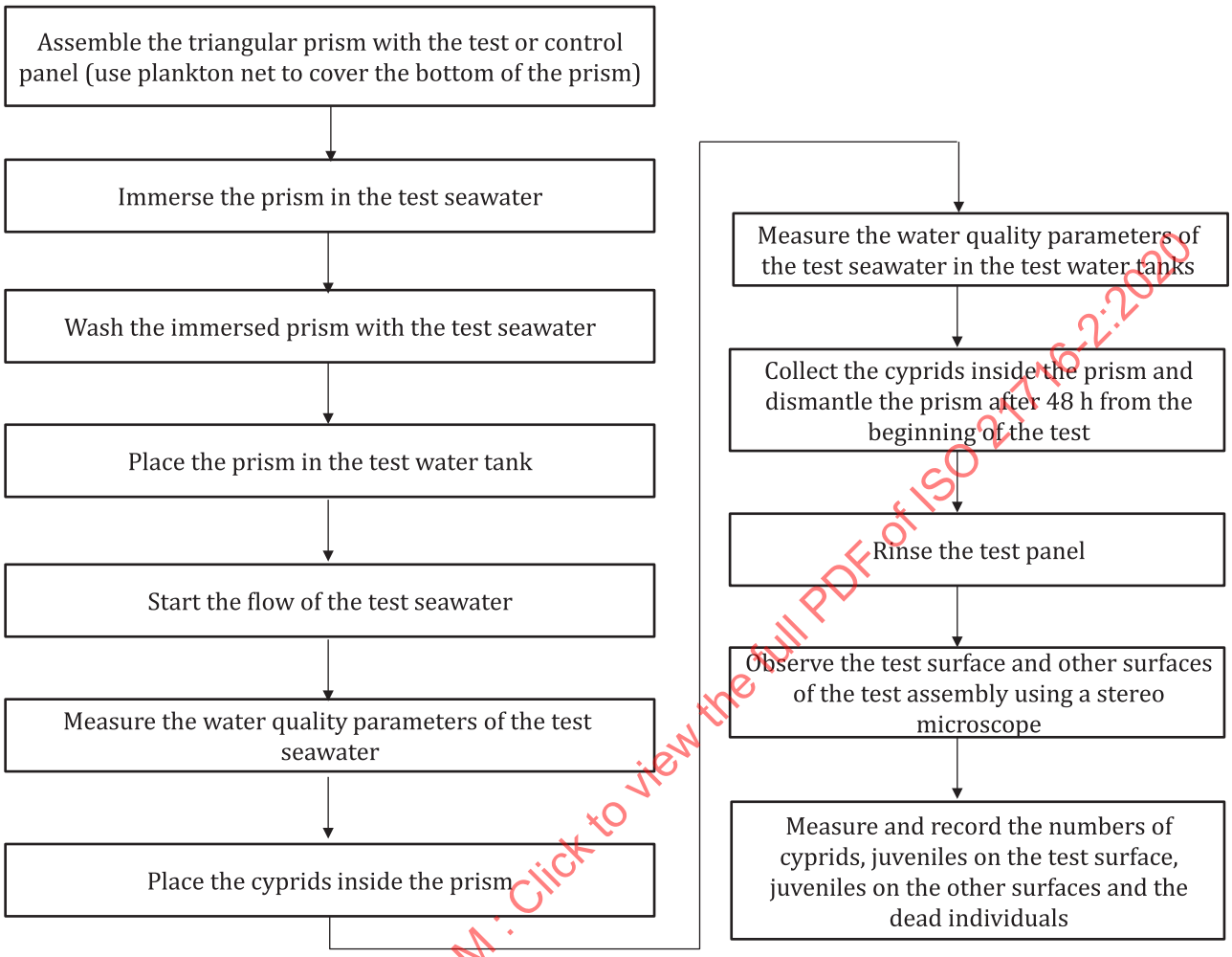
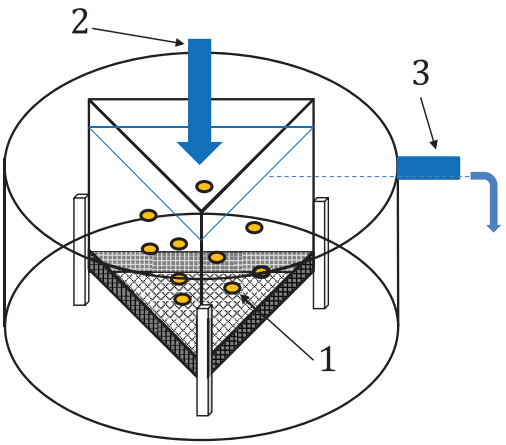
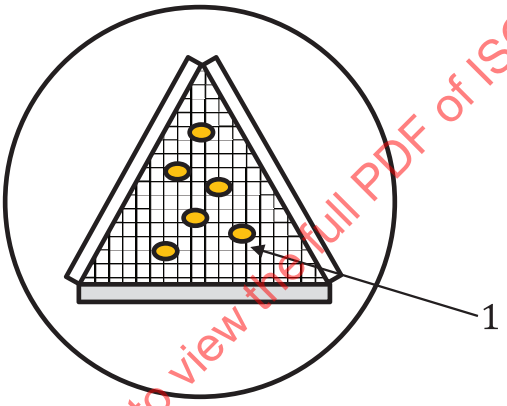


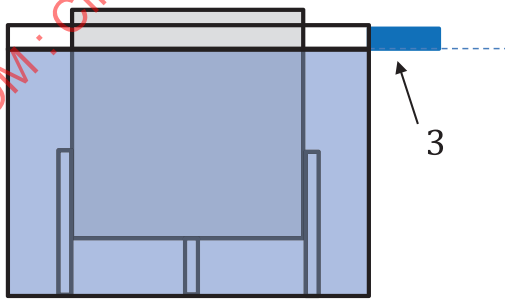
Figure 4 — Flow chart of the procedure for the test



a) Triangular prism



b) Top view



c) Side view

Key

1 cyprids of the barnacle
2 charging test water
3 discharging test water

Figure 5 — Setting of the triangular prism in the test seawater tank

9 Validation of the test

9.1 General

The results of the bioassay are validated using arithmetic tests to confirm that the viability of the cyprids used in the bioassay meet a minimum threshold value (see 9.2), and to confirm that the settlement rate for the control group also meets a minimum threshold value (see 9.3).

The results of the bioassay shall only be considered valid if both criteria are met.

9.2 Requirements of the cyprids viability test

The degree of settlement is calculated from the results of the cyprid viability test (see 8.1). The degree of settlement for each well of the microtiter plate for each run is calculated using Formula (1). The results are recorded using e.g. Table 3 to one decimal place.

$$R_v = \frac{a}{a+b+c} \times 100 \quad (1)$$

where

R_v is the degree of settlement for verifying the test (%);

a is the number of juveniles on the surface of the wells of microtiter plates;

b is the number of unsettled and live cyprids;

c is the number of dead individuals.

The average number of settlement rate shall be calculated from the values of R_v for each run. The viability of the cyprids shall be considered to be acceptable if the overall average value of R_v for all runs is 70 % or higher. If not, the test shall be rejected.

Table 3 — Example of compilation of the data for verifying the test

	No.	Number of juveniles a	Number of unsettled and live cyprids b	Number of dead individuals c	$a + b + c$	Settlement rate %	Average %
Run 1	1						
	2						
	3						
	4						
	5						
Run 2	1						
	2						
	3						
	4						
	5						

Table 3 *(continued)*

	No.	Number of juveniles *a*	Number of unsettled and live cyprids *b*	Number of dead individuals *c*	*a* + *b* + *c*	Settlement rate %	Average %
Run 3	1						
	2						
	3						
	4						
	5						

9.3 Requirements of the bioassay

The settlement rate for the control panel is calculated from the results of the bioassay test (see 8.2). Calculate the settlement rate for each control panel from each triangular prism in each run using Formula (2). Record the results using e.g. Table 4 to one decimal place.

$$R_c = \frac{S_c}{S_c + a + b + c} \times 100 \tag{2}$$

where

R_c is the settlement rate for the control panel (%);

S_c is the number of juveniles on the test surface of a triangular prism;

a is the number of juveniles on the other surfaces of a triangular prism;

b is the number of unsettled and live cyprids in a triangular prism;

c is the number of dead individuals in a triangular prism.

The average value of the settlement rate in the control panels from each triangular prism in each run is calculated using Formula (3), and the results are recorded in e.g. Table 4 to one decimal place.

$$A_c = \frac{\sum_{j=1}^{j} (R_{c1}^j + R_{c2}^j + \cdots R_{cn}^j)}{\sum_{j=1}^{j} n^j} \tag{3}$$

where

A_c is the average value of the settlement rate in the control groups (%);

j is the run number;

R_{cn}^j is the settlement rate of the n-th control panel on the j-th run (%);

n^j is the number of the control panels on the j-th run.

The settlement on the control panels shall be considered to be acceptable if A_c is 40 % or higher.

10 Settlement rates

10.1 General

The evaluation procedure for screening anti-fouling paint is given in 10.2 and 10.3. The final results are obtained by comparing the settlement rate between the test and control groups. The test data should be recorded using e.g. Table 4.

10.2 Calculation of the settlement rate for the test panel

Calculate the settlement rate for the test panel from each triangular prism in each run using Formula (4) and record the results using e.g. Table 4 to one decimal place.

$$R_t = \frac{S_t}{S_t + a + b + c} \times 100 \tag{4}$$

where

R_t is the settlement rate for the test panel (%);

S_t is the number of juveniles on the test surface of a triangular prism;

a is the number of juveniles on the other surfaces of a triangular prism;

b is the number of unsettled and live cyprids in a triangular prism;

c is the number of dead individuals in a triangular prism.

Calculate the average settlement rate for the test and control panels from each triangular prism in each run using Formula (5) and record the results using e.g. Table 4 to one decimal place.

$$A_t = \frac{\sum_{j=1}^{j} (R_{t1}^j + R_{t2}^j + \cdots R_{tn}^j)}{\sum_{j=1}^{j} n^j} \tag{5}$$

where

A_t is the average value of the settlement rate in the test groups (%);

j is the run number;

R_{tn}^j is the settlement rate of the n-th test panel on the j-th run (%);

n^j is the number of the test panels on the j-th run.

10.3 Data treatment and interpretation of the results

If A_t is less than A_c, this can indicate that the barnacle settlement on the test group is less than that of the control group. However, further analysis of the results is required in order to determine if the difference of the result between test and control groups is statistically significant. There are many possible ways to perform the statistical analysis. Typical examples are shown in Annex A.

Table 4 — Example of compilation of the data

	Sample name	Number of juveniles on the test surface S_t	Number of juveniles on the other surfaces a	Number of unsettled and live cyprids b	Number of dead individuals c	$S_t+a+b+c$	Settlement rate %
Run 1	Control panel 1	53	3	42	0	98	54,08
	Control panel 2	17	2	72	0	91	18,68
	Control panel 3	69	7	7	0	83	83,13
	Test panel 1	14	17	45	7	83	16,87
	Test panel 2	8	17	36	2	63	12,70
	Test panel 3	12	35	20	2	69	17,39
Run 2	Control panel 1	22	6	34	1	63	34,92
	Control panel 2	42	15	24	1	82	51,22
	Control panel 3	48	24	15	0	87	55,17
	Test panel 1	6	1	80	4	91	6,59
	Test panel 2	16	13	58	0	87	18,39
	Test panel 3	16	1	83	1	101	15,84
Run 3	Control panel 1	36	7	31	1	75	48,00
	Control panel 2	55	17	9	0	81	67,90
	Control panel 3	46	6	22	0	74	62,16
	Test panel 1	4	24	65	4	97	4,12
	Test panel 2	14	24	52	0	90	15,56
	Test panel 3	4	8	73	4	89	4,49
Results: A_c : 52,8 A_t : 12,4							

11 Test report

Table 5 specifies the minimum required information for the test report. The test results shall be reported using Table 5.

Table 5 — Minimum required information for the test report

Information		Requirement
Materials and dimensions of substrate to be painted		×
General specifications and process for paint	Biocides contained	×
	Name of paint	×
	Undercoat	(×)
	Surface treatment	(×)
	Dry film thickness[a]	×
Methods and time (number of days) for aging test panels[a]		×
General information on the test organisms[a]	Identification (species name and identifier)	×
	Date of sampling/collection	(×)
	Place of sampling/collection	(×)
	Rearing condition of adults (water temperature, diet and frequency of filtration/water change)	×
	Culturing condition of nauplius larvae (water temperature, light condition, aeration condition)	×
	Storage condition of cyprids (temperature and storage period in refrigerator)	×
	Results of verification of the activity and physiological condition of cyprids	×
Initial test conditions[a]	Starting date	×
	Number of test and control panels	×
	Size of the test seawater tank	×
	Water quality parameters (temperature, pH, salinity), light condition, rate of water exchange	×
	Other information on the test procedure and experimental system	(×)
Test conditions after 24 h[a]	Water quality parameters (temperature, pH, salinity), rate of water exchange	×
	Accidental/unexpected items observed during the test	(×)
Test conditions after 48 h[a]	Water quality parameters (temperature, pH, salinity), rate of water exchange	×
	Number of settled juveniles, cyprids without settlement and dead individuals	×
	Accidental/unexpected items observed during the test	(×)
Settlement rates for the test and control groups		×
Statistical analysis method used		(×)
Is there a statistically significant difference between the bioassay results for the test and control groups, Yes/No?		(×)

x: required; (x): optional.

[a] Information required for each run.

Annex A
(informative)

Statistical analysis — Examples

A.1 Introduction

It is stated in the Scope that 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. It can be useful to perform statistical analysis to determine if the difference in barnacle settlement is statistically significant. A typical example of statistical analysis is given in this Annex, based on Reference [2]. Selection of an optimal statistical analysis method from different approaches should be made depending on the purpose and the data distributions. This Annex describes some statistical analysis methods as examples.

A.2 Statistical analysis to compare a test group and a control group

A.2.1 Introduction

This clause indicates some statistical analysis methods to calculate the difference between a control group and a test group, as examples. Figure A.1 shows an example for this process. Statistical analysis of significance difference between the control and test groups is conducted using a one-way ANOVA (analysis of variance) followed by a multiple comparison test. In this case, Dunnett's multiple comparison test is recommended for comparing several treatments with the control groups (Reference [3]). Probability values (p-value) less than 0,05 are considered significant.

A.2.2 One-way analysis of variance[4]–[8]

One-way analysis of variance (ANOVA) is a collection of statistical models and their associated estimation procedures (such as the "variation" among and between groups) used to analyse the differences among group means in a sample. The ANOVA is based on the law of total variance, where the observed variance in a particular variable is partitioned into components attributable to different sources of variation. In its simplest form, ANOVA provides a statistical test of whether two or more population means are equal, and therefore generalizes the t-test beyond two means.

The one-way ANOVA test is a way to find out if survey or experiment results are significant. In other words, they help to figure out the need to reject the null hypothesis or to accept the alternate hypothesis. Basically, testers analyse groups to see if there is a difference between them. One-way refers to the number of independent variables in the analysis of variance test. One-way has one independent variable (with 2 levels).

The ANOVA test tells whether testers have an overall difference between testers' groups, but it does not tell testers which specific groups differed — post hoc tests do. Because post hoc tests are run to confirm where the differences occurred between groups, they should only be run when testers have shown an overall statistically significant difference in group means (i.e., a statistically significant one-way ANOVA result). Post hoc tests attempt to control the experiment-wise error rate (usually $\alpha = 0,05$) in the same manner that the one-way ANOVA is used instead of multiple t-tests. Post hoc tests are termed a posteriori tests, that is, performed after the event (the event in this case being a study).

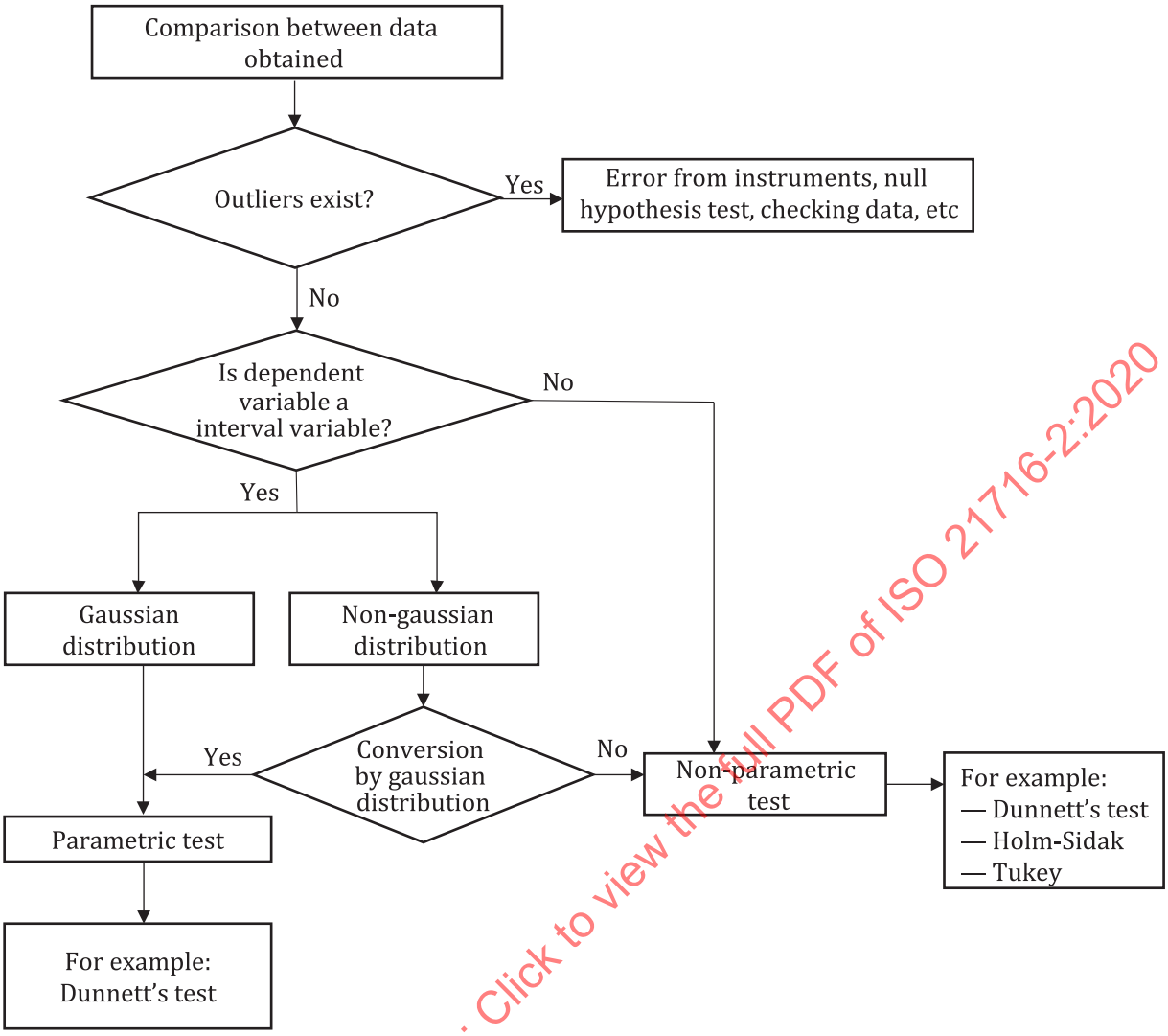


Figure A.1 — Example of a statistical analysis process

A.2.3 Indication of the result of one-way ANOVA

Statistical analysis, including one-way ANOVA, non-parametric tests in the settlement assay and variance analysis are performed. Pairwise comparison between control and test groups is performed to establish significance of the difference, where the symbols *, ** and *** correspond to $p < 0,05$ (significant), $p < 0,01$ (very significant) and $p < 0,001$ (extremely significant), respectively.

A.3 Example of statistical analysis

A.3.1 Introduction

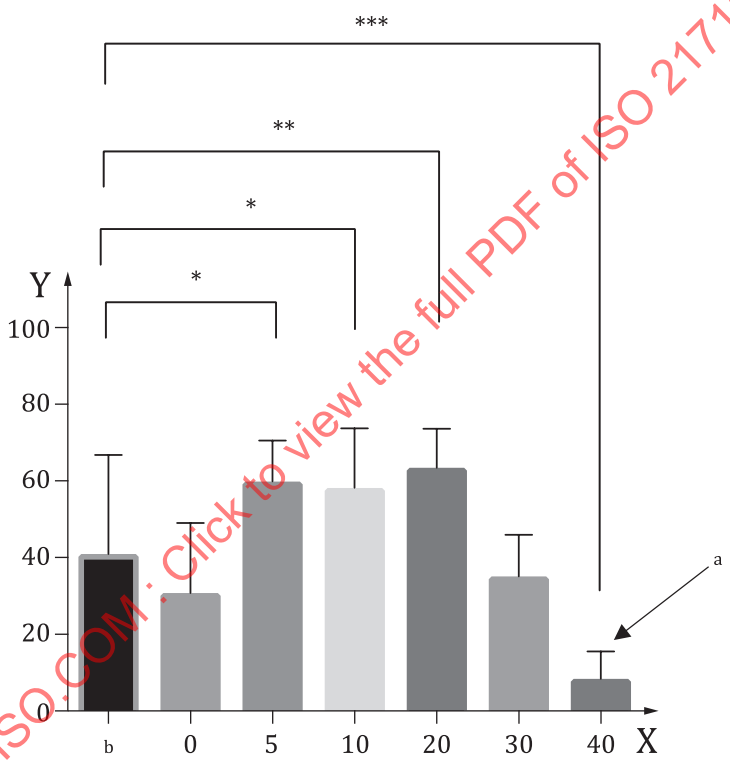
Six panels coated with anti-fouling paints containing 0, 5 %, 10 %, 20 %, 30 %, and 40 % mass fraction of Cu_2O were prepared and compared with vinyl copolymer coated panels. The test panels applied with the six anti-fouling paints were aged for 45 days by rotating cylinder apparatus holding the test panels at a speed of 10 kn (knots) with continuously flowing seawater.

The settlement behaviour of 3-day-old cyprids released inside triangular prisms made of the test panels was observed. Statistical analyses, including one-way analysis of variance (ANOVA), non-parametric tests in the settlement assay and variance analysis were performed. Statistical differences of settlement rates between the control and test groups were calculated. The pairwise comparison of the test results

for test and control groups was performed using Holm-Sidak's multiple comparison test ($p < 0,05$) in the settlement assay (Reference [2]).

A.3.2 Calculation results

Figure A.2 shows the settlement rates for the control and test groups. Error bars indicate standard deviations (SDs). The settlement rate for the control group (A_c) was 40 %, and settlement rates for the test groups (A_t) with a 5 %, 10 %, and 20 % mass fraction of Cu_2O were higher than that of the control group. The settlement rates for test groups with a 5 % and 10 % mass fraction of Cu_2O were significantly different from A_c ($p < 0,05$). For the group with a 20 % mass fraction of Cu_2O, the difference between A_t and A_c was very significant ($p < 0,01$), but there was no significant difference between A_c and A_t for the group with a 30 % mass fraction of Cu_2O. At a 20 % mass fraction of Cu_2O or below, settlement is promoted, whereas an inhibition of settlement was clearly observed at a 40 % mass fraction of Cu_2O, where the difference in settlement rate as compared to the control group was extremely significant ($p < 0,001$) (Reference [2]).



Key

X mass fraction of Cu_2O [%]

Y settlement ratio [%]

a Error bars indicate SDs.

b Control.

NOTE Pairwise comparison between control and test groups was performed to establish significance of the difference, where *, ** and *** corresponds to $p < 0,05$ (significant), $p < 0,01$ (very significant) and $p < 0,001$ (extremely significant), respectively (Reference [2]).

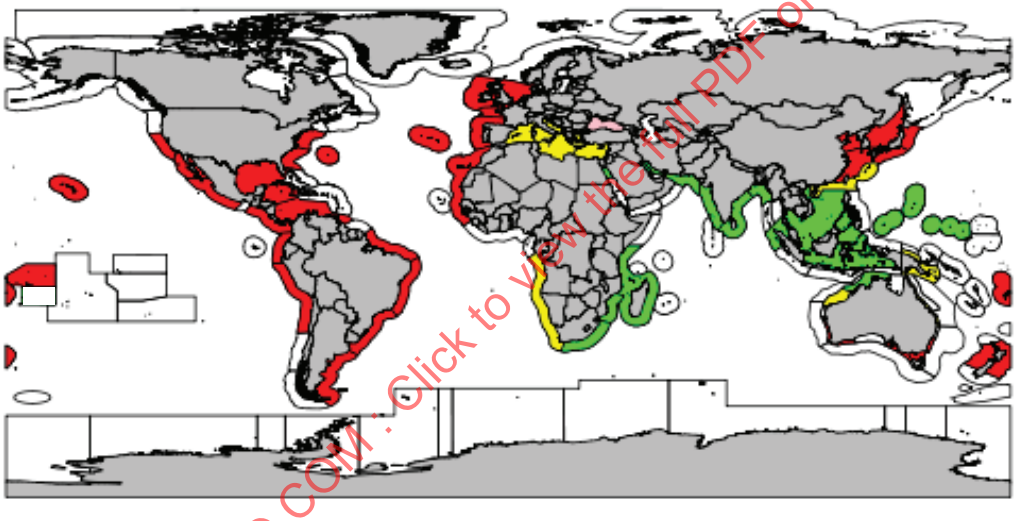
Figure A.2 — Settlement rates of *Amphibalanus amphitrite* in the control and test groups

Annex B
(informative)

The barnacle *Amphibalanus amphitrite*

B.1 Information of *Amphibalanus amphitrite*

Amphibalanus amphitrite is a cosmopolitan barnacle that naturally occurs in almost every tropical, subtropical and temperate sea (References [9], [10], [11], [12], [13]). It is one of the typical fouling species that can reach different places mainly by attaching themselves to ship hulls, and known as introduced species in many parts of the world. It is uncertain where *Amphibalanus amphitrite* originated but it can be native throughout the West Pacific and in the Indian Ocean (Reference [14]). Abundant fossil records in Guam may imply these regions are native to the species (Reference [15]). It has now spread to most of the tropical, subtropical and temperate ocean of the world (see Figure B.1).



Key

green	native region
red	introduced region
yellow	cryptogenic region
pink	failed region

SOURCE: NEMESIS databases (Reference [14]).

Figure B.1 — Distribution map of *Amphibalanus amphitrite*

B.2 Studies of *Amphibalanus amphitrite*

The establishment of the larval rearing method in recent years has made the cyprids of *Amphibalanus amphitrite* available for the laboratory use all year round. They are now used worldwide as model species in larval settlement studies of barnacles. Many laboratory studies using cyprids have been conducted to elucidate the settlement mechanism of barnacles and to seek new biocides. Most reported studies have been carried out under closed or isolated experimental conditions (References [9], [10], [11], [16], [17], [18]). As anti-fouling paints containing biocides are designed to leach the biocides into seawater, the biocide can accumulate in the seawater when such closed or isolated test conditions are

employed. It is therefore essential to use a flow-through system when performing bioassays on such anti-fouling paints to control the concentration of biocides in the seawater and avoid affecting the results.

B.3 Preparation of the test organism

B.3.1 Introduction

The cyprids should be prepared by collecting and rearing adult barnacles followed by obtaining nauplius larvae of the barnacles. The whole process of preparing test organisms consists of collecting and rearing, drying and immersing, hatching nauplius and culturing nauplius larvae, and storing cyprids as shown in Figure B.2.

B.3.2 Collecting and rearing adults

Adults attached on natural or artificial substrates in intertidal zones on the coast should be collected and be subsequently reared in the following manner:

— collected adults are transferred into a tank filled with filtered seawater at 25 °C ± 1 °C;

— *Artemia* larvae are fed to the adults;

— seawater is replaced to keep the water clean.

B.3.3 Preparing nauplius larvae

Nauplius larvae should be obtained in the following manner:

— the adults are taken out from the tank and kept dry inside an incubator at 25 °C ± 1 °C for at least 6 h;

— dried adults are submerged in the test seawater at 25 °C ± 1 °C to induce the hatching of nauplius larvae;

— the nauplius larvae gathering around the test seawater surface irradiated with light are collected with pipettes and placed in the cultured seawater.

NOTE 1 It is known that the drying process induces adults to hatch nauplius larvae.

NOTE 2 Nauplius larvae have a nature of positive phototaxis, which facilitates collecting the nauplius larvae.

B.3.4 Culturing nauplius larvae

Nauplius larvae should be subsequently cultured to induce metamorphosis into cyprids. In order to obtain cyprids, nauplius larvae should be cultured in containers, typically clear glass or plastic beakers of one or two litres, under the conditions summarized in Table B.1. It normally takes 5 days to metamorphose from nauplius larvae into cyprids.

Table B.1 — Culturing condition for nauplius larvae

Description	Condition
Quality of water	Culturing seawater should be prepared by refining the test seawater. The test seawater, as specified in 6.3, should be filtered again using 0,22 µm filters. Its salinity should be adjusted to 28 ± 0,5 using purified water, as defined in 3.5. Streptomycin of 30 µg/ml and penicillin G (sodium salt or free acid) of 20 µg/ml should be added. The following reagents are applied for culturing nauplius larvae. Reagents of analytical grade should be used unless otherwise specified:
NOTE These conditions are applied according to Reference [12].	

Table B.1 *(continued)*

Description	Condition
	— streptomycin: CAS number: 57-92-1; — penicillin G (free acid): CAS number: 61-33-6; — penicillin G (sodium salt): CAS number: 69-57-8.
Density of larvae	2 to 3 of nauplius larvae per 1 ml
Diet and density	The diatom *Chaetoceros gracilis* (200 000 cells/ml to 400 000 cells/ml), other diatoms can also be used, such as *Skeletonema costatum* (1 000 000 cells/ml to 2 000 000 cells/ml).
Water temperature	25 °C ± 1 °C
Light condition	A 12-hour light and 12-hour dark periods are given alternately, using a light with approximately 3 000 lx.
Aeration condition	Approximately 20 ml/min of air flow by a quantitative pump
NOTE These conditions are applied according to Reference [12].	

B.3.5 Storing cyprids

The cyprids for the test should be collected with pipettes. After washing the cyprids with culturing seawater, they are transferred into another container filled with culturing seawater and stored in a refrigerator at 5 °C ± 1 °C, where they should be left undisturbed for three days prior to the test. This ensures to reduce the activity of the cyprids to improve handling them in the following test procedures. It also improves the settlement of the cyprids during the test.

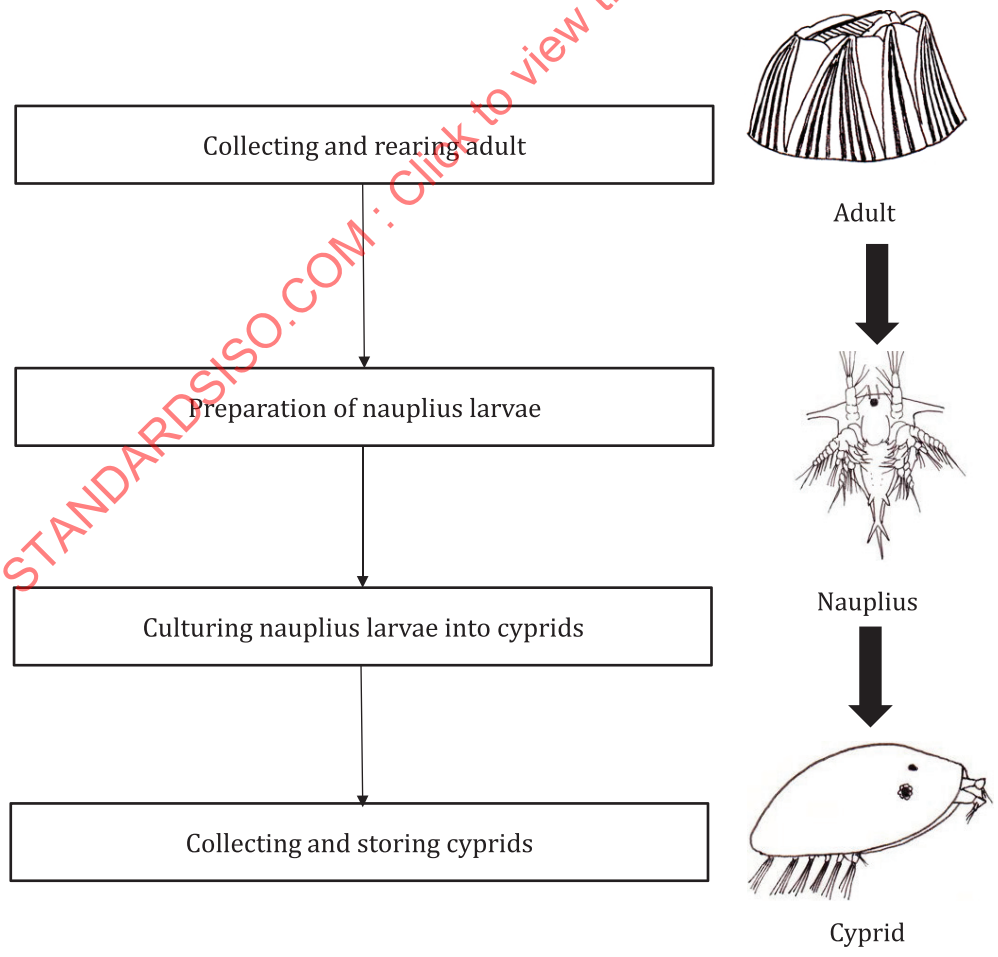


Figure B.2 — Flow chart of the preparation of test organism

Annex C
(informative)

Life cycle of a barnacle

C.1 Introduction

The life cycle of a barnacle is composed of two distinct phases: planktonic phase and sessile phase. The planktonic phase includes six feeding nauplius stages and one non-feeding cyprid stage; the sessile phase includes juvenile and adult stages. Embryos brooded within the mantle cavity of the adult are liberated when mature as the first stage of nauplius. Development then proceeds through five planktotrophic nauplius stages before metamorphosis to the lecithotrophic, settlement stage of cyprid. The cyprid is highly specialized for its role of finding a suitable place to attach prior to metamorphosis to the juvenile. The attached juvenile grows into the adult stage. The life cycle is shown in Figure C.1.

C.2 Life cycle of *Amphibalanus Amphitrite*

C.2.1 Nauplius stage (nauplius I-VI)

The hatched nauplius grows and molts five times into nauplius VI. A pelagic, suspension-feeding nauplius has one nauplius eye. During nauplius VI, a pair of compound eyes develop.

C.2.2 Cyprid stage

A pelagic, non-feeding cyprid has one nauplius eye and a pair of compound eyes (cyprid eyes). The cyprid has a pair of antennules with attachment organs to explore potential surfaces to settle with.

C.2.3 Juvenile stage

An attached cyprid metamorphoses into a juvenile barnacle. The metamorphosed juvenile loses compound eyes at the time of molting, and does not feed until cirri (feathery legs) appear.

C.2.4 Adult stage

Many adult barnacles, such as *A. amphitrite*, have six hard calcareous plates to surround and protect their bodies. The adult barnacle firmly accretes to the substrate, and captures planktons using the six pairs of cirri.