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

**Part 3:
Mussels**

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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).

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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 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 for maintaining 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 antifouling products are in service is routinely undertaken instead (see Reference [35]).

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.

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

This test method utilizes young mussels to assess settling behaviour in the presence of treated panels. Young mussels are used because they have higher byssus threads production activity as compared to the adults. More information is provided in [Annex B](#).

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

Part 3: Mussels

1 Scope

This document specifies a laboratory test method for screening anti-fouling paints in a flow-through system using mussels 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 mussel settlement on painted test panels compared with mussel settlement on inert non-toxic control panels under the conditions of the test. Examples of statistical analysis to determine if the difference in mussel 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:

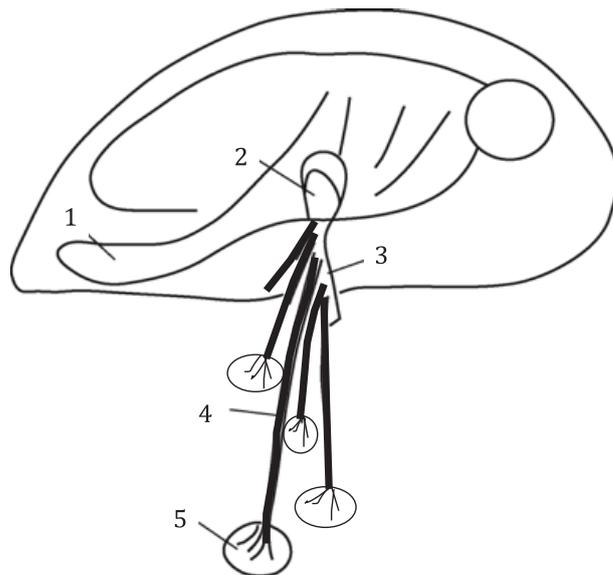
- ISO Online browsing platform: available at <https://www.iso.org/obp>;
- IEC Electropedia: available at <http://www.electropedia.org/>.

3.1

byssus

attachment organ secreted by a mussel, consisting of stem, byssus threads and adhesive discs

Note 1 to entry: See [Figure 1](#).



Key

- 1 foot
- 2 root (in byssus gland)
- 3 stem
- 4 byssus thread
- 5 adhesive disc (or plaque)

Figure 1 — Attachment organ of a mussel

3.2 shell length

longest linear distance between two points on the outside edge of the shell of a mussel

3.3 purified water

water with an electric conductivity of 2 $\mu\text{S}/\text{cm}$ 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 2](#):

- preparation of the test organism and the test seawater;
- preparation of the test panel and control panel;
- operation of the test;
- validation of the test; and
- data treatment and interpretation of the results.

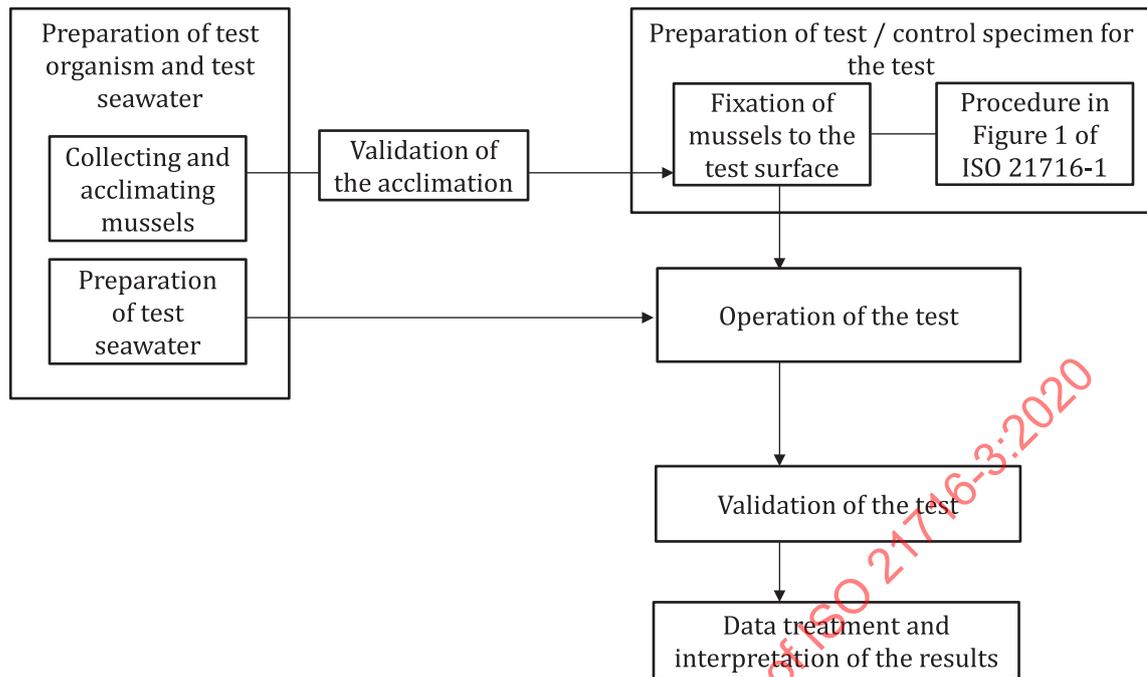


Figure 2 — Schema of the test procedure

Each bioassay shall consist of three runs at 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 validation criteria are met, then the number of byssus threads for 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.

Table 1 — List of material used

Material	Remarks
Adhesive of surgical grade	E.g., cyano-acrylate adhesive
Control panels	50 mm square of PVC is recommended.
Cultured stock of live young mussels	At least 30 live mussels, <i>Mytilus galloprovincialis</i> sp., with a shell length of 8 mm to 10 mm, that are attached to natural or artificial substrates. Other mussel species may be used if <i>Mytilus galloprovincialis</i> sp. mussels are not available.
Natural seawater	Defined in ISO 21716-1:2020, 3.8
Purified water	Defined in 3.3
Scissors	
Small piece of paper	Filter paper may be used as spacer.
Test panels	Specified in ISO 21716-1:2020, 4.2. 50 mm square is recommended.
1 µm filters	Used to prepare test seawater.

Table 2 — List of apparatus used

Apparatus	Remarks
Light	White fluorescence or LED
Light intensity meter	Accuracy: ±10 lx

Table 2 (continued)

Apparatus	Remarks
pH meter	Accuracy: $\pm 0,1$
Salinometer	Accuracy: $\pm 0,1$
Stereo microscope	Magnification: 5-30X with fiber light
Thermometer	Accuracy: $\pm 0,1$ °C
Water flow-through system	Specified in ISO 21716-1:2020, 5.2, with a means of maintaining the test seawater tank at $20\text{ °C} \pm 1\text{ °C}$ and alternately illuminating the test seawater tank with a light intensity of 3 000 lx [Clause 8, d), light conditions] and with a light intensity of <5 lx [see Clause 8, d), dark conditions].

6 Preparation of the test organism and the test seawater

6.1 General

The cultured stock of live mussels is used to perform the bioassay test in sea water.

6.2 Preparation of the test organism

Live mussels are generally prepared by collecting wild mussels and acclimatizing them in the laboratory prior to testing. Guidance on this process and on storing mussels can be found in [Annex B](#).

Information on the life cycle of mussels can also be found in [Annex B](#), and information on the identification of *M. galloprovincialis* sp. mussels can be found in [Annex C](#).

6.3 Preparation of test seawater

Pass natural seawater through a 1 μm filter unit and adjust to salinity $30,0 \pm 0,5$ using purified water.

7 Preparation of the test panels and control panels

7.1 General

The same test and control groups shall be used throughout the whole test.

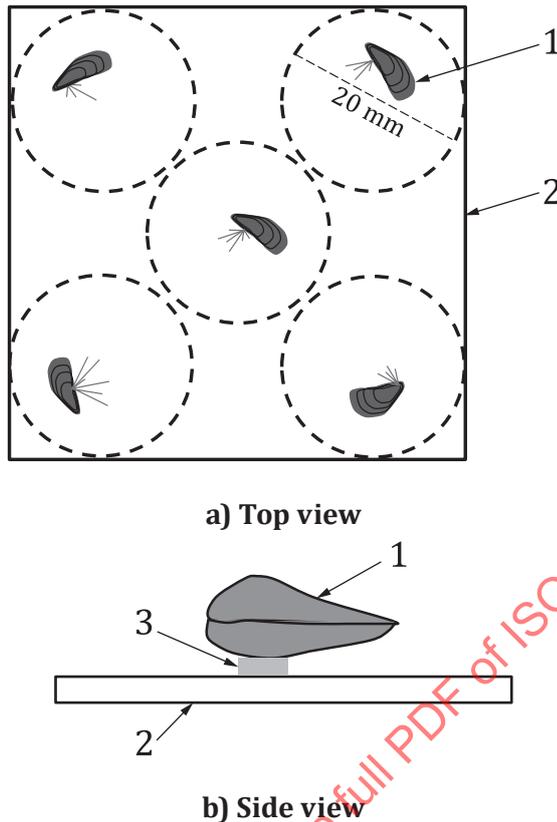
7.2 Preparation of the test panels

Test panels shall be prepared following the specifications described in ISO 21716-1:2020, Clause 4.

7.3 Affixing of mussels to the test panels and control panels

Separate the live mussels from their substrate by cutting the byssus threads with scissors, taking care to avoid damage to other tissue and organs. Affix five mussels to each test with the adhesive, using filter paper as a spacer between the shell of the mussel and the surface of the panel, providing a 20 mm diameter circular separation zone around each mussel [see [Figure 3 a](#)].

NOTE A spacer of adhesive-infiltrated filter paper can be used to prevent excessive spreading of the adhesive and improve adhesion of the mussel to the test and control panels [see [Figure 3 b](#)].

**Key**

- 1 mussel
- 2 test/ control panel
- 3 spacer of adhesive-infiltrated filter paper

Figure 3 — The mussels on the test or control panel

8 Operation of the test

The bioassay shall be simultaneously performed on the test group and the control group as follows (see [Figure 4](#)).

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) Fill the test seawater tank with test seawater and provide a continuous flow of test seawater from the seawater storage tank. Maintain the temperature of the test seawater at $20\text{ °C} \pm 1\text{ °C}$ for the duration of the test. The flow rate should be set to achieve at least about 0,8 turnover per hour of the water of test seawater tank.

NOTE If the flow rate is too low, the result can be affected by the concentration of biocide in seawater of the test seawater tank.

- b) Place the test and control panels in the test seawater tanks, ensuring all the panels are fully immersed in the test seawater.
- c) Measure and record the temperature, pH and salinity. Measure and record those parameters again after 24 h from the beginning of the test.
- d) Illuminate the test seawater tank for 12 h with a light intensity of 3 000 lx, and then leave the test seawater tank in darkness for 12 h.

- e) Carefully remove the test and control panels from the test seawater tanks after 24 h from the beginning of the test.
- f) Using a stereo-microscope, count the number of byssus threads for each live and attached mussel on each test and control panel, and record the results using e.g. [Table 3](#).

Count the number of dead or detached mussels on each test and control panel, and record the results using e.g. [Table 3](#).

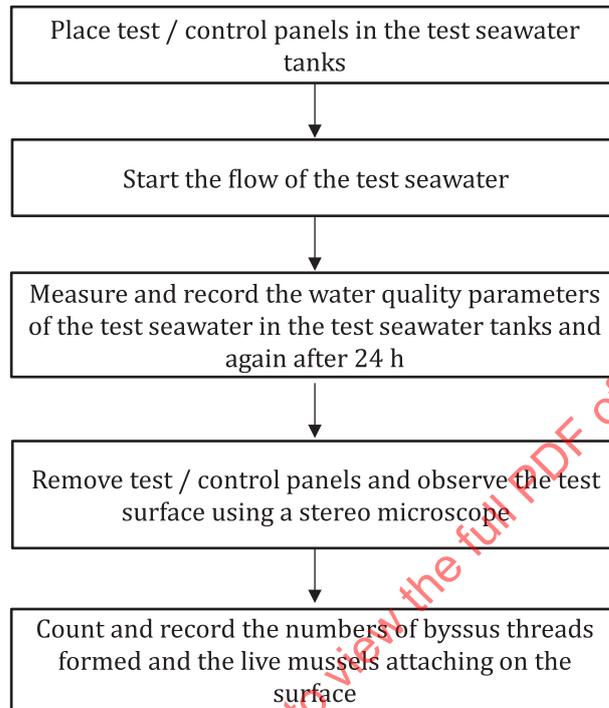


Figure 4 — Flow chart of the procedure for the test

9 Validation of the test

The results of the bioassay are validated by assessing if the results of the test meet the following four criteria.

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

- The average number of byssus threads formed by living individual in the control panels in each run is ten or more.
- The mortality of the mussels in the test panels in each run is 10 % or less.
- The mortality of the mussels in the control panels in each run is 10 % or less.
- The percentage of individuals detached from the test surface in each run is 5 or less.

10 Byssus threads formation

10.1 General

The evaluation procedure for screening anti-fouling paint is given in [10.2](#) and [10.3](#). The anti-fouling paint is evaluated by comparing the number of byssus threads between the test and control groups. The test data should be recorded using e.g. [Table 4](#).

10.2 Calculation of the average number of byssus threads formed

Calculate the average number of byssus threads formed by the mussels on the control group using [Formula \(1\)](#) and record the results using e.g. [Table 3](#) to one decimal place.

$$A_c = \frac{\sum_{j=1}^j (N_{c1}^j + N_{c2}^j + \dots + N_{cn}^j)}{\sum_{j=1}^j n^j} \quad (1)$$

where

A_c is the average number of byssus threads in the control groups;

j is the run number;

N_{cn}^j is the number of byssus threads formed by living individual mussel attaching on the control panels on the j -th run;

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

Calculate the average number of byssus threads formed by the mussels on the test group using [Formula \(2\)](#) and record the results using e.g. [Table 3](#) to one decimal place.

$$A_t = \frac{\sum_{j=1}^j (N_{t1}^j + N_{t2}^j + \dots + N_{tn}^j)}{\sum_{j=1}^j n^j} \quad (2)$$

where

A_t is the average number of byssus threads in the test groups;

j is the run number;

N_{tn}^j is the number of byssus threads formed by living individual attaching on the test panels on the j -th run;

n^j is the number of living mussels attaching on 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 byssus 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 3 — Example of compilation of the data

	Sample name	Number of byssus threads							Total	Average	Number of dead mussel (%)	Number of detached mussel (%)
		Mussel 1	Mussel 2	Mussel 3	Mussel 4	Mussel 5	Subtotal					
Run 1	Control panel 1	25	22	36	20	18	121	341	22,73	0 (0,00)	1 (3,3)	
	Control panel 2	21	31	11	34	19	116					
	Control panel 3	15	14	25	20	30	104					
	Test panel 1	10	4	5	10	*	29	112	8,00	0 (0,00)		
	Test panel 2	12	5	8	10	5	40					
	Test panel 3	7	9	3	9	15	43					
Run 2	Control panel 1	25	17	*	20	31	93	270	19,28	0 (0,00)	1 (3,3)	
	Control panel 2	34	22	26	14	13	109					
	Control panel 3	15	15	8	15	15	68					
	Test panel 1	8	*	4	8	3	23	72	5,14	1 (6,7)		
	Test panel 2	8	4	9	0	3	24					
	Test panel 3	2	2	8	11	2	25					
Run 3	Control panel 1	16	12	30	22	23	103	241	18,54	1 (6,7)	1 (3,3)	
	Control panel 2	*	8	14	10	22	54					
	Control panel 3	16	15	41	12	*	84					
	Test panel 1	3	1	4	6	0	14	42	3,00	1 (6,7)		
	Test panel 2	6	*	2	4	2	14					
	Test panel 3	1	3	2	4	4	14					
* Dead or detached mussel (excluded from the table).												
Results: A _c : 20,2 A _t : 5,4												

11 Test report

Table 4 specifies the minimum required information for the test report. The test results shall be reported using Table 4.

Table 4 — Minimum required information for the test report

Information	Requirement	
Materials and size of substrate to be painted	×	
General specifications and process for paint	Biocides contained	×
	Name of paint	×
	Undercoat	(×)
	Surface treatment	(×)
	Dry film thickness	×
Methods and time (number of days) for aging test panels ^a	×	
x: required; (x): optional.		
^a Information required for each run.		

Table 4 (continued)

Information		Requirement
General information on the test organisms ^a	Identification (species name and identifier)	×
	Date of sampling/collection	(×)
	Place of sampling/collection	(×)
	Water quality parameters at the time of sampling (temperature, salinity, pH)	×
	Acclimatization condition of the mussel (temperature, salinity, pH, light condition, acclimatization period, survival rate)	×
Initial test condition ^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 test device	(×)
Test condition after 24 h ^a	Water quality (temperature, pH, salinity), light condition	×
	The mortality of mussels in the test surface, the percentage of individuals detached from the test surface	×
	Accidental/unexpected items observed during the test	(×)
Average number of byssus threads		×
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 mussel settlement on painted test panels compared with mussel 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 mussel settlement is statistically significant. A typical example of statistical analysis was given in this Annex, based on Reference [1]. 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 [2]). Probability values (p-value) less than 0,05 are considered significant.

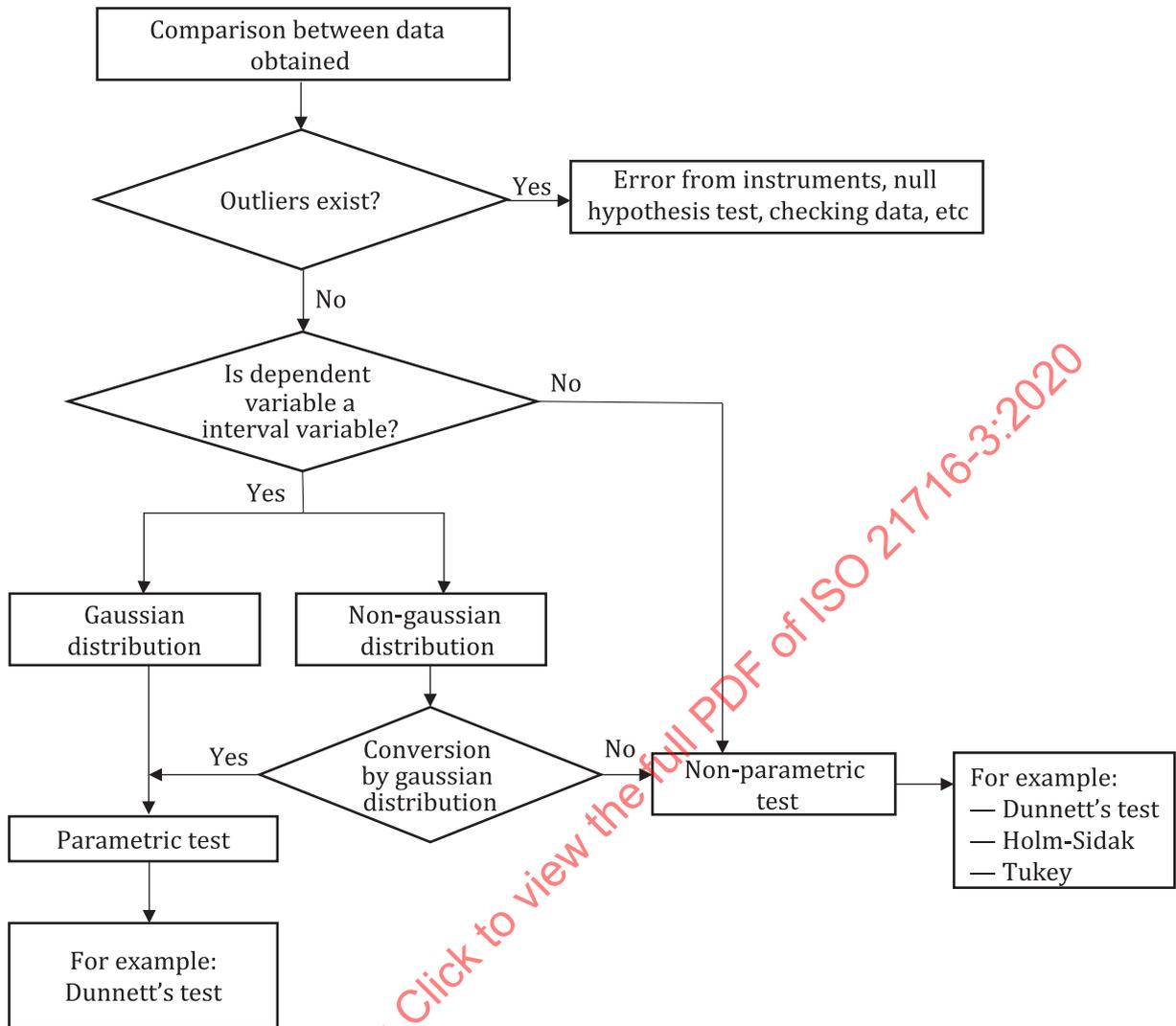


Figure A.1 — Example of a statistical analysis process

A.2.2 One-way analysis of variance^{[3]-[7]}

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, a tester analyses 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).

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 **** corresponds to $p < 0,05$ (significant), $p < 0,01$ (very significant), $p < 0,001$ (extremely significant) and $p < 0,000 1$ (extremely significant), respectively.

A.3 Example of statistical analysis

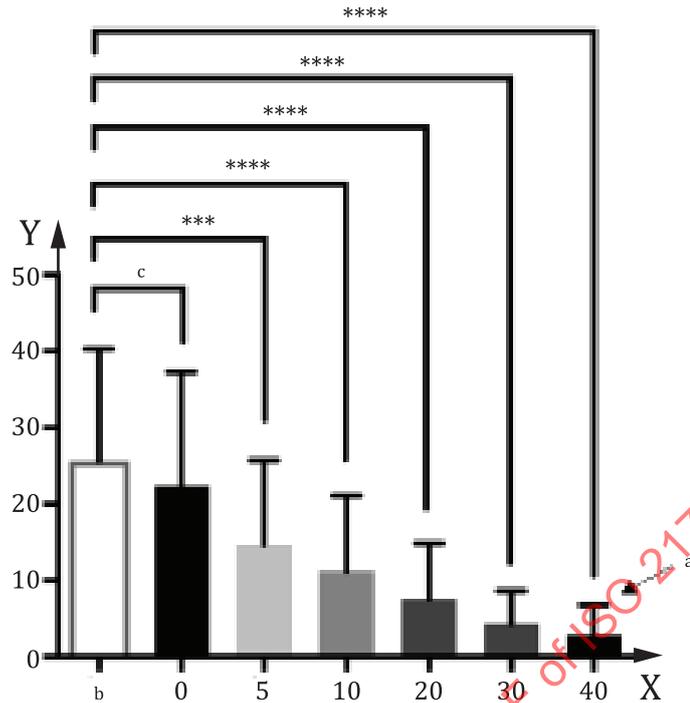
A.3.1 Introduction

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 plates 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 young mussels put on the test plates was observed. Statistical analysis, including one-way analysis of variance (ANOVA), non-parametric tests in the settlement assay and variance analysis were performed. Statistical difference in the number of byssus threads between the control and test groups was calculated. The pairwise comparison of the test results for test and control groups was performed using Kruskal-Wallis test ($p < 0,05$) in the settlement assay (see Reference [1], [8]). The Kruskal-Wallis test is a non-parametric test that was used to compare three or more unmatched groups (see Reference [9]).

A.3.2 Calculation results

Figure A.2 shows the correlation between the number of byssus threads formed and Cu_2O concentration in the test groups. Error bars indicate standard deviations (SDs). The inhibiting effect of Cu_2O on byssus threads formation was significant from 5 % mass fraction of Cu_2O ($p < 0,001$) or higher. Byssus threads production at 10 % mass fraction of Cu_2O ($p < 0,000 1$) decreased by more than 50 %. Byssus threads formation further decreased at concentrations of 20 % mass fraction of Cu_2O or higher (see Reference [1]).



Key

- X mass fraction of Cu₂O [%]
- Y number of byssus threads [*n*]
- a Error bars indicate SDs.
- b Control.
- c Not significant.

NOTE Pairwise comparison between control and test groups was performed to establish significance of the difference, where the symbols *** and **** corresponds to $0,0001 \leq p < 0,001$ (extremely significant) and $p < 0,0001$ (extremely significant), respectively (Reference [1]).

Figure A.2 — Number of byssus threads of *M. galloprovincialis* in the control and test groups

Annex B (informative)

General information of mussel, *Mytilus galloprovincialis*

B.1 Introduction

Mussels are regarded as fouling organisms because not only do they attach themselves to ship hulls, but they also attach to submerged marine structures and inside conduit pipes of facilities with seawater. Such biofouling of ships leads to increasing fuel consumption and can possibly cause introduction of non-indigenous species to another marine environment where they can cause significant change in the local ecosystem.

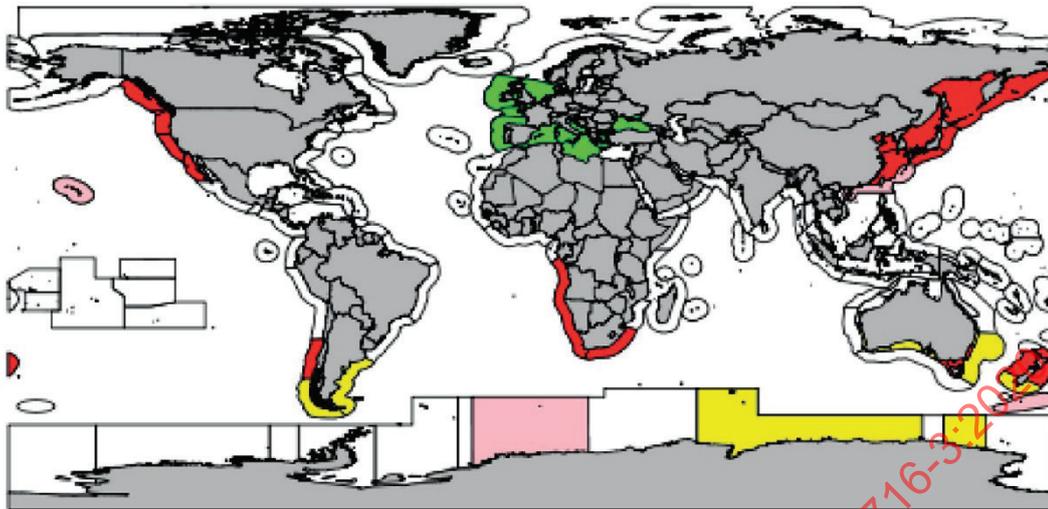
In the life cycle of the mussel, eggs are fertilized by sperm released from adults in seawater, and fertilized eggs develop to planktonic larvae. Larvae grow to the pediveliger stage when each larva competes to attach. Larvae select appropriate substrates where they attach to the substrate using their byssus, metamorphose into young mussels and begin their adult life. A mussel can attach to the substrates with pairs of a byssus thread and an attachment disc. The mussel byssus has numerous byssus threads, each formed within a groove in the mussel foot. The mussels are able to regulate byssus threads production depending on the environmental condition via their foot imprinting behaviour, which is temporarily affixing the tip of their feet to the substrate. The byssus threads production by mussels has been widely used as a standard indicator for evaluating anti-fouling efficacy against mussels in inhibition assays (Reference [10]). Settlement inhibition of mussels has been investigated using the byssus threads formation of mussels under closed or isolated experimental conditions (References [10], [11]). This means that 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.2 Information of *Mytilus galloprovincialis*

Mytilus galloprovincialis (the Mediterranean mussel) is one of the three principals in the *Mytilus edulis* complex, which are collectively and widely distributed on the temperate to subarctic coasts of both the Northern and Southern Hemispheres, and are often dominant inhabitants on hard substrates of the intertidal and nearshore habitats. *Mytilus galloprovincialis* often hybridizes with its sister taxa, the closely related *M. edulis* and *Mytilus trossulus*, when they are found in the same locality. *Mytilus galloprovincialis* is considered the most warm-water-tolerant species among the three and has the most southerly distribution in Europe and North America.

Mytilus galloprovincialis can be found from exposed rocky outer coasts to sandy bottoms in its native range (Reference [12]). As an invader, it typically requires rocky coastlines with a high rate of water flow. In fact, unlike the other 27 Asian and Atlantic molluscs introduced into Pacific shores of North America, only the Mediterranean mussel *Mytilus galloprovincialis* occurs in open coast, high energy environments on the Pacific coast; all remaining species are restricted to bays and estuaries (Reference [13]).

Mytilus galloprovincialis is native to the Mediterranean coast and the Black and Adriatic Seas. It has succeeded in establishing itself at widely distributed points around the globe (see Figure B.1), with nearly all introductions occurring in temperate regions and at localities where there are large shipping ports (Reference [14]). Ship hull fouling and transport of ballast water have been implicated in its spread, and its impact on native communities and native mussels has been suggested by a number of studies and observations (Reference [13], [15], [16]).



Key

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

SOURCE: NEMESIS Databases (Reference [17]).

Figure B.1 — Distribution map of *Mytilus galloprovincialis*

B.3 Preparation of test organism

B.3.1 Collection of mussels

Mussels with a shell length of 8 mm to 10 mm that are attached on natural or artificial substrates in intertidal zones on the coast should be collected. The number of mussels collected at the same point shall be at least 31 individuals to verify the test for each run.

B.3.2 Acclimatization for mussels

Acclimation for mussels shall be performed prior to the test under the conditions in [Table B.1](#).

Table B.1 — Acclimatization conditions for mussels

Description	Condition
Quality of water	The test seawater, specified in 6.3 , with dilution by purified water, defined in 3.3 , within a salinity range of $30 \pm 0,5$
Diet and density	The diatom <i>Chaetoceros gracilis</i> (200 000 cells/ml) and / or other diatoms such as <i>Skeletonema costatum</i>
Acclimatization period	5 days or more
Water temperature	$20 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$
Light condition	A 12 h light and 12 h dark periods given alternately as 3 000 lx
Aeration condition	Approximately 20 ml/min. of air flow by a quantitative pump
If the water temperature at the time of collection differs from the test seawater temperature by 5 °C below or above, mussels are gradually adapted to the water temperature by adjusting the temperature at a rate of 1 °C per day until the water temperature is close to $20 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$.	

B.3.3 Validation of the acclimatization

After the acclimatization period, living mussels shall be counted (e.g., by examining whether their shells are open or not). Mussel groups having a survival rate at 95 % or higher shall be used for the fixation process given in [7.3](#).

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