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Water quality — Growth inhibition test with the marine and brackish water macroalga *Ceramium tenuicorne*

Qualité de l'eau — Essai d'inhibition de croissance sur la macro algue d'eaux marine et saumâtre Ceramium tenuicorne

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ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

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Foreword

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

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 10710 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Introduction

The red macroalga *Ceramium tenuicorne* belongs to Ceramiaceae, Rhodophyta. The species can be used as a model organism for the near coastal ecosystem. This species is found in temperate marine waters in both the northern and southern hemispheres and is thus relevant for large areas. As primary producers, they are a food source for many invertebrates and serve as living habitat for bacteria, invertebrates, and juvenile fish. They also serve as substrate for many oviparous fish species.

Water quality — Growth inhibition test with the marine and brackish water macroalga *Ceramium tenuicorne*

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

IMPORTANT — It is absolutely essential that tests conducted according to this International Standard be carried out by suitably trained staff.

1 Scope

This International Standard specifies a method for the determination of the inhibition of growth of the macroalga *Ceramium tenuicorne* by substances and mixtures contained in seawater or by waste water with salinities between 4S and 32S. This method is applicable to substances that are easily soluble in water.

NOTE With modifications as described in ISO 14442[4] and ISO 5667-16[2], the inhibitory effects of poorly soluble organic and inorganic materials, volatile compounds, metals, waste water, marine water samples, and elutriates of sediments can be tested.

2 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

2.1
algal length
length from the first division to the most distant tip of the plant

NOTE The algal length is expressed in millimetres.

See Figure 1.

2.2
control medium
combination of dilution water and/or nutrient medium used in the test

[ISO 20079:2005[5], 3.6]

2.3
control batch
control medium including organisms used for testing

[ISO 20079:2005[5], 3.5]

2.4
effective concentration
E_rC_x
concentration of test sample at which an effect of x % is measured, if compared to the control

[ISO 20079:2005[5], 3.9]

2.5
growth medium
mixture of natural seawater and nutrients in which algal plants are cultivated, which is used for pre-cultures

NOTE Adapted from ISO 8692:2004[3], 3.3.

2.6
growth rate
μ
⟨water quality⟩ proportional rate of increase in algal length per day

NOTE See Clause 9.

2.7
salinity
practical salinity
S
⟨seawater⟩ ratio K_{15} of the electrical conductivity of the seawater sample, at the temperature of 15 °C and a pressure of one standard atmosphere, to that of a potassium chloride solutions in which the mass fraction of KCl is 32,435 6 × 10^{-3}, at the same temperature and pressure

NOTE Adapted from Reference [14], p. 12.

2.8
test medium
mixture of seawater, nutrients and test sample

NOTE Adapted from ISO 8692:2004[3], 3.5.

2.9
test sample
⟨water quality⟩ aqueous sample, e.g. chemical substance, mixture of chemicals or waste water, for which the inhibitory effects on the growth of algae are determined

NOTE Adapted from ISO 8692:2004[3], 3.4.

3 Principle

Algal tips from monocultures of ceramium female gametophytes are grown in defined test conditions and in a defined medium containing a range of concentrations of the test sample. The test solutions are incubated for a period of 7 d after which the increase in length is measured and the growth rate is calculated. The growth inhibition is determined as a reduction in growth rate, relative to control cultures grown under identical conditions.

When toxicity of samples is to be compared to the toxicity of other chemicals or waste waters, tests can be performed in artificial seawater. If the purpose of the testing is to assess and to predict effects in a specific receiving water body, the tests can be conducted with algae adapted to the salinity in the receiving water body. In this case, natural seawater from an uncontaminated site of the same properties is used.

4 Test organisms, nutrients, media, and materials

Unless otherwise stated, use only reagents of recognized analytical grade and distilled or demineralized water or water of equivalent purity.

4.1 Test organisms

Use either of the following marine algal clones:

a) *Ceramium tenuicorne* Kützing Waern (7S clone originating from the Baltic Sea);

b) *C. tenuicorne* Kützing Waern (20S and 30S clone originating from the Oslo fjord).

This alga is a widely distributed macroalgae species (phylum Rhodophyta) in estuarine and coastal areas. The strains recommended are available in unialgal, non-axenic cultures[1)].

NOTE 1 This growth inhibition test is based on two clones, which were formerly regarded as two different species. These species were the marine *Ceramium strictum* Harvey sensu Kylin and the brackish water species *C. tenuicorne* Kützing Waern. Complete interfertility (References [11][12]) and DNA data (Reference [10]) have shown that the two entities belong to the same species, with *C. tenuicorne* as the valid name. The marine clone (formerly *C. strictum*) used in this test was isolated in 1973 and originates from the Oslo fjord (20S to 25S). It has been maintained as a laboratory culture for over 30 years. The brackish water clone was isolated in 1995 and originates from the Baltic Sea, 20 km south of the Askö laboratory in northern Baltic proper (6S to 7S). Cultures can be maintained in the medium specified in Clause 5. Regular subculturing is necessary.

NOTE 2 Among the red algae, changes occur between haploid and diploid generations. In the growth inhibition test, the female gametophytic generation is used since it has an even dichotomous growth pattern and the fastest growth rate. In nature it is difficult to distinguish between male and female plants. This can be done in the laboratory where spermatangia are found on the branches of the male plants and trichogynes can be seen on the tips of the claws on the female plants.

NOTE 3 The Baltic Sea clone can be adapted and used in tests in salinities between 4S and 12S. The marine clone can be used as test organism in salinities between 12S and 32S.

4.2 Natural and artificial seawater

4.2.1 General

Natural seawater is used for the cultivation of the algae and either natural or artificial seawater should be used for testing. The type of seawater to be used depends on the objective of the test. When natural seawater is used, care shall be taken to ensure that it is not polluted. Take special care to avoid contamination of the water by inorganic or organic substances during preparation and storage. Equipment made of copper shall not be used.

4.2.2 Artificial seawater

Prepare the stock solutions for artificial seawater according to Table 1.

Start with about one third of the desired volume of water, add the weighed quantities of chemicals in accordance with Table 1 and make up to volume with water.

This stock solution with a salinity of 100S (equivalent to 10 % mass per volume) has a durability of at least six months, if stored in darkness at room temperature. Before use, the stock solution should be diluted with water to the desired salinity. Adjust the pH to 8,0 ± 0,2 with 1 mol/l HCl or 1 mol/l NaOH.

The artificial seawater shall be sterilized by autoclaving or sterile filtration (pore size, 0,2 µm) before use. Re-check the pH after sterilization, and adjust if necessary to 8,0 ± 0,2 with 1 mol/l HCl or 1 mol/l NaOH, before use.

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1) Suitable suppliers are: a) ITM, Department of Applied Environmental Research of Science, Stockholm University, S-106 91 Stockholm, Sweden; b) University of Oslo, Department of Biology, P.O. Box 1066 Blindern, N-0316 Oslo, Norway. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these suppliers.

Table 1 — Artificial seawater with a salinity of 10 % mass per volume or $100S$
(adapted from Reference [13])

Substance	Quantity per 1 l medium g/l	Quantity per 5 l medium g/5 l	Quantity per 10 l medium g/10 l
NaCl	70,1	351	702
Na_2SO_4	11,7	58,7	117
KCl	2,03	10,2	20,3
KBr	0,293	1,47	2,93
$Na_2B_4O_7{\cdot}10H_2O$	0,113	0,567	1,13
$MgCl_2{\cdot}6H_2O$	31,7	158	317
$CaCl_2{\cdot}6H_2O$	6,6	33	66
$SrCl_2{\cdot}6H_2O$	0,066	0,334	0,668

4.2.3 Natural seawater

Natural seawater shall be collected from an uncontaminated site. Filter to remove larger particles. Dilute as necessary with water. Salinity should be increased by addition of natural seawater of a higher salinity or with artificial seawater (see Table 1). Check the pH and adjust if necessary to 8,0 ± 0,2 with 1 mol/l HCl or 1 mol/l NaOH.

The natural seawater shall be sterilized by autoclaving or sterile filtration (pore size, 0,2 µm) before use. Re-check the pH after sterilization, and adjust if necessary to 8,0 ± 0,2 with 1 mol/l HCl or 1 mol/l NaOH, before use.

NOTE 1 A paper filter of around 30 µm mesh size is sufficient.

NOTE 2 Natural seawater can be stored frozen at temperatures below –18 °C for several years before use.

4.3 Nutrients

Prepare the six nutrient solutions in water, with the compositions given in Table 2.

Solutions 1, 2, 3, 4 and 6 in Table 2 are prepared in 100 ml one-mark volumetric flasks (6.14). A 1 l one-mark volumetric flask (6.14) is recommended for the preparation of stock solution 5, due to the low masses of the trace element reagents. Precipitation in the trace element solution is avoided by adjustment with NaOH to pH 8. The trace element stock solution (solution 5) shall be diluted 10 times before use in the cultivation media (see Table 3). After this dilution, a freshly prepared iron solution 3 may be added to trace element solution 5 to increase the durability of the iron.

The iron solution shall not be older than one month.

These stock solutions will eventually be diluted according to Table 3 to obtain the final nutrient concentrations in the growth and test media. The final concentrations in the media are given in the two rightmost columns of Table 2.

4.4 Media

Additions of stock solutions to salt water for preparation of media are shown in Table 3. For cultivation, testing in natural seawater, and testing in artificial seawater, additions shall be made in accordance with columns A, B, and C, respectively.

Table 2 — Nutrient stock solutions (adapted from Reference [9])

Reagent	Compound mass concentration	Levels in the medium after additions according to Table 3 as	
		elemental mass concentration	elemental amount of substance concentration
		µg/l	µmol/l
1 — Nitrogen solution			
KNO_3	5 000 mg/100 ml	3 462 (N)	247 (N)
2 — Phosphorus solution			
KH_2PO_4	680 mg/100 ml	775 (P)	25 (P)
3 — Iron solution			
$FeCl_3 \cdot 6H_2O$	100 mg/100 ml	103 (Fe)	1,9 (Fe)
4 — Carbon solution			
$NaHCO_3$	5 760 mg/100 ml	16 480 (C)	1 370 (C)
5 — Trace element solution			
Na_2EDTA	6 000 mg/l		
$MnSO_4 \cdot H_2O$	620 mg/l	10 (Mn)	0,18 (Mn)
$ZnSO_4 \cdot 7H_2O$	250 mg/l	2,84 (Zn)	0,043 (Zn)
$Na_2MoO_4 \cdot 2H_2O$	130 mg/l	2,58 (Mo)	0,027 (Mo)
$CoSO_4 \cdot 7H_2O$	4 mg/l	0,042 (Co)	0,000 7 (Co)
$CuSO_4 \cdot 5H_2O$	4 mg/l	0,05 (Cu)	0,000 8 (Cu)
6 — Vitamins			
Thiamine (B_1)	10 mg/100 ml	50	
Cyanocobalamin (B_{12})	0,1 mg/100 ml	0,5	
Biotin	0,1 mg/100 ml	0,5	

Table 3 — Additions of stock nutrient solutions to seawater for preparation of growth and test medium

Stock solutions	A Growth medium in natural seawater for cultivation ml/l	B Test medium in natural seawater ml/l	C Test medium in artificial seawater ml/l
1 — Nitrogen solution	0,5	0,5	0,5
2 — Phosphorus solution	0,5	0,5	0,5
3 — Iron solution	0,5	0,5	0,5
4 — Carbon solution	—	—	2
5 — Trace element solution diluted 10 times	0,5	—	—
6 — Vitamin solution	0,5	—	—

Preparation of 1 l of growth medium or test medium is shown in Table 3. For the cultivation of stock cultures, sterile natural seawater shall be used. For the growth inhibition test, either sterile artificial or natural seawater may be used depending on the objective (see Clause 4).

5 Cultivation

Stock cultures of female gametophytes of *C. tenuicorne* are cultivated in natural seawater (see 4.2.3) where nutrients have been added according to Table 3, column A. The brackish water clone is cultivated at a salinity of 7S and the marine clone (former *C. strictum*) at salinities of 20S and 30S.

The maintenance of the cultures is facilitated by working under aseptical conditions. *C. tenuicorne* should be grown in sterile plastic or glass Petri dishes (e.g. 90 mm diameter, 15 mm height). The algae are cultured at 22 °C ± 2 °C, at a light intensity of 35 µmol m^{-2} s^{-1} ± 7 µmol m^{-2} s^{-1} and a light regime of 14 h light and 10 h darkness. These conditions can be achieved on a laboratory bench with an ordinary time-regulated lamp at about 350 mm distance.

To maintain actively growing female gametophytes, plants have to be transferred to fresh medium each week. The procedure is to fill sterile Petri dishes with approximately 25 ml sterile culture medium according to Table 3 column A. Approximately 20 to 30 tips of length 10 mm to 20 mm from female plants are transferred to each dish.

If the test is intended to be performed at a salinity other than 7S, 20S or 30S, the algae need to be adapted to the new salinity prior to the start of the test. Seawater of other salinities is prepared according to 4.2.2 and 4.2.3. The algae should be adapted successively by transferring the algae every other day into fresh media with an increase or decrease in salinity of 3S. The algae shall be cultivated for at least two weeks in the final test salinity prior to the start of the test.

NOTE 1 To increase the stability of iron, iron solution 3 can be added to the diluted trace element solution.

NOTE 2 Back-up cultures can be held for up to two months without refreshing if cultivated under lower light intensities at room temperature. If the temperature is at approximately 10 °C, the algae can be kept for up to three months.

6 Apparatus

All equipment that has contact with the test medium shall be made of glass or other chemically inert material.

Usual laboratory equipment and in particular the following.

6.1 Temperature-controlled cabinet or room, with a white fluorescent light providing even illumination, suitable for the lighting requirements specified for the test in 7.5.

6.2 Fluorescent tubes of the daylight or “warm white” type, capable of providing a light intensity of (70 ± 7) µmol m^{-2} s^{-1} for the exposure period, and around (35 ± 7) µmol m^{-2} s^{-1} for cultivation.

6.3 Timer, to control a light regime of 14 h light and 10 h darkness.

6.4 Light meter, which measures photons or energy (expressed in micromoles per square metre per second), within the photosynthetic range 400 nm to 700 nm, is preferred.

6.5 pH meter, for the measurement and adjustment of pH during the preparation of culture and test solutions and to measure pH at the termination of a test.

6.6 Salinity meter or **conductivity meter**, to measure and adjust the salt content of the culture and dilution water.

6.7 Stereomicroscope, with a magnification of 6 times to 10 times, for cutting female plants prior to a test and for measuring the length of the plants.

6.8 Sterilization equipment. All equipment and solutions should be sterile to prevent contamination. This can be done in an autoclave. Solutions can also be sterilized by filtration (pore size, 0,2 µm). Glassware can be sterilized in a muffle furnace at 150 °C for 3 h.

6.9 Gas burner or **spirit lamp**. To maintain the plants as a monoculture, sterile microbiological techniques should be employed for all work in connection with the algae. Bottles and instruments should be flamed when working with the cultures and preparing solutions.

6.10 Scalpel, **scissors**, and a **pair of pincers** are useful tools for cutting and handling algae.

6.11 Culture dishes. Sterile and inert plastic or glass Petri dishes with lids, with a diameter of 90 mm and a height of 15 mm containing about 25 ml medium may be suitable.

6.12 Exposure dishes. Sterile and inert plastic or glass Petri dishes and lids, with a diameter of 50 mm and a height of 10 mm containing about 10 ml medium may be suitable.

6.13 Trays, for simplifying the handling of Petri dishes.

6.14 One-mark volumetric flasks, capacities 100 ml and 1 l, ISO 1042[1] class A.

7 Procedure

7.1 Algae material

The algae (4.1) shall be cultivated in natural seawater enriched as specified in 4.4 for at least a week prior to the start of a test. To ensure that the algae are growing actively, transfer to fresh medium 3 d to 4 d before the start of a test. Transfer 20 to 30 female plants, of length about 10 mm to 15 mm, to Petri dishes containing 25 ml of culture medium each. Three to four such dishes produce sufficient material for a test.

At the start of a test, tips are cut from the female plants of *C. tenuicorne* using a scalpel (6.10). For the marine clone, tips shall be cut as shown in Figure 1 a), i.e. with two levels of forking, two initial “claws”, and a length from the base forking to the top of 0,6 mm to 1,2 mm. To start the test with the brackish clone, larger starting pieces are used. These algae shall have an appearance as shown in Figure 1 b) with three levels of forking, four initial “claws”, and a length from the base dividing to the top of 2 mm to 3,5 mm. The cutting of algal pieces is best done under a stereomicroscope (6.7). The algal pieces are collected in a separate Petri dish with seawater of appropriate salinity. With graph paper beneath the Petri dish, the initial size of at least 20 algae shall be measured under a stereomicroscope.

Uniform length gives a more precise result. It is therefore recommended that a surplus of algae be cut and the most deviating plants discarded from the collecting dish. About 60 algae pieces are needed to carry out one test as specified in this International Standard.

Dimensions in millimetres

a) **b)**

Figure 1 — Starting size for a) the marine clone and b) the brackish water clone

7.2 Preparation of dilution water

7.2.1 Preparation of seawater

Prepare the seawater medium for a test by adding nutrients to the desired type of sterile seawater in accordance with Table 3.

7.2.2 Preparation of solutions for assessment of chemicals and products

7.2.2.1 Preparation of stock solution for assessment of chemicals and products

Preparation of a stock solution shall be carried out by dissolving a known amount of test sample in a known amount of medium (the same medium that is used as control). If the stability of the test substance is known and does not change with time, the stock solution can be prepared in advance. The stock solution should then be kept in darkness in a refrigerator at or below +4 °C.

7.2.2.2 Preparation of test media for assessment of chemicals and products

Prepare each test concentration by mixing known quantities of the stock solution and nutrient-enriched (see Table 3) sterile seawater. The pH should be measured (6.5) in the control and in the lowest and highest test concentration.

7.2.3 Preparation of solutions for assessment of waste water

7.2.3.1 Preparation of stock solution for assessment of waste water

The conductivity of the sample should be measured (6.6) and the salt content adjusted by the addition of NaCl (solid) to the desired salinity. This means that, if a fresh water sample requires testing, to achieve 20S, 2 g NaCl is added per 100 ml sample and, for a test at 7S, 0,7 g NaCl added per 100 ml sample.

Measure the pH of the sample. Waste water with a pH which deviates from 7 to 9 shall be adjusted to within these limits with 1 mol/l HCl or 1 mol/l NaOH.

Add nutrients to the waste water in proportions in accordance with Table 3 to ensure sufficient nutrients in all test concentrations. This water is regarded as 100 % waste water.

7.2.3.2 Preparation of test media for assessment of waste water

Prepare each test concentration by mixing known volumes of the waste water (prepared as described above) and nutrient-enriched (see Table 3) sterile seawater (natural or artificial depending on the purpose of the investigation). For dilution, use the same medium that is used as control medium. The test solutions (i.e. the concentration series) should be prepared on the day they are to be used.

7.3 Choice of test concentrations

Algae should be exposed to concentrations of the test sample in a geometric series. If possible, the concentrations shall be chosen to obtain several levels of inhibition ranging from less than 10 % to greater than 90 % inhibition of growth. Algae should be exposed to concentrations of the test substance in a geometric series with a ratio not exceeding 3,2 (e.g. 1,0 mg/l, 1,8 mg/l, 3,2 mg/l, 5,8 mg/l and 10 mg/l).

NOTE A suitable concentration range is best determined by carrying out a preliminary range-finding test covering several orders of magnitude of test concentrations. Replication of test concentrations is not a requirement in the preliminary test.

7.4 Start of test

Solutions for the test are prepared in accordance with 7.2.2 or 7.2.3 on day 0. Volume of 10 ml of solutions at the test concentrations are run into the test Petri dishes (sterile Petri dishes with lid and a diameter of 50 mm are recommended). Prepare four replicates for each test sample concentration and for the controls. Two algae plants should be added to each Petri dish (6.11) from the collecting algal Petri dish (see 7.1). The plants shall first be added to the controls and then in increasing order of concentration. To avoid taking the largest plants first, the Petri dish containing the starting material should be gently mixed between each concentration and then all plants should be collected from a randomly selected region. A piece of graph paper beneath the Petri dish will aid this procedure. Place the Petri dishes randomly on a tray before placing them in the incubation room.

NOTE If there is sufficient technical justification, the test design can be altered to increase the number of concentrations and reduce the number of replicates per concentration.

7.5 Incubation

Incubate the Petri dishes for 7 d at 22 °C ± 2 °C, under (70 ± 7) µmol m^{-2} s^{-1} (≈ 3 600 lux) (6.2), and with a regime of 14 h light and 10 h darkness.

In modern laboratories, the ventilation can be so high that too much of the media evaporates during the exposure time. If this occurs, cover the tray laden with Petri dishes with plastic film.

NOTE When using cool fluorescent light, 1 lux = 0,014 µmol m^{-2} s^{-1}.

7.6 Measurement

Growth shall be measured as length after 7 d. The length of the plants shall be measured from the first forking point to the most distant tip of each plant, e.g. with a stereomicroscope (6.7) with a graph paper beneath the Petri dish. Measure the pH (6.5) of the controls and in the lowest and highest test concentrations.

7.7 Test with reference substances

Carry out a test using 3,5-dichlorophenol and/or zinc sulfate. E_rC_{50} should be in the range 1,2 mg/l to 3,4 mg/l for 3,5-dichlorophenol and 0,01 mg/l to 0,08 mg/l for zinc sulfate. Reference substances may be tested to check the test procedure and sensitivity. It is advisable to test the reference substances regularly and to use control charts for measuring within-laboratory precision and monitoring culture health. Guidance on preparation of control charts is available in ISO/TS 20281[6].

NOTE The range for 3,5-dichlorophenol and zinc sulfate is based on data of the international interlaboratory test of this International Standard and for zinc when additional published data were included (see Annex B).

8 Validity criteria

Consider the test valid if the following conditions are met:

a) the increase in length in 7 d in the controls has increased by a factor of more than three compared to the starting length;

b) the coefficient of variation of the control growth rates does not exceed 30 %;

c) the control pH has not changed to become lower than 6,5 or higher than 9,5.

9 Evaluation of results

9.1 Calculations

Calculate the mean increase in length for each replicate over the test duration by subtracting the mean algae start length from the mean final length of the algae in each replicate. The growth measured as increase in length is nearly linear for *C. tenuicorne* during the 7 d test period (see Annex A). Thus, the increase in length, i.e. the growth rate, μ, in millimetres per day, is calculated using Equation (1):

$$\mu = \frac{l_7 - l_0}{t_7 - t_0} \tag{1}$$

where

l_7 is the length, in millimetres, at the final measurement on day 7;

l_0 is the length, in millimetres, at the start;

t_7 is the time, in days, at the final length measurement on day 7;

t_0 is the time, in days, at the first length measurement.

Calculate the mean value of μ for the control and each test concentration. From these values, calculate the percentage inhibition (growth rate) for each test concentration i, $I_{\mu i}$, from Equation (2):

$$I_{\mu i} = \frac{\bar{\mu}_c - \bar{\mu}_i}{\bar{\mu}_c} \times 100 \tag{2}$$

where

$\bar{\mu}_i$ is the mean growth rate for test concentration i;

$\bar{\mu}_c$ is the mean growth rate for the control.

9.2 Determination of E_rC_x

Tabulate values of $I_{\mu i}$ against the corresponding test concentrations, and plot these values on semi-logarithmic paper (with the test concentration on the logarithmic scale) as appropriate. Fit a line to the data by eye and read the EC_{50} (the test concentration corresponding to 50 % inhibition) and EC_{20} (the test concentration corresponding to 20 % inhibition) from this graph.

Alternatively, calculate the EC_{20} and EC_{50} by a linear regression analysis technique offered by software programs.

9.3 Expression of results

If applicable, denote EC_{20} and EC_{50} values based on growth rate as E_rC_{20} and E_rC_{50} and the method of determination. Also indicate clearly the time span used for the determination, for example E_rC_{50} (0 d to 7 d). Quote E_rC_{20} and E_rC_{50} values to two significant digits, normally in milligrams per litre.

NOTE When testing waste water by means of a graduated dilution, the most concentrated test batch tested, at which an inibition (for ceramium assay < 5 %) is observed, is expressed as lowest ineffective dilution (LID). This dilution is expressed as the reciprocal value of the volume fraction of waste water in the test medium [e.g. if the waste water content is 1 part in 4 (25 % volume fraction), the dilution level is D = 4]; see ISO 5667-16:1998[2], Annex A.

9.4 Interpretation of results

EC_{20} and EC_{50} values are toxicological data derived from a laboratory experiment carried out under defined standard conditions. They give an indication of potential hazards, but should not be used directly to predict effects in the natural environment.

10 Reproducibility

In a ring test performed in 2006, six laboratories participated. Two reference substances were tested, i.e. zinc ion as $ZnSO_4 \cdot 7H_2O$ and 3,5-dichlorophenol. Most laboratories performed the test twice with each substance. The result is shown in Table 4. More details can be found in Annex B.

Table 4 — Result of ring test of the growth inhibition method with *Ceramium tenuicorne* 20*S*

Substance	No. of laboratories	No. of valid tests	Outliers	Mean E_rC_{50}[a] mg/l	Standard deviation[a]	Coefficient of variation[a] %
$ZnSO_4 \cdot 7H_2O$	6	9	2	0,049	0,023	48
3,5-Dichlorophenol	6	10	0	2,3	0,55	25
[a] Outliers are not included in the calculations						

11 Test report

This test report shall contain at least the following information:

a) the test method used, together with reference to this International Standard (ISO 10710:2010);

b) test substance chemical identification data;

c) test organism: species, origin, strain number, method of cultivation;

d) test details:

1) start date and duration,

2) method of preparation,

3) nominal and measured concentrations tested,

4) composition of medium,

5) culturing apparatus and incubation procedure,

6) light intensity and quality,

7) temperature,

8) pH of test solutions at the start and end of the test;

e) results:

1) mean algal length for each test concentration (and control) at each measuring point,

2) growth curves (algal length against time),

3) relationship between concentration and effect (percentage inhibition values against concentration) in table or graphical representation: e.g. percentage inhibition against concentration on logarithmic-scaled abscissa,

4) E_rC_{20} value and method of determination,

5) E_rC_{50} value and method of determination,

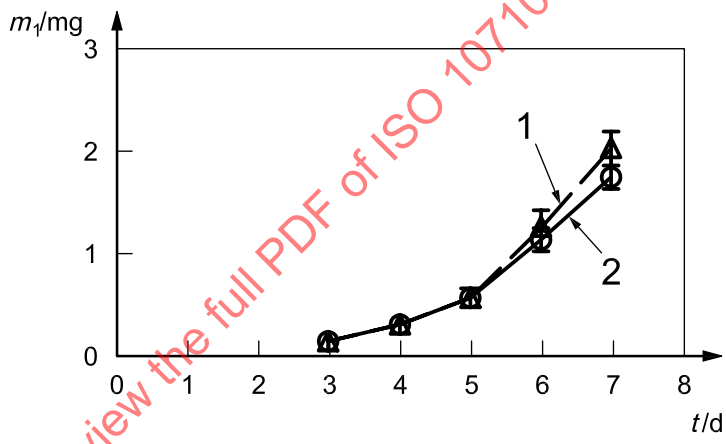
6) other observed effects.

Annex A
(informative)

Growth pattern of *Ceramium tenuicorne* in 7S and 20S

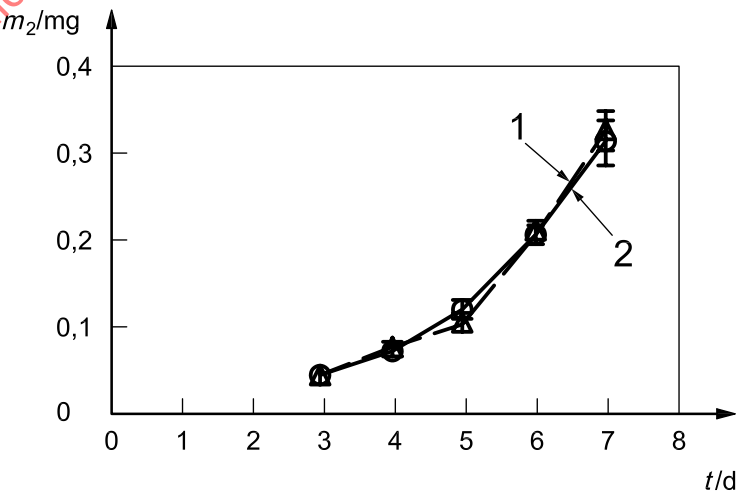
Figures A.1 and A.2 show the relationship between growth in number of days and wet mass, dry mass, and length for the red macroalga *Ceramium tenuicorne* grown at room temperature, under 70 µmol m^{-2} s^{-1} (≈ 3 600 lux), with a regime of 14 h light and 10 h darkness at two salinities, namely 7S and 20S. The growth was performed in artificial seawater and in natural seawater — from Reference [8].

Key
1 artificial seawater
2 natural seawater
m_1 wet mass per plant
t time



a)

Key
1 artificial seawater
2 natural seawater
m_2 dry mass per plant
t time



b)

Figure A.1 — Growth of female *C. tenuicorne* in 7S (*continued*)