TECHNICAL REPORT

ISO/TR 11044

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Water quality — Scientific and technical aspects of batch algae growth inhibition tests

Qualité de l'eau — Aspects scientifiques et techniques des essais d'inhibition de croissance d'un lot d'algues

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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.

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.

In exceptional circumstances, when a technical committee has collected data of a different kind from that which is normally published as an International Standard ("state of the art", for example), it may decide by a simple majority vote of its participating members to publish a Technical Report. A Technical Report is entirely informative in nature and does not have to be reviewed until the data it provides are considered to be no longer valid or useful.

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/TR 11044 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Introduction

The growth of microalgae in batch cultures follows a well known pattern, with a lag phase followed by an exponential growth phase, a phase of declining growth rate, a stationary phase, and ultimately a death phase (Reference [9]). The characteristics of these phases are dependent on the environmental conditions including the chemical composition of the growth medium, which provides the basis for using batch cultures of algae as bioassays to investigate growth stimulating or inhibiting properties of constituents of the growth medium.

The first systematic application of microalgae bioassays for which standard protocols were developed was for assessment of nutrient status and identification of growth limiting nutrients. Skulberg (Reference [50]) developed a bioassay with the green alga *Selenastrum capricornutum* Printz, which was used to assess fertilizing influences of pollution in inland waters. The nutrient bioassay with *S. capricornutum* was further developed and standardised in Reference [55]. The strain of *S. capricornutum* used as test organism in the nutrient bioassays was originally isolated from the river Nitelva in southeast Norway in 1959. It has become the most commonly used test algae for bioassays and is available from most major culture collections. Due to taxonomic revisions, it was first renamed *Raphidocelis subcapitata* and later *Pseudokirchneriella subcapitata* (Korshikov) Hindak (Reference [20]).

It was early recognized that bioassays of microalgae could be used to study the growth-inhibiting effects of toxic chemicals and waste waters, and a modification of the algal assay procedure for toxicity studies was made in Reference [43]. However, based on compilations of early algae toxicity test data some authors claimed that the sensitivity of algae generally was low (Reference [26]). The environmental relevance of results of the tests was also questioned because of the significant interspecies variation in response and lack of field-validation of results of algal toxicity tests (Reference [28]). On the other hand, microalgae are generally the most important primary producers in aquatic ecosystems. Excluding the assessment of toxicity to this group of organisms in risk assessment and environmental management cannot be justified. Development and standardisation of methods have therefore been undertaken to increase the reproducibility and relevance of toxicity tests with microalgae. Standardised growth inhibition tests with algae are now a cornerstone in the environmental management and risk assessment of chemicals. Recent reviews (e.g. Reference [57]) show that they are often the most sensitive of the "base-set" tests which include also acute toxicity tests with fish and *Daphnia*.

In addition to several national organisations, the Organisation for Economic Co-operation and Development (OECD) and the International Organization for Standardization (ISO) took on the work of developing guidelines and standards for growth inhibition with microalgae in the late 1970s. The OECD guidelines aim to test chemical substances, while ISO documents cover tests for composite water samples, such as waste water and elutriates. However, harmonisation of the procedures was an objective as the two series of documents were developed in parallel by the two organisations. The development of the freshwater test was initiated by ISO in 1978. Three ring tests were organised between 1980 and 1982 and included in ISO 8692:1989, revised as ISO 8692:2004. The first draft of a marine algae inhibition test was produced in 1982, but the first ISO/DIS was not published until 1991, when the method had been ring tested. ISO 10253:1995 was revised as ISO 10253:2006. In addition to these two standards, ISO 14442:1999, guidelines for algal growth inhibition tests with poorly soluble matter, volatile compounds, metals and waste water, was revised as ISO 14442:2006. In this Technical Report, the general principles of the batch culture growth inhibition tests, and how some critical methological aspects have been addressed in the International Standards for algal growth inhibition tests, are presented.

Water quality — Scientific and technical aspects of batch algae growth inhibition tests

1 Scope

This Technical Report discusses scientific and technical aspects that have been considered in connection with the development of batch algal growth inhibition test procedures specified in ISO 8692, for freshwater, and ISO 10253, for marine waters.

Previously unpublished results of experiments performed at the Norwegian Institute for Water Research (NIVA) have been included to demonstrate various aspects.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 8692:2004, *Water quality — Freshwater algal growth inhibition test with unicellular green algae*

ISO 10253:2006, *Water quality — Marine algal growth inhibition test with* Skeletonema costatum *and* Phaeodactylum tricornutum

ISO 14442, *Water quality — Guidelines for algal growth inhibition tests with poorly soluble materials, volatile compounds, metals and waste water*

3 Terms and definitions

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

3.1
effective concentration
EC_x
concentration of test sample which results in a reduction of x % in the specific growth rate relative to the controls

[ISO 8692]

NOTE Unless otherwise stated, the form EC_x is used in this Technical Report to mean E_rC_x where “r” denotes “rate”. Effective concentrations based on area under the growth curve can be derived, and these are designated E_bC_x, where “b” denotes “biomass” (see 6.5 for further details).

3.2
specific growth rate
μ
proportional rate of increase in cell density per unit of time:

$$\mu = \frac{1}{n}\frac{\mathrm{d}n}{\mathrm{d}t}$$

where

n is the cell density, expressed in cells per millilitre;

t is the time, expressed in days.

NOTE 1 Specific growth rate is expressed in reciprocal days.

NOTE 2 Adapted from ISO 8692.

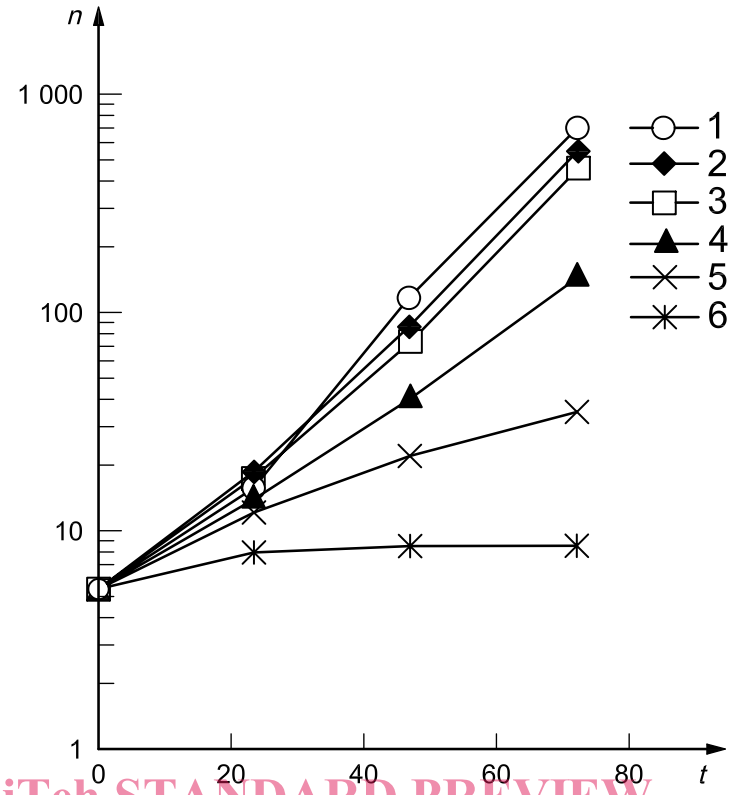
4 General principles of ISO algal growth inhibition tests

The algae growth inhibition test methods specified in ISO 8692 and ISO 10253 are based on batch cultures which are inoculated with algae from an exponentially growing inoculum culture and incubated under continuous illumination. The growth medium, inoculum biomass density, temperature, and illuminance, have been selected to allow an exponential increase in the algal biomass density during the 72 h incubation period for the recommended test species.

The experimental design of the tests includes a series of five or more concentrations of the test material in growth medium prepared in triplicate, and six control replicates without test material. After inoculation with test algae, the solutions are incubated in transparent, inert containers under continuous illumination and constant temperature. The cultures should be agitated in order to obtain a homogenous suspension of the algae and to stimulate gas exchange with the atmosphere. The biomass density in the cultures is measured by direct or indirect methods at 24 h intervals until termination of the test after 72 h.

An example of a growth inhibition test with *Pseudokirchneriella subcapitata* is shown in Figure 1. The substance tested was potassium dichromate. The growth curves show close adherence to exponential growth in the cultures, and decreasing growth rates with increasing concentration of the test substance. Average specific growth rates may be calculated as the logarithmic increase in cell density from start to 72 h. Figure 2 shows the concentration/response plot for the endpoint growth rate. A curve has been fitted to the observations by non-linear regression using a log-logistic model (REGTOX) [1]. Concentrations causing 10 % and 50 % reduction of the growth rate (EC_{10} and EC_{50} respectively) have been calculated from the regression equation.

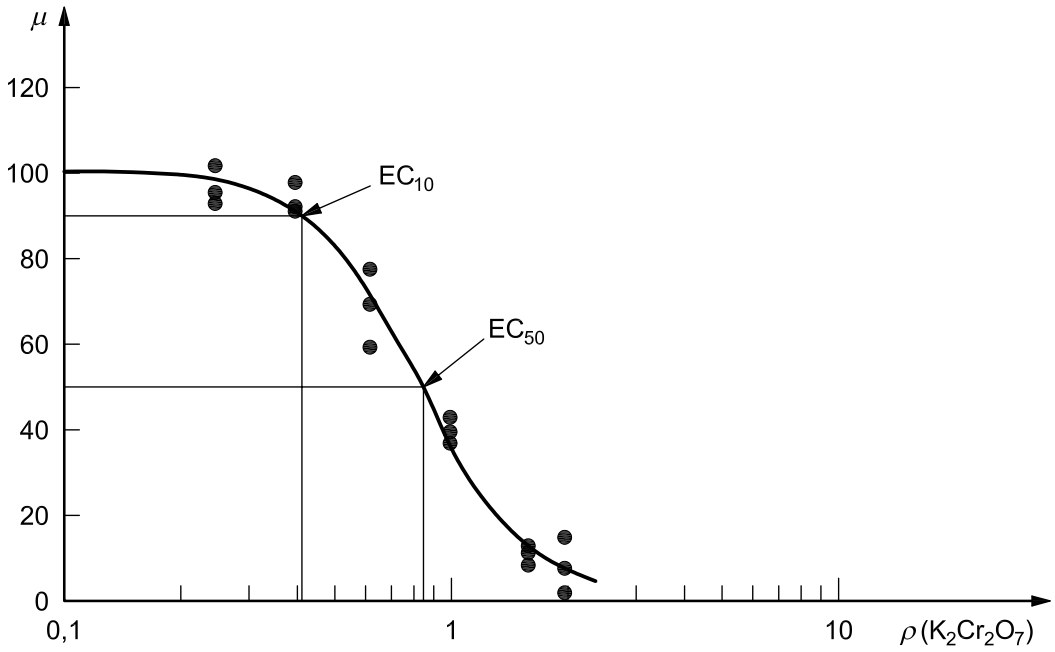
1) Available (2008-11-14) at http://eric.vindimian.9online.fr/



Key

1 $\rho(K_2Cr_2O_7)$ = 0 (control)
2 $\rho(K_2Cr_2O_7)$ = 0,25 mg/l
3 $\rho(K_2Cr_2O_7)$ = 0,4 mg/l
4 $\rho(K_2Cr_2O_7)$ = 0,63 mg/l
5 $\rho(K_2Cr_2O_7)$ = 1 mg/l
6 $\rho(K_2Cr_2O_7)$ = 1,6 mg/l
n cell density, 10^3 cells/ml
t time, h

Figure 1 — Growth curves (mean values of replicates) for cultures of *P. subcapitata* at different mass concentrations of $K_2Cr_2O_7$



Key

μ	specific growth rate as a percentage of control
EC_{10}	effective concentration at 10 % inhibition
EC_{50}	effective concentration at 50 % inhibition
$\rho(K_2Cr_2O_7)$	potassium dichromate mass concentration, mg/l

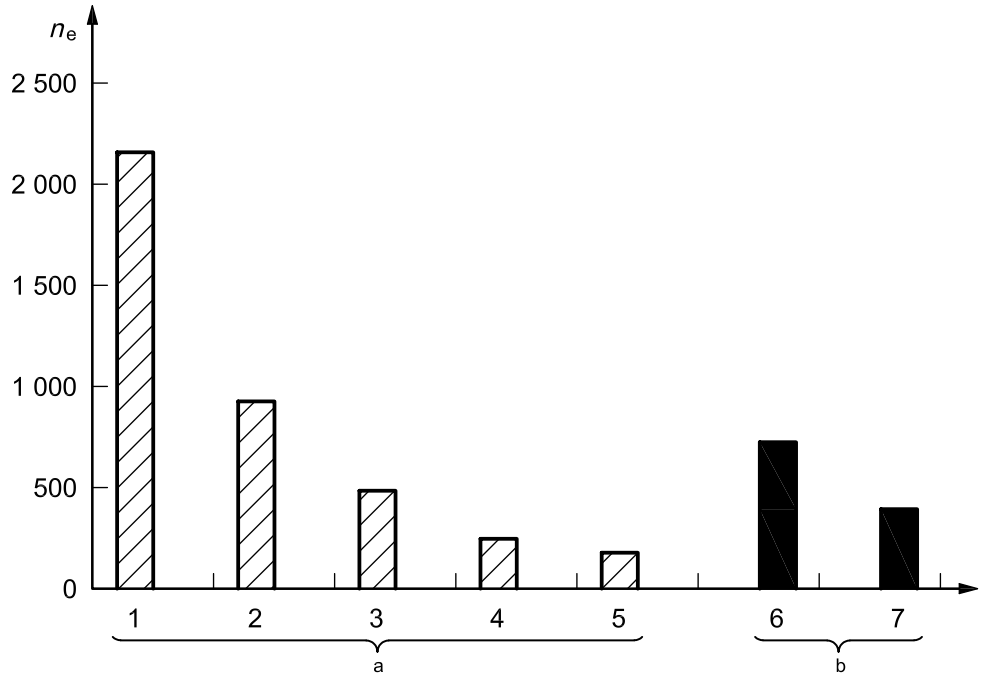
Figure 2 — Mass concentration/response plot showing the effect of $K_2Cr_2O_7$ on the growth rate of *P. subcapitata*

5 Test species

5.1 General

Microalgae constitute a phylogenetically diverse group of organisms, including the procaryotic cyanobacteria and several phyla of eucaryotic algae. It is therefore not surprising that the sensitivity among different species of microalgae to various toxic substances is highly variable. Some studies have shown that such interspecies variation in sensitivity may amount to three to four orders of magnitude (References [2], [24], [54]). This variation in sensitivity must, of course, be acknowledged when intrepreting data on algal toxicity in a risk assessment context and the use of a battery of species has been proposed to account for the variation (References [8], [21], [33], [53]).

ISO 8692 specifies two green algae — *P. subcapitata* and *Desmodesmus subspicatus* (previously known as *Scenedesmus subspicatus*) — as test species in freshwater. ISO 10253 specifies two marine diatoms, *Skeletonema costatum* and *Phaeodactylum tricornutum* for the marine algae growth inhibition test. A search for data entries on toxicity of chemicals to the algal species included in the ISO and OECD test methods in the US EPA database ECOTOX showed a total of approximately 5 000 data entries of which 42 % are from tests with *P. subcapitata,* which confirms the position of this strain as a reference alga in bioassays (see Figure 3). Among the marine species, *S. costatum* appears to be the one most frequently used.



Key

1 *P. subcapitata*
2 *Chlorella vulgaris*
3 *D. subspicatus*
4 *Anabaena flos-aquae*
5 *Navicula pelliculosa*
6 *S. costatum*
7 *P. tricornutum*
n_e number of entries

a Freshwater algae.

b Marine algae.

Figure 3 — Number of data entries on toxicity to algae in the US EPA database ECOTOX

Some characteristics of the ISO 8692 and ISO 10253 test algae are presented in Table 1. The data were obtained from batch cultures in ISO 8692 (freshwater) and ISO 10253 (sea water) media. The cultures were incubated at 21 °C and continuous illuminance of 80 µmol/m^2 s [2)] and analysed in the late exponential phase. The cell density and mean cell volume were measured using a Coulter Multisizer M3 [3)] equipped with a 100 µm orifice tube. The dry mass was measured after collection of the algae on a glass fibre filter which was dried at 104 °C until constant mass. For the marine species the mass of salts in the water adsorbed in the filters was corrected for. It should be noted that "cell" in this context refers to particles identified by the particle counter. For species forming aggregates as e.g. *D. subspicatus* and *S. costatum*, the true cell volume and mass may be less than indicated in Table 1.

2) Both ISO 8692 and ISO 10253 use the term "light intensity" rather than "illuminance". The photosynthetically available radiance (PAR) is defined as the total irradiance in the wavelength range 400 nm to 700 nm. Both ISO 8692 and ISO 10253 indicate in a note that for light-measuring instruments calibrated in the photometric unit, lux, an equivalent range of 6 000 lx to 10 000 lx is acceptable for testing.

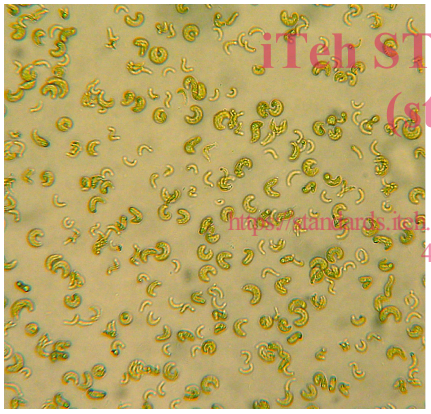
3) Example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Table 1 — Example of size and mass of cells of different ISO test algae grown in freshwater and marine growth media

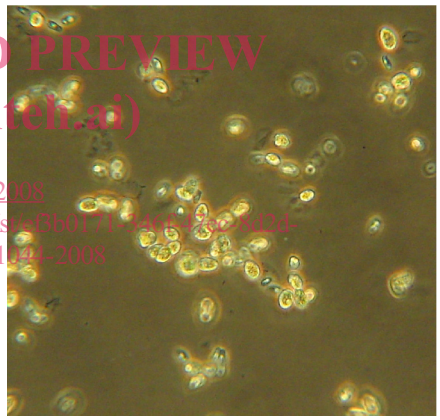
Species	Strain	Mean cell volume μm^3	Mean cell dry mass mg
P. subcapitata	NIVA/CHL 1 ≡ CCAP 278/4	72	$3{,}0 \times 10^{-8}$
D. subspicatus	NIVA/CH 55 ≡ SAG.86.81	139	$5{,}3 \times 10^{-8}$
S. costatum	NIVA/BAC 1	115	$4{,}6 \times 10^{-8}$
P. tricornutum	NIVA/BAC 2	56	$1{,}9 \times 10^{-8}$

5.2 *Pseudokirchneriella subcapitata*

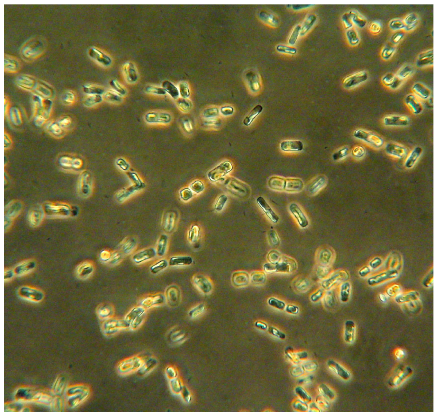
P. subcapitata is the most used test alga in growth inhibition tests and is recommended as test species in several national standards in addition to the international ISO and OECD test protocols. All cultures of this species maintained in the major culture collections (e.g. CCAP 278/4, ATCC 22662, 61.81 SAG, UTEX 1648) stem from a clone culture isolated from a Norwegian river in 1959 (Reference [50]). This is a great advantage from the point of view of reproducibility of test results which is an important aspect of standardisation. The appearance of *P. subcapitata* in culture is shown in Figure 4.



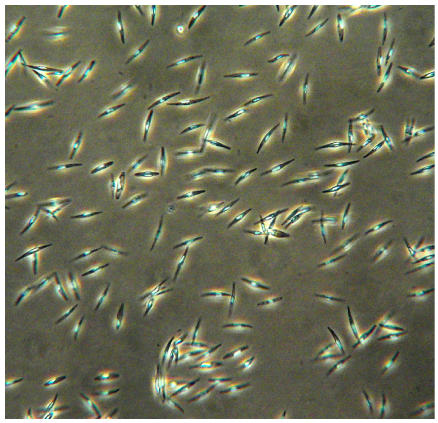
a) ***P. subcapitata***



b) ***D. subspicatus***



c) ***S. costatum***



d) ***P. tricornutum***

Figure 4 — Light microscope photographs of cultures of test algae specified in ISO 8692 and ISO 10253