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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

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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is entirely informative in nature and does not have to be reviewed until the data it provides are considered to be no longer valid or useful.

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

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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.

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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:

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

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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/

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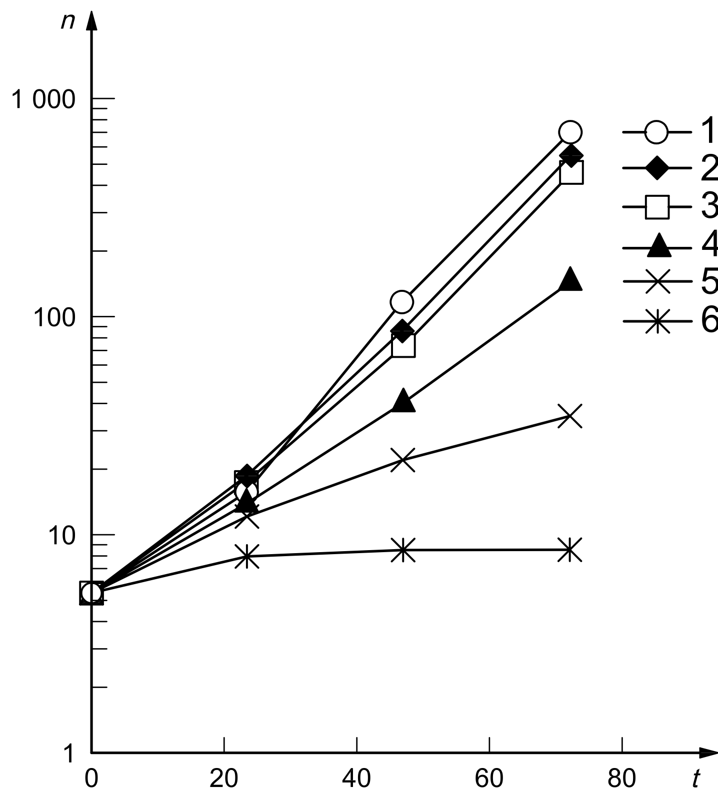

Key

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 $K_2Cr_2O_7$

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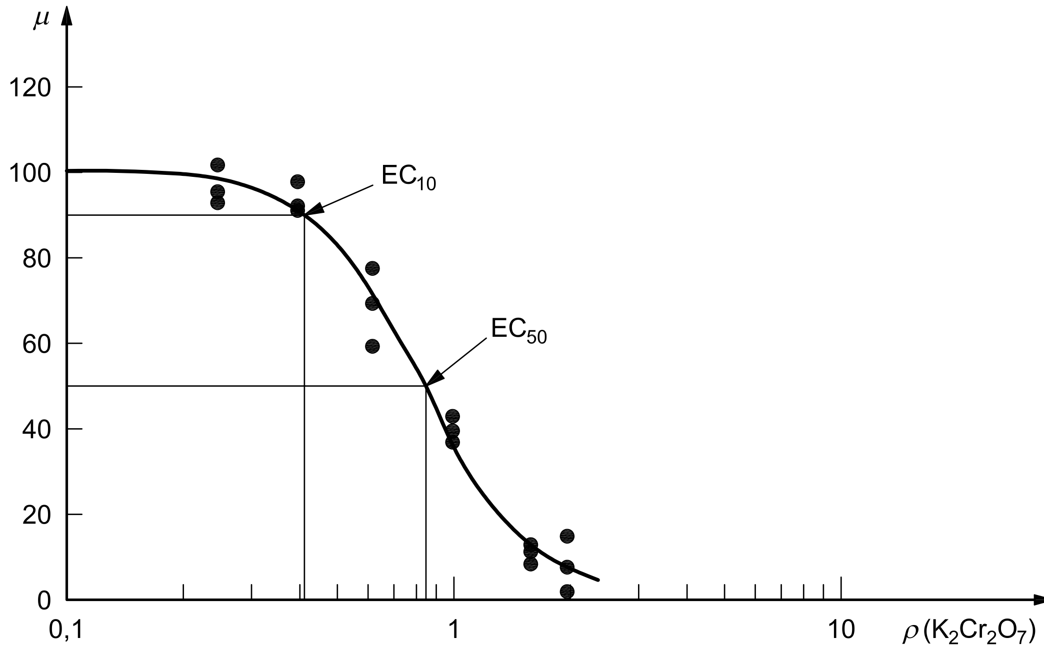

Key

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.

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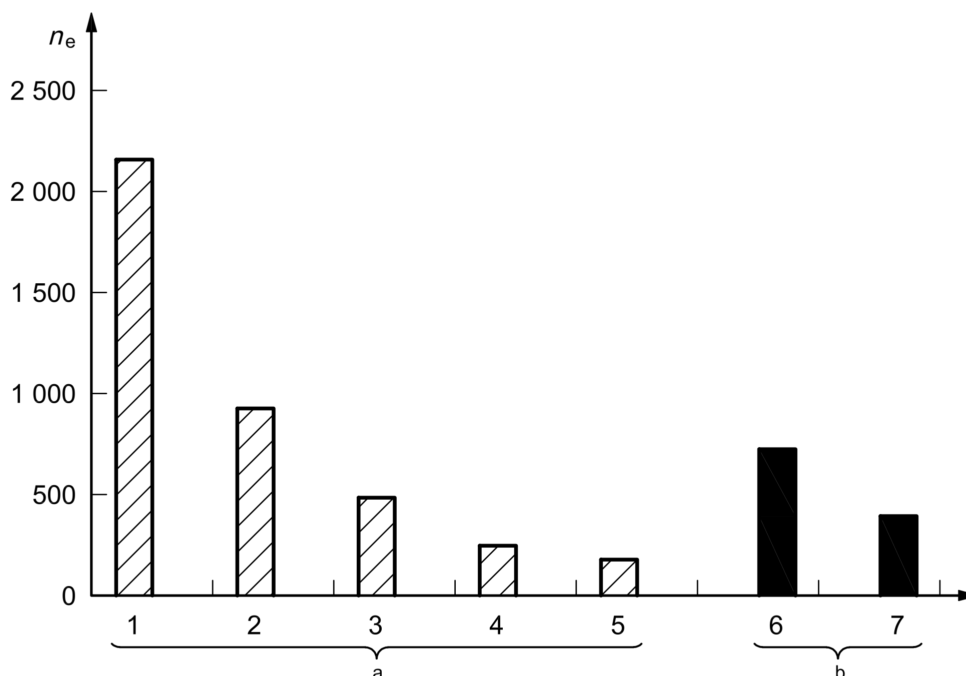

Key

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.

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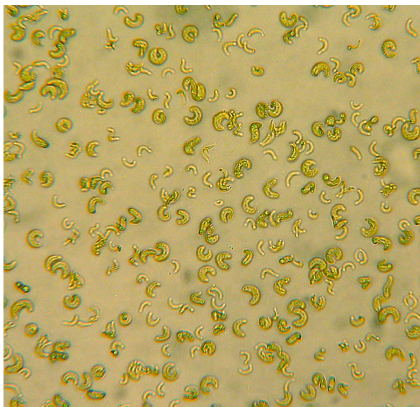
Table 1 — Example of size and mass of cells of different ISO test algae grown in freshwater and marine growth media

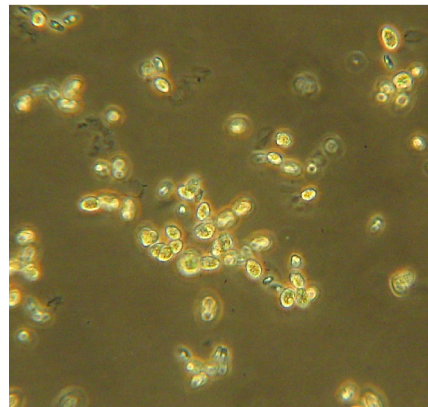
Species	Strain	Mean cell volume µm^3	Mean cell dry mass mg
P. subcapitata	NIVA/CHL 1 ≡ CCAP 278/4	72	$3{,}0 \times 10^{-8}$
D. subspicatus	NIVA/CH 55 ≡ SAG.86.81	139	$5{,}3 \times 10^{-8}$
S. costatum	NIVA/BAC 1	115	$4{,}6 \times 10^{-8}$
P. tricornutum	NIVA/BAC 2	56	$1{,}9 \times 10^{-8}$

5.2 *Pseudokirchneriella subcapitata*

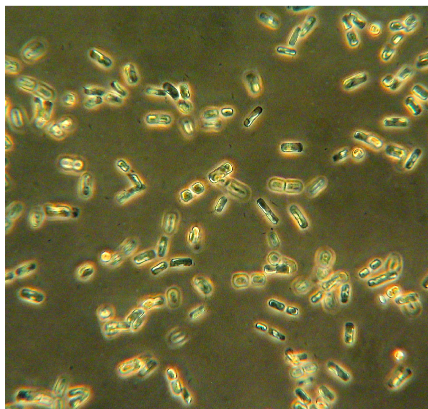
P. subcapitata is the most used test alga in growth inhibition tests and is recommended as test species in several national standards in addition to the international ISO and OECD test protocols. All cultures of this species maintained in the major culture collections (e.g. CCAP 278/4, ATCC 22662, 61.81 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.



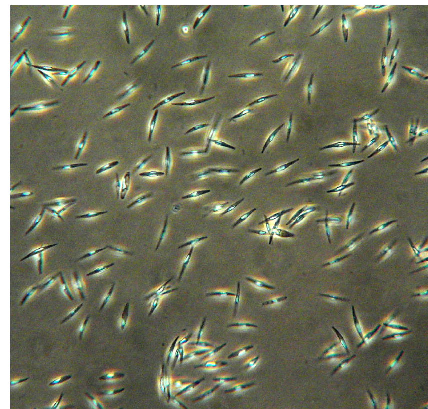
a) ***P. subcapitata***



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

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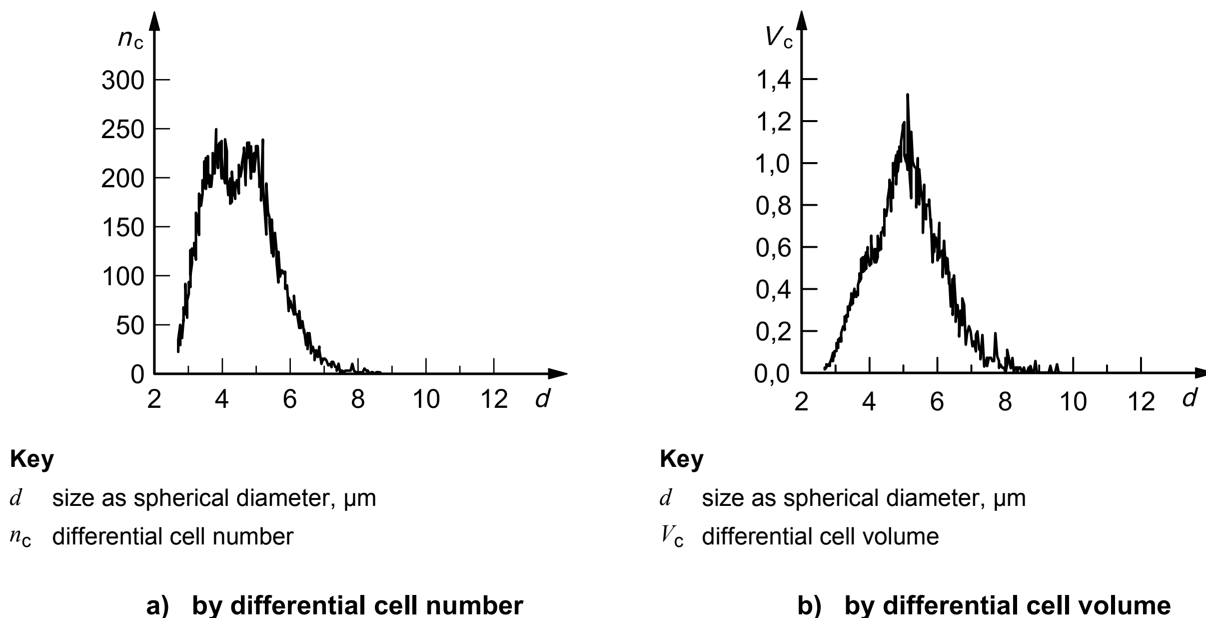
The cells are solitary and easily counted with an electronic particle counter. Up to eight autospores form within the cells and are released when the daughter cells are mature. As a result, a non-synchronous culture contains a mixture of cells of various sizes from small, recently released cells to large cells with visible autospores. An example of the size distribution of such a culture analysed with a Coulter Multisizer is shown in Figure 5.

Sometimes, partial synchronisation occurs in toxicity tests with *P. subcapitata*. In such a case, a size distribution with two peaks may be observed.



Key

d size as spherical diameter, µm

n_c differential cell number

a) by differential cell number

Key

d size as spherical diameter, µm

V_c differential cell volume

b) by differential cell volume

Figure 5 — Size distributions of a culture of *P. subcapitata* in the exponential growth phase in ISO 8692 growth medium

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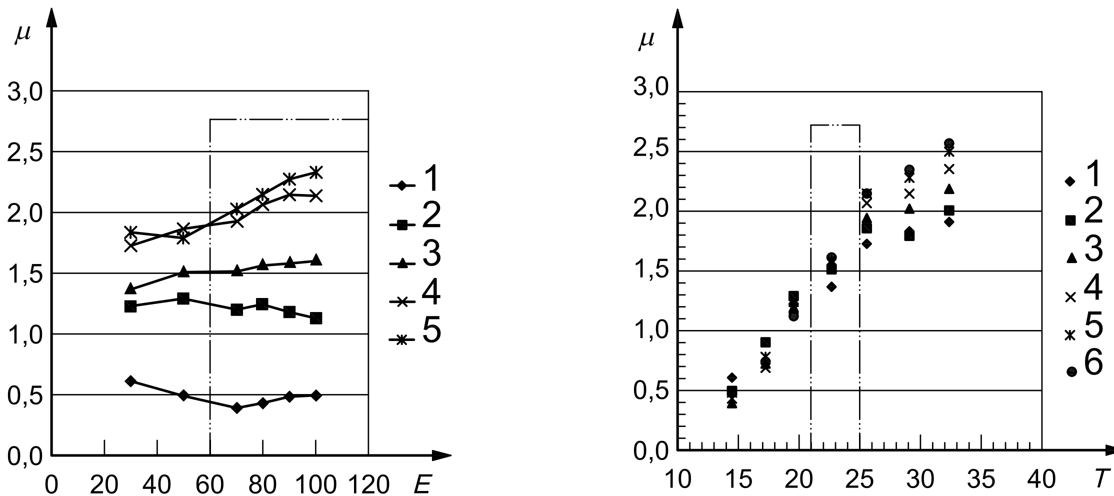
The requirement for growth of *P. subcapitata* in the control cultures in ISO 8692 is that the specific growth rate shall be at least 1,4 d^{-1}. This corresponds to an increase in cell density of a factor of 67 in 72 h. Normally the growth rate is well above this requirement under the conditions specified in ISO 8692. Results of an experiment where cultures of *P. subcapitata* were incubated on a light/temperature gradient are shown in Figure 6.



Key

1 14,5 °C
2 19,6 °C
3 22,7 °C
4 25,6 °C
5 29,1 °C
E illuminance, μmol/m^2 s
μ specific growth rate, d^{-1}

NOTE The range of illuminance specified in ISO 8692 is indicated by the frame inside the figure.

a) plotted against illuminance

Key

1 30 μmol/m^2 s
2 50 μmol/m^2 s
3 70 μmol/m^2 s
4 80 μmol/m^2 s
5 90 μmol/m^2 s
6 100 μmol/m^2 s
T temperature, °C
μ specific growth rate, d^{-1}

NOTE The range of temperature specified in ISO 8692 is indicated by the frame inside the figure.

b) plotted against temperature

Figure 6 — Effects on the growth rate of *P. subcapitata* in ISO 8692 growth medium

In this experiment, the cell density was measured twice every day for 5 d, and the growth rate calculated by regression analysis of cell density against time for the exponential part of the growth curve. The results indicate that the maximum growth rate occurs at a temperature of approximately 32 °C. In the temperature range specified in ISO 8692 (21 °C to 25 °C) a linear increase of growth rate with temperature was observed. At an illuminance of 90 μmol/m^2 s, the growth rate increased by 0,16 d^{-1} for each degree celsius between 17 °C and 27 °C (R^2 = 0,995 1). This means that the growth rate increased from 1,38 d^{-1} at 21 °C to 2,02 d^{-1} at 25 °C.

The effect of illuminance was highly temperature dependent. At temperatures below 20 °C, the growth rate was almost unaffected by illuminance in the range 30 μmol/m^2 s to 100 μmol/m^2 s. At higher temperatures, the growth rate increased with illuminance, but even at 22,7 °C, the growth rate increased only 0,2 d^{-1} between 60 μmol/m^2 s and 100 μmol/m^2 s. The growth response at higher temperatures indicates that growth is saturated at about 90 μmol/m^2 s. The results are in agreement with Reference [35], which reports growth rates of approximately 1,31 d^{-1} at 50 μmol/m^2 s and 1,51 d^{-1} at 100 μmol/m^2 s at a temperature of 20,8 °C.

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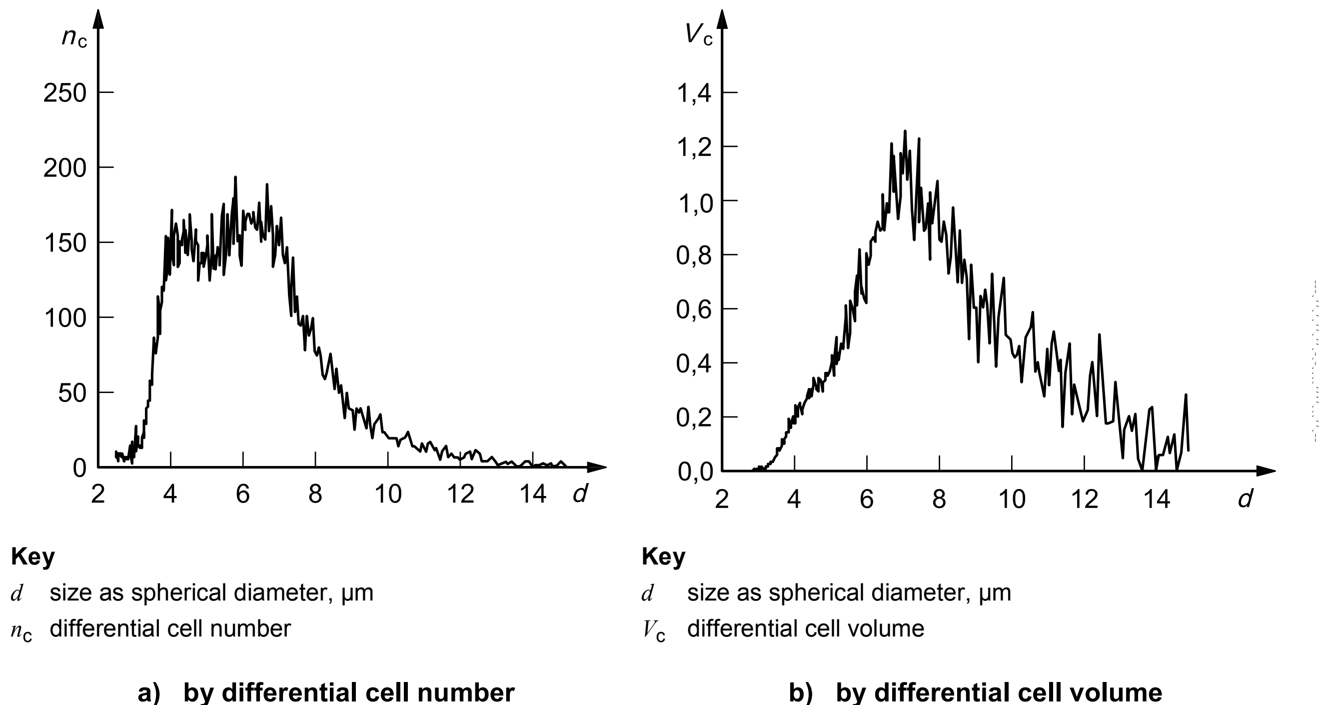
5.3 *Desmodesmus subspicatus*

Different strains of *D. subspicatus* can be obtained from the major culture collections. The strain specified in ISO 8692 (SAG 86.81 ≡ CCAP 276/22 ≡ UTEX 2594) was originally isolated by Brinkmann from an aquarium in Berlin in 1953. According to Reference [17], this strain grows mainly as single cells in culture. It may, however, also occur in colonies (coenobia) of four cells to eight cells arranged in a row or as loose aggregates without systematic organisation [see Figure 4 b)]. Cell length and width reported in Reference [17] are 3,5 µm to 5,0 µm and 4,0 µm to 6,0 µm, respectively. The size distribution of a culture in ISO 8692 medium is shown in Figure 7.



Key

d size as spherical diameter, µm

n_c differential cell number

a) by differential cell number

Key

d size as spherical diameter, µm

V_c differential cell volume

b) by differential cell volume

Figure 7 — Size distributions of a culture of *D. subspicatus* in the exponential growth phase in ISO 8692 growth medium

The size distribution plots indicate that the single cells with a spherical diameter of approximately 4 µm to 7 µm dominate. The long tail in the distributions with spherical diameters up to at least 15 µm, which is especially pronounced in the differential volume plot, is probably caused by the presence of aggregates.

5.4 *Skeletonema costatum*

Several strains under the name of *S. costatum* are available in the major culture collections. In ISO 10253, two strains are specified: CCAP 1077/1C (origin: North Sea, post-1970) and NIVA BAC 1 (origin: Oslo Fjord, 1962). In the CCAP strain catalog, two other strains (CCAP 1077/3 and 1077/5) are listed as strains used for ecotoxicity testing, while this is not the case for CCAP 1077/1C. Probably several different strains are used as test organisms. The issue is further complicated by recent taxonomic revisions which have revealed that *S. costatum* is a “species complex” rather than a true species, and several previous “*S. costatum*” strains have been renamed as *S. grethae* (e.g. CCAP 1077/3 and 1077/4) or *S. pseudocostatum* (CCAP 1077/7) (see Reference [49]). Apparently CCAP 1077/1C has not yet been examined for taxonomic revision. The stain NIVA BAC 1 has recently been examined and allocated to *S. pseudocostatum*.

The size distribution of the strain NIVA BAC 1 grown in ISO 10253 medium is shown in Figure 8. The distribution shows a fairly narrow peak around 6 µm, indicating that the culture is predominantly single celled or that the particle counter is able to distinguish each cell even if they are connected in chains [see Figure 4 c)].

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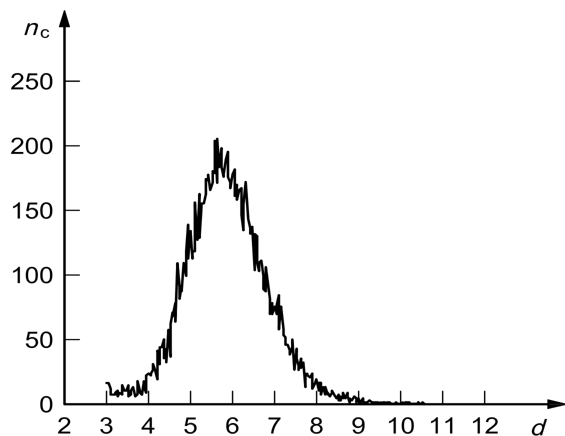
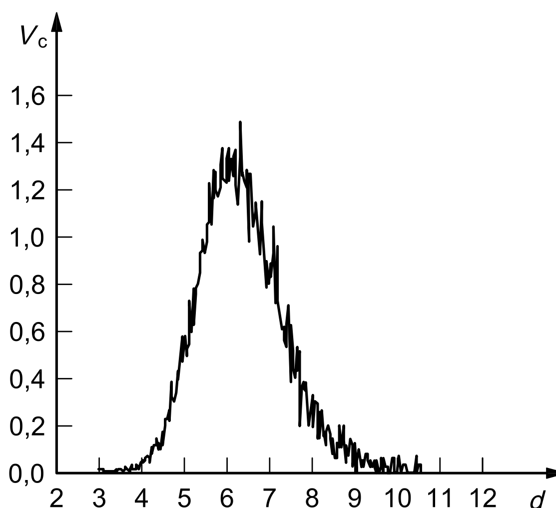


Key

d size as spherical diameter, µm

n_c differential cell number

a) by differential cell number



Key

d size as spherical diameter, µm

V_c differential cell volume

b) by differential cell volume

Figure 8 — Size distributions of a culture of *S. costatum* strain NIVA BAC 1 (*S. pseudocostatum*) in the exponential growth phase in ISO 10253 growth medium based on natural sea water

During the development of ISO 10253, a comparison of different *S. costatum* strains was performed (Reference [13]). The strains included were ISTPM P4, NIVA BAC 1, CCAP 1077/1C, CCAP 1077/3, and CCAP 1077/5. The study showed differences in cell size and chain length between the strains and suggested that these differences were correlated to differences in sensitivity to the reference substances potassium dichromate and 3,5-dichlorophenol (DCP). The results are summarized in Table 2.

Table 2 — Results of tests of reference substances with different strains of *S. costatum*

Strain	Potassium dichromate EC_{50} mg/l	3,5-Dichlorophenol EC_{50} mg/l	Control growth rate d^{-1}	Mean particle size in control μm^3/cell
ISTPM P4	6,6	1,4	2,64 to 3,36	563
NIVA BAC 1	2,6	0,95	1,68 to 2,11	120
CCAP 1077/1C	3,9	1,2	1,65 to 1,94	169
CCAP 1077/3	7,4	1,4	2,64 to 2,40	596
CCAP 1077/5	5,7	1,3	2,25 to 2,40	454

In future revisions of ISO 10253, the implications of the recent taxonomic revision of the "*Skeletonema costatum* complex" should be considered. Preferably only one strain of *S. costatum* should be recommended as test alga.

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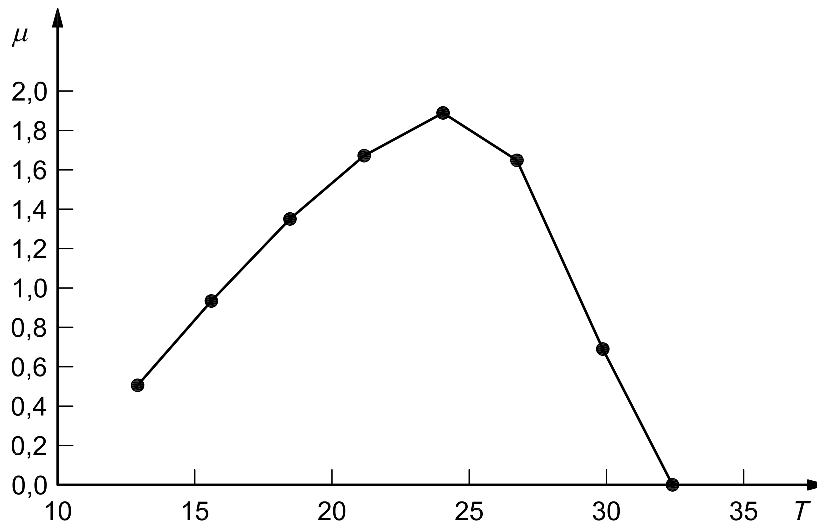
The effect of temperature on the growth rate of strain BAC 1 in ISO 10253 growth medium based on natural sea water is shown in Figure 9. The specific growth rate was calculated from the increase in biomass density (measured as total cell volume with a Coulter Multisizer) in batch cultures after 70 h incubation at 80 µmol/m^2 s. The results showed an almost linear increase in growth rate with temperature in the range 13 °C to 21 °C. Linear regression of the data show a slope of 0,141 d^{-1} per degree celsius (R^2 = 0,996 2). The optimum temperature for this strain appears to be around 24 °C and growth was completely inhibited at 32,5 °C. It should be noted that the growth rates observed in this experiment are somewhat lower than what is normally found in control cultures in toxicity tests with the same strain under similar conditions. The specific growth rates obtained in control cultures incubated at approximately 75 µmol/m^2 s and 21 °C are usually around 2,0 d^{-1}. The low calculated growth rates in the experiment are probably due to the fact that the growth rate was not exponential throughout the incubation period.



Key

T temperature, °C

μ specific growth rate, d^{-1}

Figure 9 — Effect of temperature of average growth rate (0 h to 70 h) of *S. costatum* (NIVA BAC 1) incubated in ISO 10253 natural sea water medium at 80 µmol/m^2 s

Because of the high growth rate of most *S. costatum* strains (see Table 2), exponential growth is often not maintained for 72 h under the specified test conditions, even if the inoculum density is held at 5 × 10^3 cells/ml. This may have the effect that partly inhibited cultures catch up with the control cultures during the last day of the test as shown in Figure 10 a). In this example, the significant growth inhibition that was observed in 1 mg/l of 3,5-DCP after 48 h was no longer seen after 72 h. Deviation from exponential growth of *S. costatum* was noted also in the ISO and PARCOM ringtests (References [1], [15]). It is obvious that it is more appropriate in this case to base the calculations of growth rates on the first 48 h of the test when the growth in the control cultures is still exponential. In ISO 10253:2006, such a recommendation was included.

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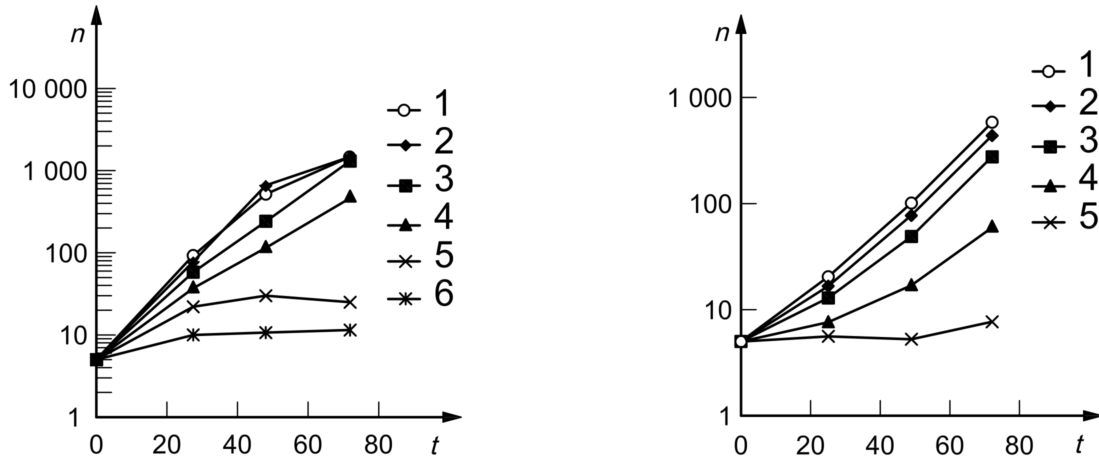

Key

1	ρ(3,5-DCP) = 0 (control)	5	ρ(3,5-DCP) = 2,5 mg/l
2	ρ(3,5-DCP) = 0,63 mg/l	6	ρ(3,5-DCP) = 4 mg/l
3	ρ(3,5-DCP) = 1 mg/l	n	cell density, 10^3 cells/ml
4	ρ(3,5-DCP) = 1,6 mg/l	t	time, h

a) *S. costatum* (NIVA BAC 1)

Key

1	ρ(3,5-DCP) = 0 (control)	5	ρ(3,5-DCP) = 4 mg/l
2	ρ(3,5-DCP) = 1 mg/l		
3	ρ(3,5-DCP) = 1,6 mg/l	n	cell density, 10^3 cells/ml
4	ρ(3,5-DCP) = 2,5 mg/l	t	time, h

b) *P. tricornutum* (NIVA BAC 2)

Figure 10 — Growth curves obtained from 3,5-dichlorophenol toxicity tests

5.5 *Phaeodactylum tricornutum*

The strains of *P. tricornutum* specified in ISO 10253 are CCAP 1052/1A (origin: off Plymouth, UK) and NIVA BAC 2. The origin of BAC 2 is not known. It was obtained from the University of Oslo and it may be identical to CCAP 1052/1A. Size distributions of a culture of the strain BAC 2, grown in ISO 10253 medium based on natural sea water, are shown in Figure 11. *P. tricornutum* appears as single cells in culture and the variation in cell size is usually small. Typical growth rates of this strain in ISO natural sea water medium are around 1,6 d^{-1} at 21 °C and 75 μmol/m^2 s.

From a technical point of view, *P. tricornutum* is an excellent test organism (low variation in cell size, does not form aggregates, intermediate growth rate). In contrast to *S. costatum*, exponential growth is usually maintained for 72 h under the test conditions specified in ISO 10253 [see Figure 10 b)]. In spite of this, *P. tricornutum* has been used less frequently than *S. costatum* in marine algae toxicity tests (Figure 3). The main reason for this is probably that *S. costatum* is considered as a more relevant species from an ecological point of view. Furthermore, the requirement to use *S. costatum* as a test organism in the PARCOM test programme for offshore chemicals has contributed to its popularity. Nevertheless, *P. tricornutum* was included in a ring test organised by PARCOM in 1991. In comparison to *S. costatum*, *P. tricornutum* was less sensitive to the reference substance 3,5-DCP (which is in agreement with the result of the ISO ring test) but more sensitive to two offshore chemicals (Reference [1]).

P. tricornutum inhabits brackish waters and has a wider salinity tolerance than *S. costatum*. It is therefore a suitable species for tests in brackish waters or for testing toxic effects of “fresh” waste waters in sea water. By selection of different species specified in ISO 8692 and ISO 10253, toxicity tests may be performed at all salinities from freshwater to sea water. Figure 12 shows the effect of salinity on the growth rate of *P. subcapitata*, *P. tricornutum* (NIVA BAC 2), and *S. costatum* (NIVA BAC 1) in ISO media prepared with different proportions of distilled water and natural sea water.

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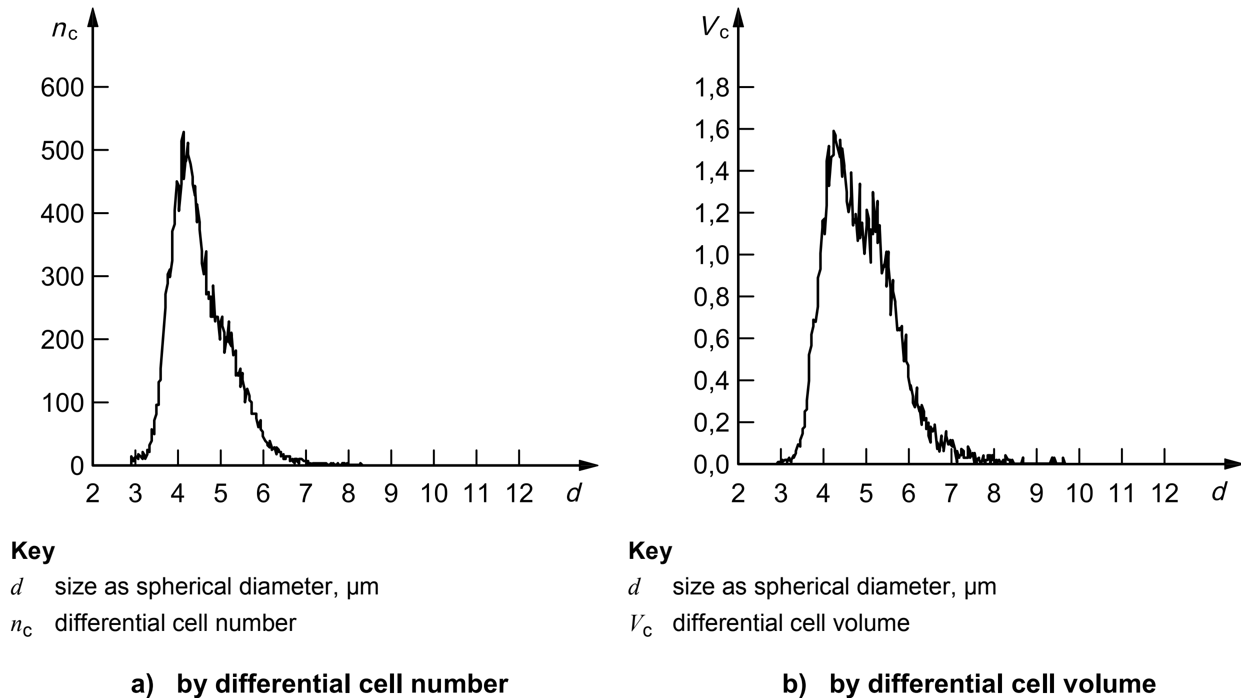

Figure 11 — Size distributions of a culture of *P. tricornutum* strain NIVA BAC 2 in the exponential growth phase in ISO 10253 growth medium based on natural sea water

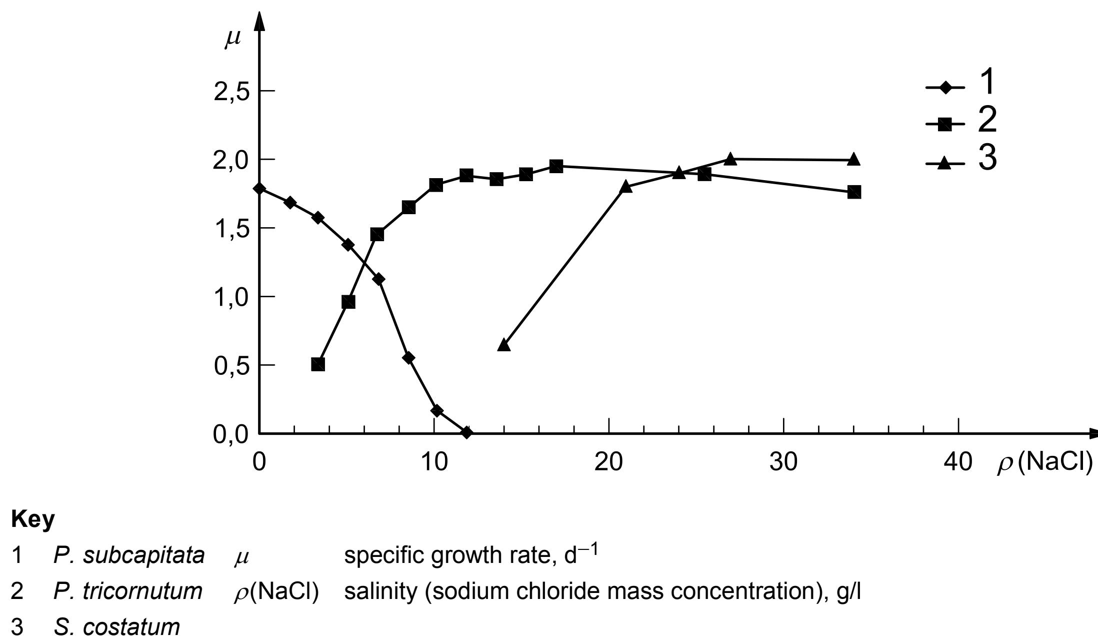

Figure 12 — Growth of *P. subcapitata, P. tricornutum* (NIVA BAC 2), and *S. costatum* (NIVA BAC 1) at different salinities

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6 Test conditions

6.1 Growth medium

The composition of the growth medium may affect the toxic response of the test algae, either because of chemical interactions that affect the biological availability of the test substance or because of its effects on the physiological status of the algae. Influence of the growth medium composition on the expression of toxicity in algal tests has in particular been demonstrated in tests of toxic metals, where pH, hardness and presence of chelating agents are important factors (Reference [22]). Of course, the same factors affect the toxicity to algae in their natural environments. A standardised growth medium can therefore not be representative to all natural environments, a fact that must be recognised when interpreting toxicity data from tests with algae as well as for other aquatic organisms.

Test media for the ISO growth inhibition tests have been designed to allow unrestricted exponential growth of the recommended test algae under the specified culturing conditions, avoiding as much as possible excessive concentrations of ingredients that may cause chemical interference with various test substances. For the freshwater test (ISO 8692) a specific growth medium was developed after experience from early ring tests indicated that differences in composition of the medium affected the test results. It was also concluded that a high buffering capacity was required in order to reduce pH variations which were identified as one of the main reasons for deviations in results between different laboratories (Reference [13]). In the third ISO ring test, a medium buffered with 150 mg/l $NaHCO_3$ and NO_3^- nitrogen source were used. Still, a substantial pH increase was observed after 96 h incubation (Reference [14]). Later it was decided to restrict the exposure to 72 h, and modifications of the medium were made, including lowering the concentration of $NaHCO_3$ to 50 mg/l and replacing K_2HPO_4 with KH_2PO_4. NO_3^- was replaced with NH_4^+ as a nitrogen source in order to counteract pH increase that tended to occur towards the end of the test when the CO_2 demand from algal photosynthesis exceeded the mass transport of CO_2 across the water/gas interface.

Chelating agents are required to avoid precipitation of iron in the growth medium. However, chelators may reduce the availability and effect of toxic metals. EDTA (ethylenediaminetetraacetate) is the most commonly used chelator in artificial algal growth media. In ISO 8692:1989, the medium contained 80 µg/l $FeCl_3 \cdot 2H_2O$ and 100 µg/l of $Na_2EDTA \cdot 2H_2O$. Later, the iron content was reduced to 64 µg/l of $FeCl_3 \cdot 2H_2O$ in order to obtain equimolar concentrations of EDTA and complexing trace metals. Still the medium is not optimal for testing metal toxicity. Different modifications of the medium have been used to overcome this shortage. In Reference [18], 32 µg/l humic acid replaced EDTA in order to create a medium with metal complexing properties more similar to natural surface waters. Some reported tests (e.g. Reference [31]) employed synthetic growth media without organic chelators. Experience has shown, however, that the growth of algae is highly variable in such media. Iron is required as a trace element in the medium, and the concentration required to support the production of biomass in a 72 h test is above the solubility of iron(III) hydroxide. The effect of excluding EDTA in the medium is demonstrated in Figure 13, which shows the growth performance of *P. subcapitata* in the ISO medium with and without EDTA. The two media were inoculated with 5×10^3 cells/ml of *P. subcapitata* grown in medium with EDTA. The average specific growth rate in six cultures with EDTA was 1,68 d^{-1} with a coefficient of variation of 2,9 %. In nine cultures without EDTA, the average growth rate was 1,15 d^{-1} with a coefficient of variation of 21 %.

Studies performed at the Danish Technical University demonstrated that the concentration of iron and EDTA could be reduced without affecting the growth performance of *P. subcapitata* and based on this finding, a recommendation for a modified medium for testing of metals was included in ISO 14442, a guidance document for tests with poorly soluble materials, volatile compounds, metals and waste water. The proposal is to use 20 µg/l (0,074 µmol/l) of $FeCl_3 \cdot 2H_2O$ and 22,6 µg (0,083 µmol/l) $Na_2EDTA \cdot 2H_2O$ in the medium and to reduce the test duration from 3 d to 2 d.

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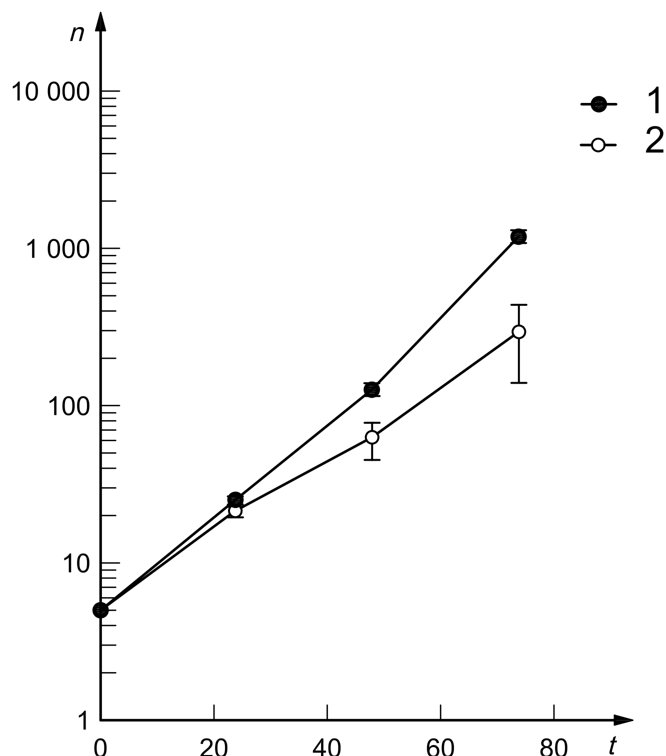

Key

1 with EDTA

2 without EDTA

n cell density, 10^3 cells/ml

t time, h

Figure 13 — Growth of *P. subcapitata* in ISO 8692 growth medium with EDTA (0,27 µmol/l) and without EDTA (mean values and standard deviation)

The ISO 10253 marine test can be performed in synthetic or natural seawater of (30 ± 5) g/kg salinity. The sea water is spiked with nutrients in order to support exponential growth of the test algae for 72 h. The nutrient medium has been adopted from ASTM E 1218[58]. Because of the high content of anions in the sea water, a higher concentration of EDTA than in the freshwater medium is required to avoid precipitation of iron. During the development of the ISO 10253 test, some work was done to investigate the possibility of reducing the content of EDTA in the medium in order to make it more suitable for testing metals. Experiments at NIVA performed in 1991 showed that the BAC 1 strain grew as well in a natural sea water medium with iron and EDTA reduced to the levels used in the freshwater medium (ISO 8692) provided that the zinc concentration was also reduced from 150 µg/l to 15 µg/l. Reduced concentrations of iron and EDTA were also used by several laboratories that participated in a ring test organised by PARCOM in 1991 (Reference [1]). Some laboratories reported, however, that *S. costatum* would not grow as well in the modified medium as in the original ISO 10253 medium. This may be due to the differences in the composition of the natural sea water used by different laboratories and the fact that different strains of *S. costatum* may have different nutrient requirements. It was found that any modifications of the ISO 10253 medium would need to be based on extensive trials in many laboratories and the original formulation with 149 µg/l iron and 15 mg $Na_2EDTA·2H_2O$ has been retained.

Since the growth inhibition tests have been designed to allow exponential growth in a batch culture, the algae will not experience nutrient limitation. This is different from the natural habitats of most microalgae, where the growth is often limited by the supply of a limiting nutrient. It has been argued that the stress from nutrient limitation may increase the sensitivity of algae to toxic substances and some studies indicate that this may be the case for the effects of metals on *P. subcapitata* (References [3], [30]). Other studies have also shown that effects on nutrient uptake by algae may occur at lower concentrations of toxicants than those affecting growth (References [38], [46]), and such effects may have important consequences for the competitive ability of algae

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in a nutrient-limited environment. However, it may also be argued that the potential for a toxicant to cause a significant effect on the growth of an alga is higher when the growth rate is high than when it is reduced as a consequence of nutrient limitation. The fact that the growth inhibition tests are carried out without the additional stress of nutrient limitation should therefore not disqualify them as a basis for environmental risk assessment. In any case, studies of growth inhibition under controlled nutrient limitation require the use of continuous cultures which is a technique demanding too many resources for the purpose of International Standard toxicity tests.

6.2 pH control

The pH of the test medium affects the toxicity of many chemicals either by its effect on speciation of the chemicals or by competitive effects of hydrogen ions on the binding to the uptake sites. Ionisable organic substances such as chlorophenols are more lipophilic and exert a higher toxicity in their un-ionised than in their ionised form (Reference [51]). For metals, the effect of pH may be very complicated since it may affect both the forming of complexes of different biological availability and the binding at the cell surface ligands (References [5], [18], [45]).

In batch algal cultures as well as in the natural environments of plankton algae, the photosynthetic activity of the algae affects the pH of the aquatic environment through displacement of the carbonate system. The algae assimilate CO_2, dissolved in the water as a carbon source for photosynthesis. The consumption of dissolved CO_2 will trigger diffusion of CO_2 from the atmosphere to restore the equilibrium concentration. In a batch culture, the CO_2 demand increases with the biomass. When the rate of CO_2 consumption exceeds the mass transfer of CO_2 through the water surface, CO_2 will be derived from HCO_3^- which results in liberation of OH^- and an increase in pH. The mass balance of carbon in algal batch cultures has been described in Reference [12].

The ISO 8692 freshwater medium is mainly buffered by HCO_3^-. The content of $NaHCO_3$ (50 mg/l) gives a pH of approximately 8,1 when the medium is in equilibrium with CO_2 in the atmosphere. When inoculated with algae and incubated as described in ISO 8692, the pH is usually fairly stable during the first 48 h. Between 48 h and 72 h an increase in pH is often observed, which is an indication that the mass transfer of CO_2 is not sufficient to support the photosynthetic demand. The methods specify validity criteria stating that the control pH shall not have increased more than 1,5 in freshwater tests (ISO 8692) and 1,0 in marine tests (ISO 10253). By selecting the appropriate incubation conditions within the recommended ranges these criteria can easily be fulfilled.

The rationale for having validity criteria for pH changes was discussed at length during the development of ISO 8692 and ISO 10253. The main argument for having a strict control of pH is to create stable and well defined chemical conditions under which the toxic effects of the chemicals are studied. This is, of course, particularly important for substances that show pH-dependent speciation.

Furthermore, a great increase in the pH value towards the end of the incubation period is a strong indication of carbon-limited growth. Under these conditions, exponential growth can no longer be maintained.

On the other hand, it has been argued that the pH changes that occur in algal toxicity tests are caused by the algae themselves and hence may be considered an inherent property of dense algal populations. Therefore it may even be desirable that the pH varies during the test.

Another reason to allow higher pH variations is that these variations will occur only in the cultures that are not, or only marginally, inhibited by the test substance. Near the EC_{50} value, the final biomass density is usually less than 10 % of the control, and consequently the CO_2 demand is not high enough to induce significant changes in the pH of the medium. Since pH changes mainly occur in the low-effect part of the response curve and during a short period of the total exposure duration, the response curve mainly reflects the toxic effect of the test substance at the initial pH value of the test medium.

Because of the importance of pH on the toxic effect of many substances there may be a need to perform tests at different pH values. Different options for such modifications of the test procedures are described in ISO 14442. Recommendations include addition of organic buffers such as MES (2-morpholinoethanesulfonic acid) or HEPES [*N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid] to the medium. Another buffer, MOPS (3-morpholinopropanesulfonic acid), has been used in studies of toxicity of metals at pH values in the range 6 to 8 (Reference [18]).

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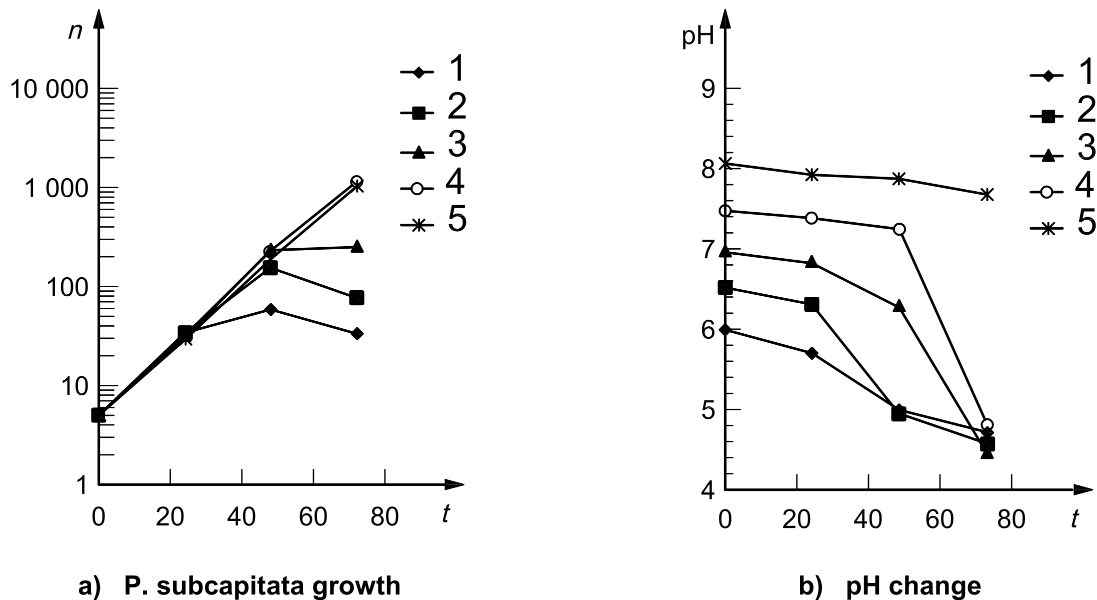
Different initial pH values in the medium may be obtained without using organic buffers by varying the concentration of HCO_3^-. With only HCO_3^- and air as a buffering system, pH values in the range 7 to 9 can be obtained. Lowering the HCO_3^- concentration, however, also leads to less stable pH levels in the growing cultures. Also bear in mind that utilisation of NH_4^+ as the source of nitrogen in the ISO 8692 freshwater medium liberates H^+ ions and causes the pH in cultures to decrease slightly with time if the mass transport conditions are good. In fact, the pH decrease caused by assimilation of nitrogen from NH_4^+ may inhibit the growth of algae if the HCO_3^- concentration is too low. This can be demonstrated by results from an experiment with *P. subcapitata* grown in ISO 8692 medium where the $NaHCO_3$ concentration was varied from 0 mg/l to 50 mg/l (see Figure 14). All cultures grew at the same rate during the first day, but pH decreased to inhibiting levels in all media with 5 mg/l or less of $NaHCO_3$. When the same experiment was performed in media where NH_4^+ had been replaced with NO_3^-, exponential growth was obtained in all media throughout the 72 h exposure period (see Figure 15). In this case, however, the pH increased in all media and the increase was inversely proportional to the concentration of HCO_3^-.



a) P. subcapitata growth

b) pH change

Key

1 $\rho(NaHCO_3) = 0$

2 $\rho(NaHCO_3) = 1,5$ mg/l

3 $\rho(NaHCO_3) = 5$ mg/l

4 $\rho(NaHCO_3) = 15$ mg/l

5 $\rho(NaHCO_3) = 50$ mg/l

n cell density, 10^3 cells/ml

pH acidity-alkalinity scale

t time, h

Figure 14 — Changes over time in ISO 8692 medium prepared with different concentrations of $NaHCO_3$

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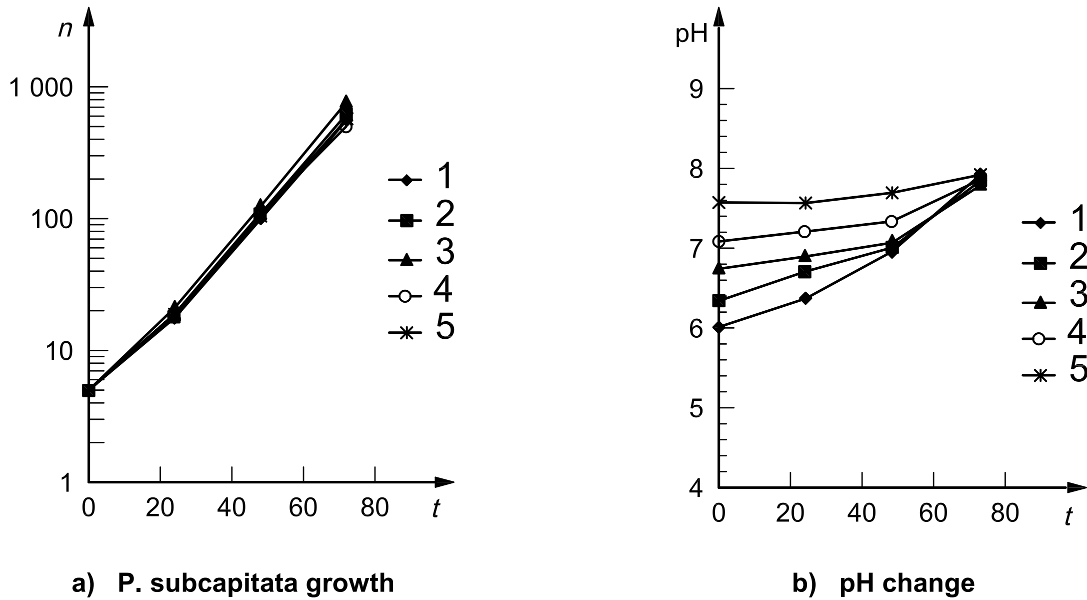

a) P. subcapitata growth **b) pH change**

Key

1 $\rho(NaHCO_3)$ = 0
2 $\rho(NaHCO_3)$ = 1,5 mg/l
3 $\rho(NaHCO_3)$ = 5 mg/l
4 $\rho(NaHCO_3)$ = 15 mg/l
5 $\rho(NaHCO_3)$ = 50 mg/l
n cell density, 10^3 cells/ml
pH acidity-alkalinity scale
t time, h

Figure 15 — Changes over time at different concentrations of $NaHCO_3$ in ISO 8692 medium where NH_4^+ has been replaced by NO_3^- as a nitrogen source

6.3 Inoculum density

For proper characterisation of the batch culture algal test system, the initial biomass must be known. The inoculum biomass density in ISO 8692 and ISO 10253 has been set as low as practical in order to minimise unwanted effects of high biomass density during the test. Ideally, the biomass concentration in a static toxicity test should be low enough not to significantly affect the concentration of the test substance in solution. For substances that strongly adsorb to or accumulate in the biomass, e.g. certain metals and lipophilic organic substances, a high biomass density may lead to depletion of the test substance from the water phase. An algal growth inhibition test is a dynamic system with exponentially increasing biomass density, which means that the portion of the test substance adsorbed to the biomass increases during exposure. Whether this process leads to a significant depletion of the water phase depends on the partition coefficient of the test substance between the water and the algae as well as the biomass density. When such depletion of the water phase occurs, further growth of the algae will lead to a dilution of the accumulated test substance in the algal cells and, consequently, a reduced toxic effect. Decreasing sensitivity of the test system with increasing inoculum density has been demonstrated in tests with copper (References [10], [36]). Similar effects may be expected to occur in tests with highly lipophilic organic substances. Due to the dependence on biomass density, the masking effect on toxicity due to dilution in the biomass will be most prominent in slightly inhibited cultures. In severely or totally inhibited cultures, the biomass density and the portion of the test substance adsorbed to the biomass remain low throughout the test.

The density of inoculum will affect the duration of the exponential growth phase in a given growth medium. It must therefore be selected to match the nutrient content of the medium and the selected growth conditions in order to allow exponential growth for 72 h. Furthermore, the CO_2 requirement for photosynthesis increases with the cell density and may lead to increase in pH in the growth medium exceeding the specified validity criteria for pH in ISO 8692 and ISO 10253.

The decisive practical aspect on inoculum biomass density is that of measuring techniques. While biomass on the dry basis ought to be selected as the reference parameter from a theoretical standpoint, in practice, the

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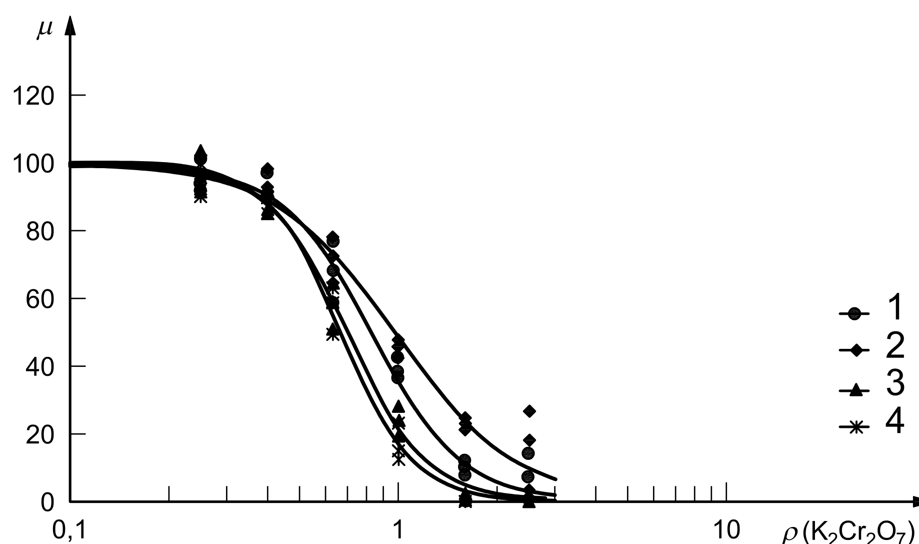
biomass concentration cannot be routinely measured but typically can be calculated from measurement of a surrogate parameter using a conversion factor. These techniques include e.g. photometric measurements of optical density, microscopic counting, counting with electronic particle counters, and fluorometric measurements of *in vivo* or *in vitro* chlorophyll. Due to variability in cell size and pigmentation, the different surrogate parameters will not always give identical growth curves. Also the response pattern may be different if the toxicant affects cell size or pigmentation. The effect of different techniques has been studied in tests of the reference substances $K_2Cr_2O_7$ and 3,5-DCP with *P. subcapitata*. Cell number and total cell volume were measured with a Coulter Multisizer, and fluorescence with a Cytofluor 2300 [4] plate scanner using 485 nm and 685 nm as excitation and emission wavelengths. DCMU-enhanced fluorescence was measured after addition of 5 µmol/l of DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea]. The response plots for the test with $K_2Cr_2O_7$ show that the apparent growth inhibition is higher when measured as fluorescence than on total cell volume (see Figure 16). Growth rates based on cell numbers give an intermediate response curve. This indicates that $K_2Cr_2O_7$ affects the chlorophyll content of the cells and that the mean volume of the cells increases with the concentration of $K_2Cr_2O_7$. The EC_{50} and EC_{10} values are listed in Table 3.



Key

μ	specific growth rate as a percentage of control
$\rho(K_2Cr_2O_7)$	potassium dichromate mass concentration, mg/l
1	cell numbers
2	total cell volume
3	*in vivo* fluorescence
4	DCMU-enhanced fluorescence

Figure 16 — Concentration/response plots for the effect of $K_2Cr_2O_7$ on *P. subcapitata*

4) 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.

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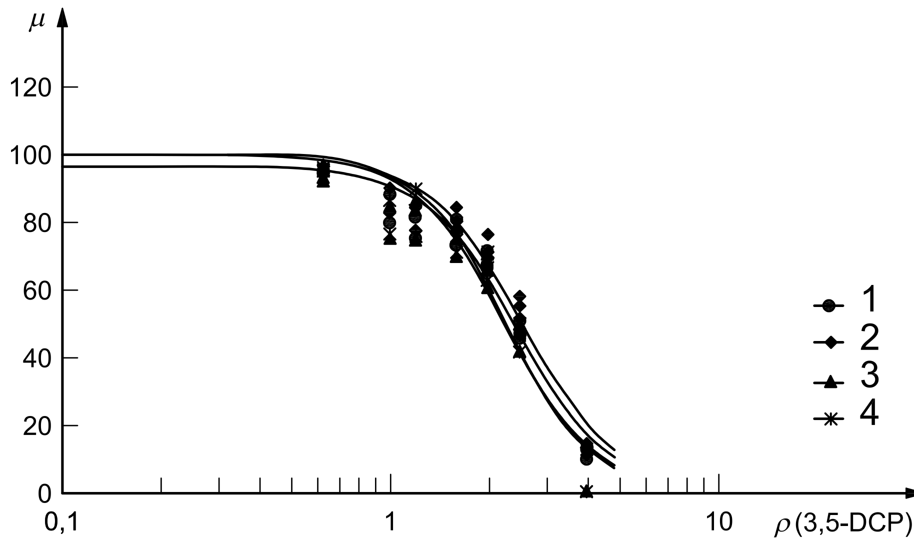
Table 3 — EC values calculated on the basis of different parameters for biomass in a test of $K_2Cr_2O_7$with *P. subcapitata*

Parameter	EC_{50} mg/l	95 % **Confidence interval**	EC_{10} mg/l	95 % **Confidence interval**
Cell numbers	0,83	0,77 to 0,88	0,41	0,34 to 0,47
Total cell volume	0,99	0,93 to 1,07	0,38	0,33 to 0,45
In vivo fluorescence	0,69	0,66 to 0,71	0,37	0,34 to 0,40
Fluorescence with DCMU	0,66	0,64 to 0,89	0,38	0,35 to 0,41

In a test of 3,5-DCP, less difference between the results based on different measuring techniques was observed (see Figure 17). The data based on cell volume overlap those based on cell number, which means that 3,5-DCP had little effect on the cell size. Fluorescence measurements showed a higher degree of growth inhibition at high concentrations of 3,5-DCP, but the calculated EC_{10} and EC_{50} values were rather independent of the measuring technique in this case (see Table 4).



Key

μ	specific growth rate as a percentage of control
ρ(3,5-DCP)	3,5-DCP mass concentration, mg/l
1	cell numbers
2	total cell volume
3	*in vivo* fluorescence
4	DCMU-enhanced fluorescence

Figure 17 — Concentration/response plots for the effect of 3,5-dichlorophenol on *P. subcapitata*

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Table 4 — EC values calculated on the basis of different parameters for biomass in a test of 3,5-dichlorophenol with *P. subcapitata*

Measurement	EC_{50} mg/l	95 % **Confidence interval**	EC_{10} mg/l	95 % **Confidence interval**
Cell numbers	2,4	2,3 to 2,6	1,2	1,1 to 1,4
Total cell volume	2,5	2,3 to 2,6	1,2	1,0 to 1,3
In vivo fluorescence	2,1	2,0 to 2,2	1,2	1,0 to 1,3
Fluoresence with DCMU	2,2	2,1 to 2,3	1,2	1,1 to 1,4

The results show that the parameters used for calculation of growth rate may have significant effects on the response curve and effective concentrations derived from it. These effects tend to be more pronounced in the most inhibited cultures. Therefore high effective concentrations (e.g. EC_{50}) are more dependent on the measured parameter than low effective concentrations (e.g. EC_{10}).

Optical density, which is another option for recording the growth, was not included in the comparison of measuring techniques reported above. Measurements of the final biomass density in strongly inhibited cultures require use of a long pathlength for optical density measurement. This introduces a risk for interference from suspended or coloured material other than algae that may be present in the cultures. Such interference is particularly likely to occur when testing waste waters. Correction for such interference by measurements in non-inoculated samples may not always be appropriate since the development of light-absorbing material in such samples may not be the same as in the inoculated cultures. Interpretation of growth curves and calculated growth rates of highly inhibited cultures must therefore be made carefully when these are based on optical density measurements.

The appropriate inoculum cell density in growth inhibition test is dependent on the cell size and growth rate of the test algae. In ISO 8692 and ISO 10253, biomass densities between 10^3 cells/ml and 10^4 cells/ml are prescribed. According to the cell masses shown in Table 1, this corresponds to 0,02 mg/l to 0,2 mg/l of *P. tricornutum* (strain BAC 2) and 0,05 mg/l to 0,5 mg/l of *S. costatum* (strain BAC 1) and *D. subspicatus*. This is a considerable variation and normalisation of the inoculum biomass density on the dry matter basis should be considered in future revisions of ISO 8692 and ISO 10253.

6.4 Incubation conditions

6.4.1 Light

Specifications on incubation conditions in the algae growth inhibition tests include illumination, temperature and agitation of the cultures. The effect of light and temperature on the toxic response in algae has not been examined in detail, but Reference [35] shows higher sensitivity of *P. subcapitata* to some toxicants at light saturating than at light limiting irradiances. Similarly, Reference [4] shows that the sensitivity of *S. subspicatus* to potassium dichromate was positively correlated to the photon flux density. Performing growth inhibition tests at light saturation may therefore be preferable to achieve maximum sensitivity, but also in order to reduce the effects on the algal growth rates caused by spatial variations in illuminance on the area where the cultures are incubated. Nevertheless, ISO 8692 and ISO 10253 specify a rather wide range of illuminance (60 µmol/m^2 s to 120 µmol/m^2 s). This was considered necessary for practical reasons because a strict requirement of a high illuminance would create problems with appropriate temperature control of the incubators used.

6.4.2 Temperature

The temperature requirement specified in the ISO 8692 freshwater test is (23 ± 2) °C, which is below the optimum temperature for the test species used. Reference [35] did not show any direct effects of temperature in the range 16 °C to 26 °C on the inhibitory response of *P. subcapitata*. Control of temperature, and in particular a uniform temperature in all cultures in a test is, however, still important since it will affect the growth rate and hence the duration of the exponential growth phase. The observed effects of light and temperature

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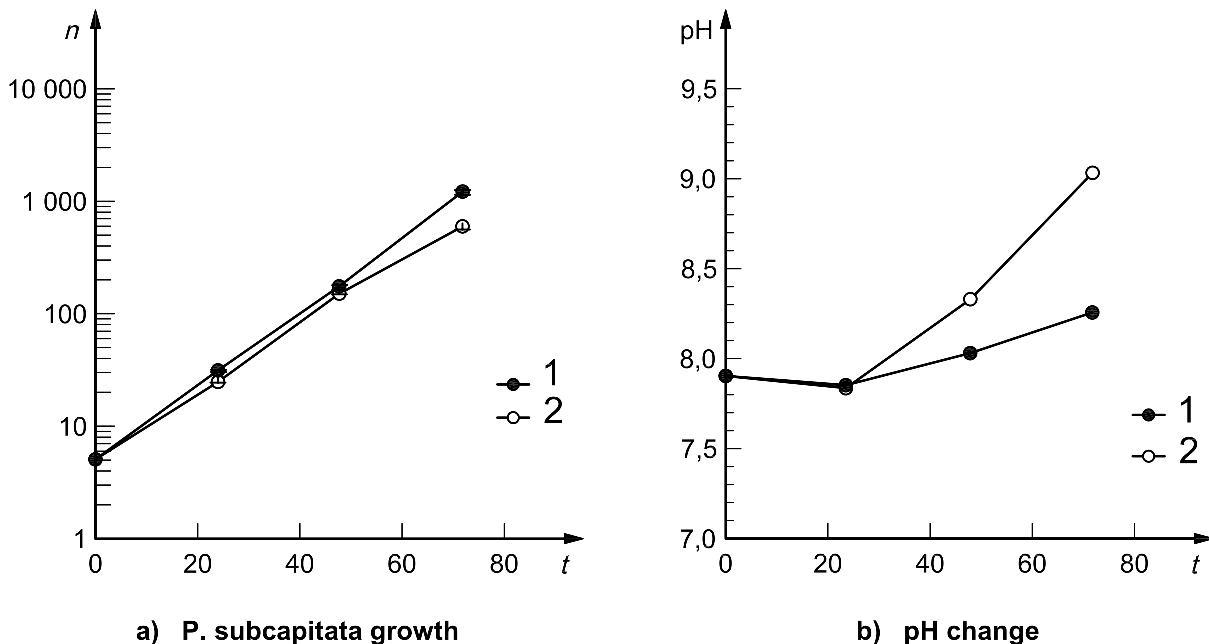
on the growth rate of *P. subcapitata* (see Figure 6) indicate that variation of exposure temperature within the specified range is an efficient way to control the growth rate (and final biomass density) at a level which does not give rise to significant pH change of the medium. According to the experience at NIVA, the pH in agitated cultures of *P. subcapitata*, remains almost constant during 72 h exposure at 21 °C and 80 μmol/m^2 s when the inoculum density is 5×10^3 cells/ml.

The incubation temperature in ISO 10253 is (20 ± 2) °C. As shown in Figure 9, the effect of temperature in this range on the growth rate of *S. costatum* is significant.

6.4.3 Agitation

According to ISO 8692 and ISO 10253, the cultures should be continuously shaken, stirred or aerated in order to keep the cells in free suspension and to facilitate CO_2 mass transfer from air to water. The necessity of this requirement has been discussed several times during the development of the procedures. It has been claimed that whether cultures are continuously agitated or not does not affect the result of the tests. In the early ring tests, it was not possible to come to any conclusion on the effect of agitation on the apparent toxicity. There is no doubt, however, that agitation has the effect of increasing the transfer of CO_2 to the cultures and hence delays the onset of pH-increase. Thus, the requirement for agitation reduces pH-drift problems that are often experienced in growth inhibition tests.

An experiment to demonstrate the effect of agitation has been performed with *P. subcapitata* in ISO 8692 medium. A culture, inoculated with 5×10^3 cells/ml was incubated in 250 ml Erlenmeyer flasks with 100 ml culture volume. One set of flasks was incubated on a reciprocating shaking table and another set on a static table, both at 21 °C and 75 μmol/m^2 s. The growth curves and pH values in the static and agitated cultures are shown in Figure 18. In the agitated cultures, growth remained exponential (μ = 1,8 d^{-1}) for 72 h and the pH increased from 7,9 to 8,25. The growth rate in the static cultures was almost the same as in the agitated cultures for the first 48 h, but lower between 48 h and 72 h. The pH in the static cultures increased after 24 h and reached 9,0 after 72 h. The importance of agitation, however, depends on other factors such as inoculum density, geometry of the test vessels and incubation conditions that affect growth rate (light and temperature).



a) P. subcapitata growth

b) pH change

Key

1 shaken

2 stationary

n 10^3 cells/ml

pH alkalinity scale

t time, h

Figure 18 — Changes over time in cultures incubated on a shaking table (shaken) and without agitation (stationary)

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6.5 Test endpoint

A number of toxicity endpoints have been employed in toxicity tests with algae, including photosynthesis (References [44], [56]), protein synthesis (Reference [41]), phosphate uptake (References [25], [38]), nitrogen uptake (Reference [46]), membrane integrity (Reference [19]), esterase activity (Reference [11], [48]), ATP-status (Reference [27]), and pulse amplitude modulation (PAM) fluorescence (Reference [23]). These endpoints are more or less specifically affected by different chemicals and may be more or less sensitive than population growth to toxic exposure. The great majority of toxicity tests with microalgae are, however, based on inhibition of growth as the endpoint. The advantages of growth inhibition as an endpoint are that it is a general and ecologically relevant toxic effect which does not require sophisticated and expensive equipment for determination. Only growth inhibition endpoints are mandatory in ISO 8692 and ISO 10253, but many of the alternative endpoints listed above can be applied in tests performed according to the same protocols.

Even if inhibition of growth has been the only endpoint in ISO 8692 and ISO 10253, different options to express growth have been used as a basis for the determination of effective concentrations. In the growth inhibition tests that evolved from the nutrient bioassays, the cell density achieved at a certain time, usually after 96 h, was used to calculate growth inhibition relative to control cultures. In the development of ISO 8692 and ISO 10253, the specific growth rate was proposed as a more appropriate endpoint for growth inhibition of exponentially growing microalgae. However, the area under the growth curve (AUGC) was included as a second, mandatory endpoint to be calculated. The arguments for AUGC were that it would reflect the time-integrated effects on growth better than the final cell density and that it did not rely on any assumption of a specific growth pattern in the cultures. In practice, the effective concentrations (e.g. EC_{50}) determined on the basis of AUGC are usually very similar to those determined from final biomass density. Effective concentrations for the AUGC were therefore denoted E_bC_x, where “b” refers to “biomass”, while effective concentrations for the growth rate endpoint were reported as E_rC_x where “r” refers to “rate”.

Effective concentrations based on specific growth rate are usually significantly higher than the corresponding effective concentrations for AUGC and final cell density in algal growth inhibition tests with 72 h incubation time. The difference in EC_{50} calculated for the various endpoints depends on the growth rate in the control cultures and the slope of the concentration/response curve as shown in Reference [37]. A universal conversion factor can therefore not be derived. Analysis of 50 tests with *P. subcapitata* performed at NIVA in accordance with ISO 8692 showed an average ratio $E_rC_{50}/E_bC_{50} = 2{,}34$ (minimum: 1,47; maximum: 10,6). Linear regression of EC values from the the same data set showed that the E_bC_{50} corresponded to E_rC_{17} (slope 0,996, $R^2 = 0{,}981$, see Figure 19). Similarly, the E_rC_{50} corresponded to E_bC_{81} (slope: 1,06; $R^2 = 0{,}996\,8$).

Analysis of data from 694 data sets for chemicals notified in the European Union confirmed that growth rate appears to be a less sensitive endpoint than AUGC in the respect that it yielded on average 40 % lower values of EC_{50} (Reference [57]). Even so, the algal growth inhibition test was still generally more sensitive than the acute toxicity tests with *Daphnia* and fish when the EC_{50} was calculated on the basis of growth rate.

The use of two different mathematical approaches to express growth inhibition is unfortunate and confusing for the users of algae toxicity data. Inhibition of growth based on growth rate, final cell density, and AUGC do not represent three independent endpoints since they are all based on the recorded changes in cell density in the cultures. Reference [37] compares the approaches from a theoretical point of view and shows that when applied on predominantly exponential growing test algae, the effective concentrations derived from final cell density and AUGC vary with time and are affected by the absolute magnitude of the specific growth rate of the test algae. For that reason, these endpoints are less suitable than the growth rate based endpoint for comparing test results from different laboratories and the sensitivity of different species (Reference [6]).

The apparent higher sensitivity of the endpoints based on final biomass density or AUGC has been the main argument for their use. From a scientific point of view, however, it may be argued that the analysis of the growth-inhibiting effect on microalgae should be based on the logarithmic increase in cell density rather than the arithmetic increase for the reason that the growth is predominantly exponential (Reference [39]). Furthermore, effects on growth rate may more readily be related to ecological effects than effects on cell density at an arbitrary point of time. Preference for use of the growth rate endpoint is also given by Reference [7], which describes procedures for risk assessment of chemicals within the European Union. For these reasons ISO has decided to use inhibition of growth rate as the only endpoint in the algae growth inhibition tests and this has been implemented in ISO 8692:2004 and ISO 10253:2006.

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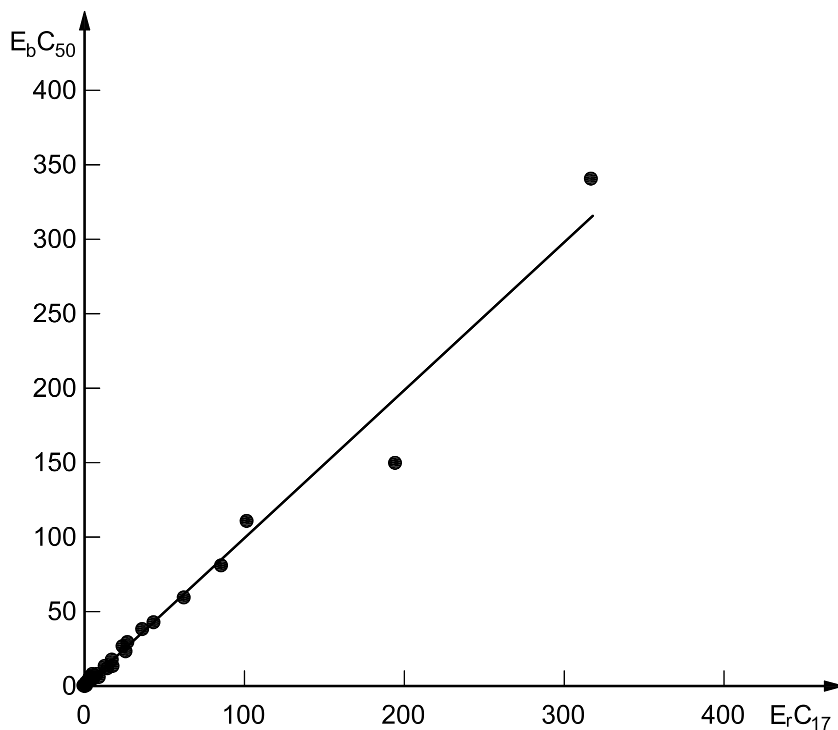

Key

E_bC_{50} 50 % effective concentration based on biomass (AUGC)

E_rC_{17} 17 % effective concentration based on growth rate

NOTE The plot shows the regression analysis with slope closest to unity (E_bC_{50}/E_rC_{17}).

Figure 19 — Relationship between EC values based on growth rate (E_rC_x) and AUGC (E_bC_x)

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