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Validation report for the draft revised Test Guideline TG 201, inclusion of a new algal strain, for the Freshwater Alga and Cyanobacteria, Growth Inhibition Test

OECD Series on Testing and Assessment

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Foreword

This document describes the results of a ring-test with Mayamaea permitis (NIES-2724) in order to replace the, no-longer available, Navicula pelliculosa (UTEX664), which was included in the original Test Guideline (TG) 201 - Freshwater Alga and Cyanobacteria, Growth Inhibition Test.

Guidelines for Testing of Chemicals are periodically reviewed and updated in the light of scientific progress. The TG 201 revision includes:

- the diatom strain Mayamaea permitis as new recommended species to replace Navicula pelliculosa (UTEX664) which is no longer available,
- an update of scientific name for the recommended strains (Pseudokirchneriella subcapitata, and Anabaena flos-aquae),
- a clarification on the calculation of CV of the section-by-section specific growth rates.

The revised TG and this accompanying validation report are the result of Test Guidelines Programme (TGP) project 2.67, which was included in the TGP workplan in 2021, and led by Japan.

The Validation Management Group for Ecotoxicity testing (VMG-Eco) has provided feedback and discussed the proposed revisions during several of its meetings, with a final review of this validation report in September-October 2025.

The subsequent Working Party of National Coordinators of the Test Guidelines Programme (WNT) review took place from December 2025 – January 2026, and the WNT approved the revised TG and this validation report at its 38th meeting in April 2026.

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INTRODUCTION

Pennate diatom strain UTEX664 (Navicula pelliculosa) is one of the recommended test strains in OECD TG 201 (Freshwater Alga and Cyanobacteria, Growth Inhibition Test)1) and has been widely used for environmental risk assessment worldwide. Diatoms belong to the stramenopile group, which occupies a taxonomic position markedly different from that of other algal groups such as green algae and red algae. This taxonomic distinction suggests that their sensitivity to chemical substances may also differ from that of other algal species. However, UTEX664 is currently no longer commercially available from any algal culture collections worldwide. Therefore, it is essential to propose and validate new diatom strains as potential alternatives for inclusion in the revised test guideline.

UTEX664 was originally isolated in Alaska by R.A. Lewin in 1951 and identified by him as *Navicula pelliculosa*. However, in 1997, Lange-Bertalot moved *Navicula pelliculosa* into the genus *Fistulifera* (Lange-Bertalot 1997)[2)], making *Fistulifera pelliculosa* the currently accepted name for this strain. More recently, a study by Tsuji et al. (2021)[3)] suggested that UTEX 664 had been misidentified as *Navicula pelliculosa* (= *Fistulifera pelliculosa*) and that clearly belongs to genus *Mayamaea*. Although molecular phylogenetic analysis could not be performed for UTEX664 because this stain was not available, the authors suggested that this strain is *Mayamaea permitis*, based on remaining scanning electron microscope (SEM) images of UTEX664 (Fig. 1-1) (Tsuji et al. 2021) [3)]. Therefore, *Mayamaea permitis* is considered the primary candidate to replace UTEX664, along with two closely related strains to *M. permitis*, *M. pseudoterrestris* and *M. terrestris* (Fig. 1-1).

Search across all algal culture collections worldwide has revealed that one strain of *M. permitis* (NIES-2724) is available from the NIES culture collection (Table 1). Additionally, two closely related strains—*M. pseudoterrestris* (NIES-4280=UTEX 661) and *M. terrestris* (NIES-4281=UTEX B673)—are also available from both UTEX and NIES culture collections (Table 1). In the future, these strains are expected to be shared among UTEX and other algal culture collections.

In our preliminary study on the growth rates of these strains, M. permitis (NIES-2724) was found to fulfill three validity criteria for the control culture as defined by OECD TG 201 using the OECD standard medium (Table 1-2). On the contrary, the growth rates of M. pseudoterrestris (NIES-4280=UTEX 661) and M. terrestris (NIES-4281=UTEX B673) were significantly lower when using the OECD standard medium compared to that of M. permitis (NIES-2724) (Table 1-2). Thus, these two strains did not meet validity criteria of undergoing more than a factor of 16 within 72 hours (Table 1-2).

Nevertheless, when a modified OECD medium (see Table 2-2) was used, the growth of M. pseudoterrestris (NIES-4280=UTEX 661) improved (Table 1-2). However, even with the modified OECD medium, the growth rate of M. terrestris (NIES-4281=UTEX B673) did not reach the minimum requirement regarding growth within 72 hours (Table 2).

A Comparison of EC50 values for two reference chemicals and six herbicides demonstrated that there is no significant difference in sensitivity among the Mayamaea genus (Table 1-3). Therefore, based on the results of these preliminary studies, M. permitis (NIES-2724) has been proposed as candidate for alternative strain of UTEX 664 in OECD TG201.

1 Results of a Ring-test with *Mayamaea permitis* (NIES-2724) in the algal growth inhibition test

Project title:

The Revision of OECD TG 201 “Freshwater Alga and Cyanobacteria, Growth Inhibition Test” relating to algal strains

Organization of the Ring Test (in alphabetical order):

Chemical Evaluation and Research Institute (CERI), Japan; German Environment Agency (UBA), Germany; Kumiai Chemical Industry CO., LTD., Japan; Mitsubishi Chemical Research Corporation, Japan; National Institute for Environmental Studies, Japan; National Institute for Industrial Environment and Risks (INERIS), France

Financial Support: Ministry of Environment, Japan

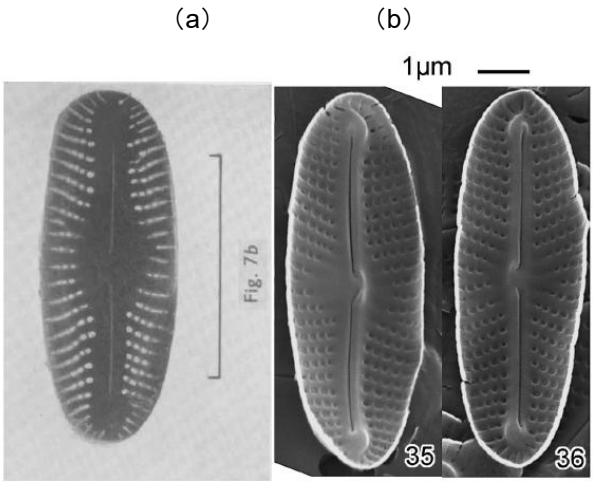
Report: Takahiro Yamagishi, Hiroshi Yamamoto (National Institute for Environmental studies, Japan)

Summary

It is urgent to introduce new diatom strains for the algal growth inhibition test, *as* recommended strain *Navicula pelliculosa* (UTEX 664) has been lost from all algal culture collections worldwide and is therefore no longer available. In our preliminary study evaluating the growth rates of alternative strains, *Mayamaea permitis* (NIES-2724), a closely related strain to UTEX 664, was assessed and found to meet the three validity criteria for control cultures as defined by OECD TG 201 when grown in the OECD standard medium (Table 2). Based on these preliminary findings, *M. permitis* (NIES-2724) was proposed as a candidate replacement for UTEX 664.

This international ring test aims to identify potential issues associated with tests using diatom strain (NIES-2724) and to propose optimized testing, culturing, and handling methods to ensure highly reproducible results. The test was primarily conducted following the OECD Test No. 201 guideline, *Freshwater Alga and Cyanobacteria, Growth Inhibition Test* (2011). Two reference substances specified in TG201—3,5-dichlorophenol and potassium dichromate—were used as test substances, and ECx values are compared to assess the consistency and reliability of the results.

This ring test demonstrated that the growth inhibition test using NIES-2724 can reliably detect toxic effects, with a variation of approximately twofold, provided that the test is conducted under established conditions.



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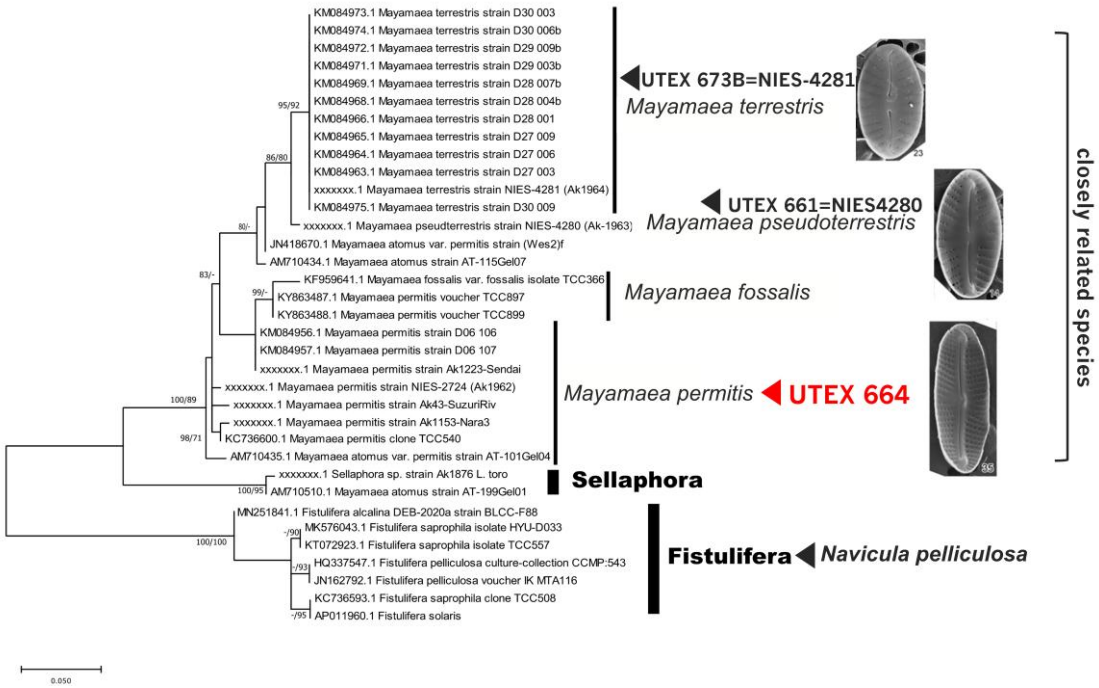


Fig.1-1: (a) TEM image of UTEX664, (b) SEM image of NIES-2724, (c) Maximum likelihood (ML) phylogenetic tree based on 37 sequences of the rbcL gene showing the structure within Mayamaea and related genera, modified from Tsuji et al. (2021). The Tamura 3-parameter model with gamma distributed (G) rate variation and complete deletion was used for the analysis. Bootstrap values greater than 70% with ML/NJ methods are indicated at the nodes.

Table 1-1: Information of the candidates for an alternative strain to UTEX 664, belonging to genus *Mayamaea*

Strain number	UTEX 664(lost strain)	NIES 2724
Phylum	Heterokontophyta	Heterokontophyta
Class	Bacillariophyceae	Bacillariophyceae
Scientific name	Mayamaea sp.	Mayamaea permitis
	(misidentified as Navicula pelliculosa)	
Common name	Pennate diatom	Pennate diatom
Origin	Alaska, USA	Tsukuba, Ibaraki, Japan
Habitat	Terrestrial(snow)	Freshwater
Isolation	Lewin, R.A. (1951)	S. Mayama (2005)
Status	Axenic	Axenic
Culture condition	Medium:CSi	Medium:CSi
	Temperature:20?	Temperature:20?
	Light intensity:24 mol/m^2/sec	Light intensity:24 mol/m^2/sec
	Light cycle:10L:14L	Light cycle:10L:14L
	Duration:1M	Duration:1M
Strain number	UTEX 661 = NIES-4280	UTEX B673 = NIES-4281
Phylum	Heterokontophyta	Heterokontophyta
Class	Bacillariophyceae	Bacillariophyceae
Scientific name	Mayamaea pseudoterrestris	Mayamaea terrestris
	(misidentified as Navicula pelliculosa)	(misidentified as Navicula pelliculosa)
Common name	Pennate diatom	Pennate diatom
Origin	Connecticut, USA	Devon, UK
Habitat	Terrestrial	Terrestrial
Isolator	Lewin, R.A. (1951)	Lewin, R.A. (1955)
Status	Axenic*	Axenic*
Culture condition	Medium:CSi	Medium:CSi
	Temperature:20?	Temperature:20?
	Light intensity:24 mol/m^2/sec	Light intensity:24 mol/m^2/sec
	Light cycle:10L:14L	Light cycle:10L:14L
	Duration:1M	Duration:1M

*NIES-4280 and NIES-4281 that are originated from UTEX 661 and UTEX B673 respectively, were made free from bacteria recently. UTEX661 and UTEX B673are not bacterial-free strain.

Table 1-2: Growth characteristics of three candidate strains for replacing UTEX 664

		Growth factor within the 72-hour test period	Mean coefficient of variation for section-by-section specific growth rate	Coefficient of variation of average specific growth rates
Strain	Medium	>16 times	<35%	<7%
Mayamaea permitis (NIES-2724)	OECD medium ＋Si	125±12.5	13.7±8.15	1.63±0.97
Mayamaea pseudoterrestris (NIES-4280)	OECD medium ＋Si	**12.2±1.71**	11.6±0.68	0.99±0.54
	Modified OECD medium *	25.2±3.22	10.2±0.74	1.23±0.41
Mayamaea terrestris (NIES-4281)	OECD medium ＋Si	**7.32±2.21**	22.7±1.68	3.86±1.46
	Modified OECD medium *	**11.3±8.21**	22.7±1.68	3.86±1.46

n=3, mean±SD

*Iron citrate is used instead of Fe-EDTA at the modified OECD medium (See Table 4).

Table 1-3: Comparison of EC50 values on two typical toxicants and six herbicides

	***Mayamaea permitis* (UTEX-664)**	***Mayamaea permitis* (NIES-2724)**	***Mayamaea pseudoterrestris* (NIES-4280)**	***Navicula pelliculosa* (strain code is unknown)**
3,5-DCP	-	1.24 mg/L	1.0 mg/L	-
Cr(VI)	-	0.31 mg/L	0.51 mg/L	-
Mefentrifluconazole	1.35 mg/L (data base)	1.45 mg/L	1.28 mg/L	-
Chlorsulfuron	> 126 mg/L (data base)	160 mg/L	132 mg/L	-
Dimoxystrobin	0.0078 mg/L (data base)	0.012 mg/L	0.0052 mg/L	-
Simazine	-	0.15 mg/L	0.19 mg/L	0.09 mg/L (data base)
Flumetsulam	-	40.2 mg/L	38.2 mg/L	41.6 mg/L (data base)
Boscalid	-	2.3 mg/L	2.8 mg/L	1.8 mg/L (data base)

TEST CONDITIONS AND METHODS FOR STATISTICAL EVALUATION

Participants

6 laboratories participated in this ring test (Table2-1), but one laboratory retired on the way. In this report, each laboratory will be named with an individual laboratory code (Lab A-Lab F).

Table 2-1 Participating laboratories (in alphabetical order)

No.	Laboratory	Status
1	Chemicals Evaluation and Research Institute (CERI), Japan	Done
2	German government environmental agency (UBA), Germany	Did not finish
3	Kumiai Chemical Industry CO., LTD., Japan	Done
4	Mitsubishi Chemical Research Corporation, Japan	Done
5	National Institute for Industrial Environment and Risks (NIES), Japan	Done
6	National Institute for Industrial Environment and Risks (INERIS), France	Done

Test chemicals

Two specific chemicals, 3,5-dichlorophenol and potassium dichromate, are utilized as test substances

Test design and test conditions

Test algae

Axenic cultures of *Mayamaea permitis* (NIES-2724) were utilized for this validation test. The strain was obtained from the NIES collection (https://mcc.nies.go.jp/) and provided to all participating laboratories.

Medium

The OECD standard medium, supplemented with Na_2SiO_3 (100 mg/L) (OECD medium +Si), was utilized for *M. permitis* (NIES-2724). The preparation of the medium followed the guideline outlined in OECD Test guideline 201 (2011)[1)]. However, it is important to note that the pH of the medium must be adjusted to 7.0 ± 0.1 using HCl, as the addition of Na_2SiO_3 (100 mg/L) can cause the pH of the medium to increase to approximately 10. For stock culture, CSi medium (https://mcc.nies.go.jp/02medium-e.html) was useful, but OECD medium + Si may also be used for stock culture.

Table 2-2: Composition of the modified OECD medium for NIES-2724

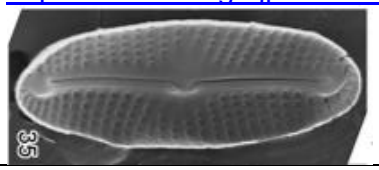
	OECD medium		Modified OECD medium	
Component	mg/L	mM	mg/L	mM
NH_4Cl	15	0.280	15	0.280
$NaNO_3$	-	-	-	-
$MgCl_2 \cdot 6(H_2O)$	12	0.0590	12	0.0590
$CaCl_2 \cdot 2(H_2O)$	18	0.122	18	0.122
$MgSO_4 \cdot 7(H_2O)$	12	0.0609	12	0.0609
KH_2PO_4	1.6	0.00919	1.6	0.00919
$FeCl_3 \cdot 6(H_2O)$	0.064	0.000237	0.064	0.000237
$Na_2EDTA \cdot 2(H_2O)$	0.1	0.000269	0.1	0.000269
$FeC_6H_5O_7 \cdot nH_2O$	-	-	-	-
H_3BO_3	0.185	0.00299	0.185	0.00299
$MnCl_2 \cdot 4(H_2O)$	0.415	0.00210	0.415	0.00210
$ZnCl_2$	0.003	0.0000220	0.003	0.0000220
$CoCl_2 \cdot 6(H_2O)$	0.0015	0.00000630	0.0015	0.00000630
$Na_2MoO_4 \cdot 2(H_2O)$	0.007	0.0000289	0.007	0.0000289
$CuCl_2 \cdot 2(H_2O)$	0.00001	0.00000006	0.00001	0.00000006
$NaHCO_3$	50	0.595	50	0.595
$Na_2SiO_3 \cdot 9H_2O$			**100**	**0.352**
pH	8.1		**7.0**	

Components different from the standard OECD medium in the modified OECD medium were indicated with bold style.

Test design and test concentration

The test design included three replicates at each test concentration and six replicates in the control group.

Table 2-3 Summary of the conditions applied for the international validation test.

Test strain	*Mayamaea permitis* (NIES-2724) https://mcc.nies.go.jp/index_en.html 
Endpoint	Growth inhibition
Initial cell density	5,000-10,000 cells/mL*
Measurements	• Cell count using electronic particle counter, microscope, or a flow cytometer. • Fluorescence (ex: 430 nm, em: 670 nm) or optical density (680 nm)
Test condition	• Axenic • Temperature: 23±2℃ • Light intensity: 20—60 μ mol m^{-2} s^{-1} * • Cycle: continuous light
Medium	• NIES-2724: OECD medium+Si
Test duration	72 hours
Validity criteria in the control cultures	The biomass within the 72-hour: ≥ a factor of 16 The mean CV for section-by-section specific growth rates < 35% The CV of average specific growth rates in replicates < 7.0%
Test chemicals	• 3,5-DCP (4 mg/L, 2 mg/L, 1 mg/L, 0.5 mg/L 0.25 mg/L) • Potassium dichromate (VI) (1 mg/L, 0.5 mg/L, 0.25 mg/L, 0.13 mg/L, 0.063 mg/L as Potassium dichromate)**

* The type of flask cap can affect the growth in algae. For example, aluminum caps tend to promote higher growth compared to silicon caps. In such a case, levels of light intensity or initial cell density should be reduced.

** Different concentrations of potassium dichromate (VI) were used for Lab C: 0.2 mg/L, 0.1 mg/L, 0.05 mg/L, 0.025 mg/L, and 0.13 mg/L.

Test procedure

1. Preparation of Inoculum culture:

   To achieve highly reproducible results, a pre-culture for 3-4 days was conducted. During the pre-culture, cell counting was performed every 24 hours to confirm whether the cultures had reached their exponential growth stage. If first pre-culture was insufficient to reach this phase, a second propagation step of the pre-culture was performed. Additionally, if extensive cell aggregation was observed, pre-culture flask was directly sonicated for 3 to15 minutes at 40–50 kHz in an ultrasonic bath prior sampling for cell counting every 24 hours. This sonication treatment every 24 hours was effective to maintain stable exponential growth of cultures. In our previous study, we confirmed that this treatment does not adversely affect the diatom growth.

2. Preparation of test solutions:

   To prepare a test solution of maximum concentration, 4.0 mg/L of 3,5-DCP and 1.0 mg/L of potassium dichromate were directly added to the medium (OECD medium + Si). After stirring for 30 minutes, prepared solutions were sterilized by passing through a filter with a pore size of 0.22 or 0.45 μm. To prepare test solution of lower concentrations, the prepared test solution of maximum concentration was diluted with sterilized medium.

3. Incubation:

   OECD TG201 mentions that UTEX664 (the lost strain) had a tendency to aggregate under certain growth conditions and accumulate in the surface film due to the production of lipids, which made accurate cell counting difficult. Similar issues were observed in both NIES-2724 and NIES-4280 (UTEX-661) strains, which also impeded accurate cell counting. TG suggests that vigorous shaking, such as using a vortex mixer, may be necessary prior to cell counting.

   In our previous study, we found that using a reciprocating shaker with 100-120 rpm significantly reduced aggregation and surface accumulation, supporting exponential cell growth during the test period. Conversely, the use of a rotary shaker resulted in more aggregation and surface accumulation compared to a reciprocating shaker. Therefore, the use of a reciprocating shaker was recommended for tests involving diatom strain. if a reciprocating shaker is not available or extensive cell aggregation was observed, each test flask was directly sonicated for 3 to15 minutes at 40–50 kHz in an ultrasonic bath prior to sampling for cell counting every 24 hours. This sonication treatment facilitated stable exponential growth during testing as in pre-culture.

Measurements

Before counting the number of cells, one mL aliquot of culturing solution was subjected to 2 minutes of sonication in an ultrasonic bath to separate individual cells. If extensive cell aggregation in test flask was observed, each test flask was directly sonicated for 3 to15 minutes at 40–50 kHz in an ultrasonic bath prior to sampling for cell counting every 24 hours during testing phases as described in “Incubation” section.

Chemical analysis

Chemical analyses of test solutions were preferably conducted at each testing laboratories to avoid the loss of chemicals during the shipment. The samples of both before and after exposure, were analyzed using a liquid chromatography tandem mass spectrometry (LC/MS/MS) and induced coupled plasma mass spectrometry (ICP-MS) for 3,5-DCP and potassium dichromate (VI), respectively. If there is no proper analyzer is available for the determination of these chemicals, the samples were shipped to NIES for the measurement (refer to ANNEX 1).

Validity Criteria

Validity criteria were set following the guideline of OECD Test No. 201, Freshwater Alga and Cyanobacteria, Growth Inhibition Test (2011)[1)].

- The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72-hour test period.
- The mean coefficient of variation for section-by-section specific growth rates in the control cultures must not exceed 35%.
- The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7%.

Statistical Analysis

EC50

The determination of various inhibitory concentrations (IC_x) was carried out by using the open-source statistical software R (R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/). IC_x values were calculated using 2-parameter log-logistic models implemented in R, utilizing the extension package "drc" (Ritz, 2010, 2015)[4)5)]. The model function $f(x)$, as described in Equation 1, represents the relative endpoint response at concentration x, with the upper limit set at the mean response in the control (100%).

$$= \quad (1)$$

where x is concentration and b and e are parameters.

NOEC

The determination of the no-observed-effect concentration (NOEC) was carried out by using the open-source statistical software R (R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/). To estimate the NOEC, a one-tailed Dunnett's procedure was employed.

Results

Cr(VI)

Results of Control Data

Figures 3-1, 3-2, and 3-3 showed the control data results for potassium dichromate across five laboratories. Each graph presented the specific growth rate over 72 hours, the coefficient of variation (CV) of the mean specific growth rates, and the mean CV for section-by-section growth rates, respectively. The results indicated that four laboratories (Labs A, B, C and E) met all three validity criteria for the control group. However, Lab D did not meet the criterion for the mean CV of section-by-section growth rates. The independent additional test was conducted in Lab D with same test condition. The mean CV of section-by-section growth rates still did not also meet the criterion, reaching 39.1% (Annex Table 5).

Figure 3-4 showed the cell density at each concentration of Cr(VI) during testing. The results indicated that the growth rate at Lab D was low on the first day compared to the subsequent three days. This explains why the mean CV for section-by-section growth rates (days 0-1, 1-2 and 2-3, for 72-hour tests) exceeded the 35% validity criterion.

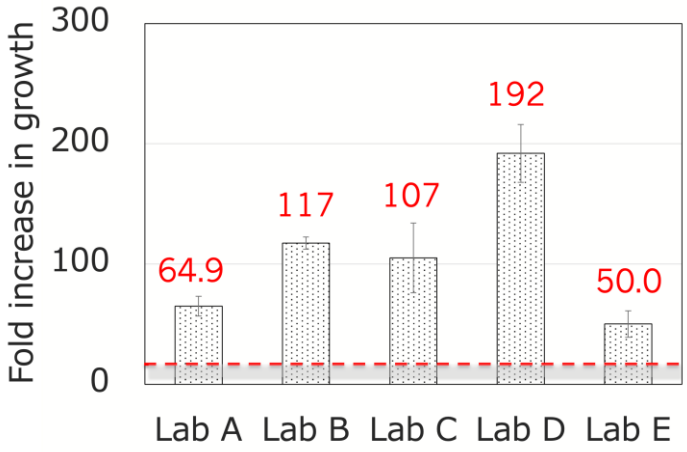


Figure 3-1 The fold increase in growth within 72 hours in the control results from the Cr(VI) tests
Bars indicate means ± SD (n=6). Dotted line indicates a 16-fold growth.

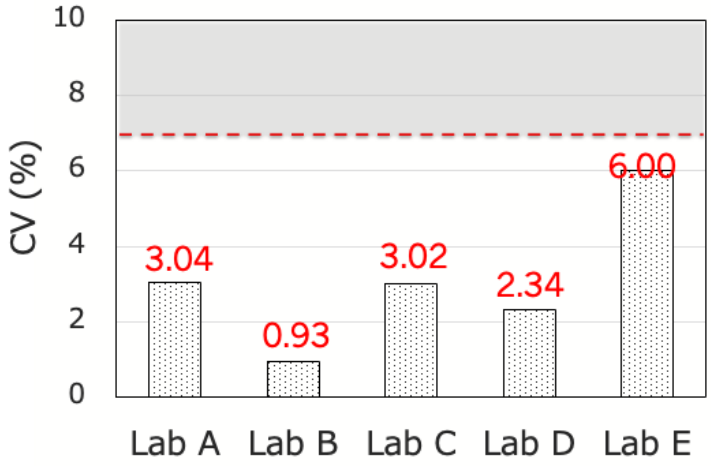


Figure 3-2 CV of the mean specific growth rates in the control results from the Cr(VI) tests
Dotted line indicates a 7% CV in the mean specific growth rate.

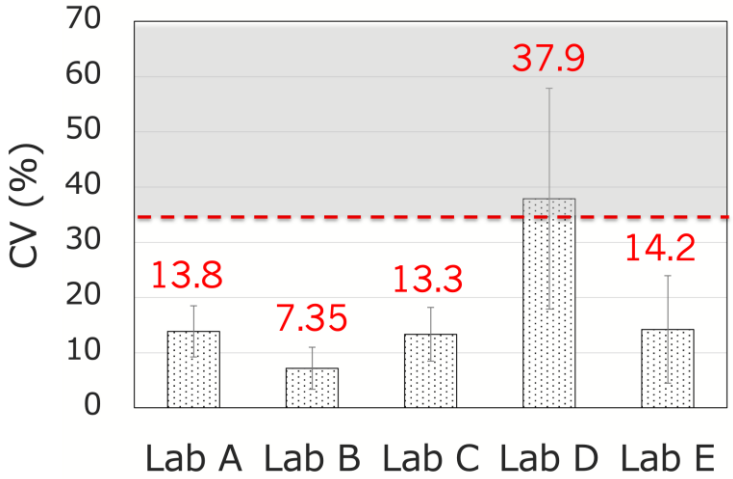


Figure 3-3 Mean CV for section-by-section growth rates in the control results from the Cr(VI) tests
Bars indicate means ± SD (n=6). Dotted line indicates a 35% mean CV for the section-by-section growth rates.

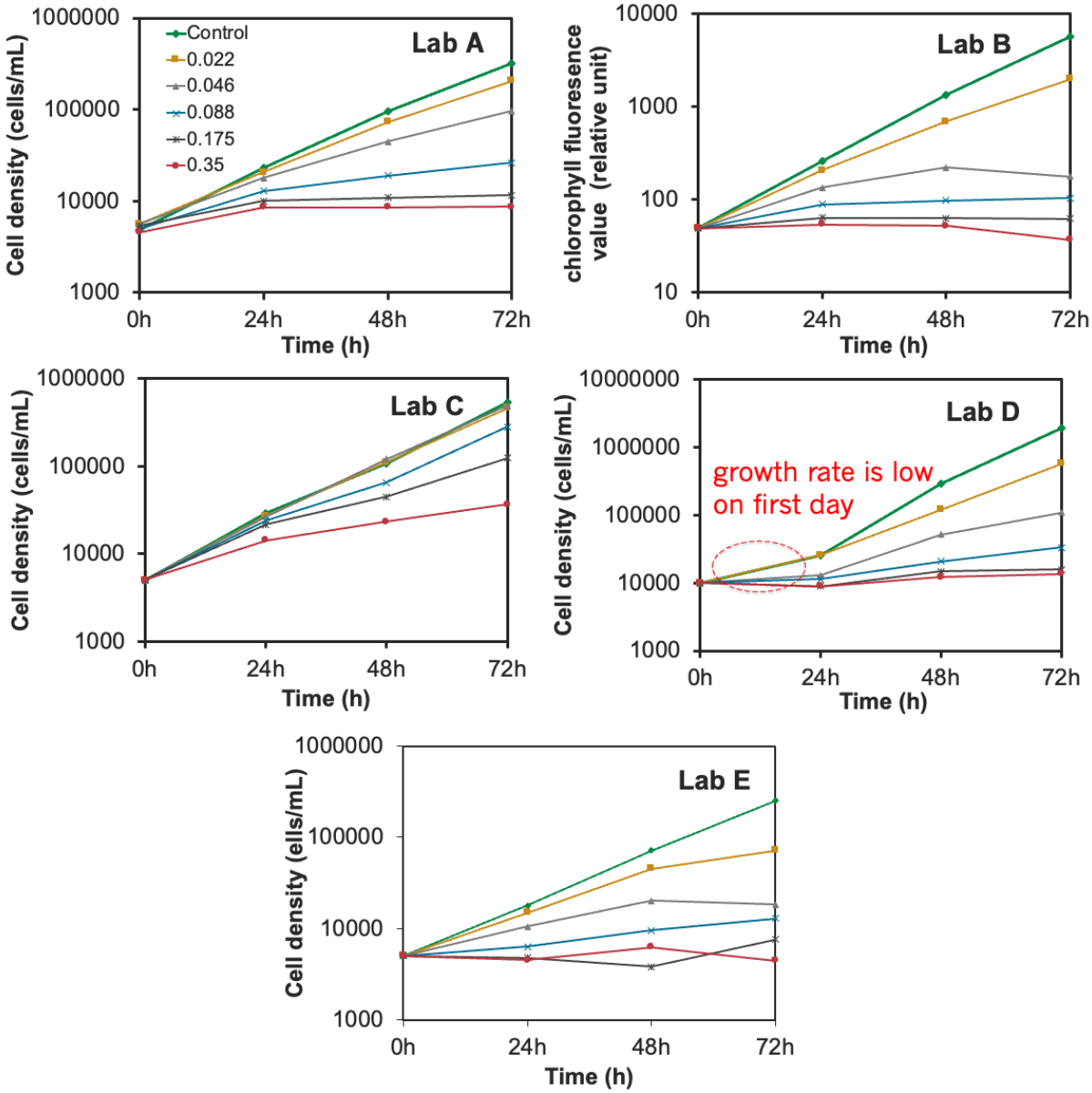


Figure 3-4 Cell density during testing (Cr(VI))

Comparison of ECs values among the participants

Table 3-1 showed a comparison of EC values obtained using $K_2Cr_2O_7$ across the testing laboratories. The EC values calculated with measured concentration of Cr(VI) were similar across the five testing laboratories, with a variation of approximately twofold (a maximum factor of 2.28), including the two results from Lab D, which did not meet the criterion for the mean CV of section-by-section growth rates.

Table 3-1 Comparison of EC value across testing laboratories (Cr(VI)) (mg/L)

Lab code	EC50	EC10	EC5	NOEC
Lab A nominal	0.072 (0.066-0.078)	0.017 (0.013-0.020)	0.010 (0.007-0.01)	<0.021
Lab A measured	0.073 (0.067-0.081)	0.018 (0.013-0.023)	0.011 (0.007-0.016)	<0.021
Lab B nominal	0.034 (0.031-0.037)	0.014 (0.011-0.017)	0.010 (0.008-0.013)	<0.021
Lab B measured	0.035 (0.032-0.037)	0.013 (0.011-0.016)	0.009 (0.007-0.012)	<0.021
Lab C nominal	0.058 (0.054-0.061)	0.014 (0.013-0.016)	0.009 (0.007-0.011)	0.009
Lab C measured	0.06 (0.056-0.064)	0.014 (0.012-0.016)	0.008 (0.007-0.01)	0.008
Lab D* nominal	0.042 (0.040-0.046) 0.046 (0.044-0.049)	0.013 (0.011-0.014) 0.018 (0.015-0.020)	0.009 (0.007-0.010) 0.013 (0.011-0.015)	<0.021 <0.021
Lab D* measured	0.042 (0.039-0.045) 0.043 (0.041-0.047)	0.012 (0.009-0.014) 0.016 (0.013-0.019)	0.007 (0.006-0.009) 0.011 (0.009-0.014)	<0.021 <0.018
Lab E nominal	0.033 (0.028-0.039)	0.007 (0.004-0.011)	0.004 (0.002-0.007)	<0.021
Lab E measured	0.032 (0.027-0.037)	0.007 (0.004-0.011)	0.005 (0.002-0.007)	<0.021
Average	0.048 ± 0.015 (nominal) 0.048 ± 0.016 (measured)	0.014 ± 0.004 (nominal) 0.013 ± 0.004 (measured)	0.009 ± 0.003 (nominal) 0.009 ± 0.003 (measured)	- -
CV (%)	31.7 (nominal) 33.3 (measured)	28.0 (nominal) 28.3 (measured)	31.9 (nominal) 27.1 (measured)	- -

*Two independent tests were performed in Lab D. The two data sets from Lab D were included in the calculation of mean and CV for the presented EC values.
The number in parentheses: 95% CI, Average: mean EC50 among testing laboratories ± SD

3,5-DCP

Results of Control Data

Figures 3-5, 3-6, and 3-7 showed the control data results for 3,5-DCP across five laboratories. Each graph presented the specific growth rate over 72 hours, the coefficient of variation (CV) of the mean specific growth rates, and the mean CV for section-by-section growth rates, respectively. The results indicated that

four laboratories (Labs A, B, C, and E) met all three validity criteria for the control group. However, Lab D did not meet the criterion for the mean CV of section-by-section growth rates.

Figure 3-8 showed the cell density at each concentration of 3,5-DCP during testing. The results indicated that the growth rate at Lab D was low on the first day. This explains why the mean CV for section-by-section growth rates exceeded the 35% validity criterion, indicating the need for an additional pre-culturing. An additional independent test by Lab was conducted by mixing two pre-cultures (3 days old for the first one and 4 days old for the second one) to initiate the test. This test met the validity criterion of the mean CV for section-by-section growth rates (Fig. 3-9) (Annex Table 8).

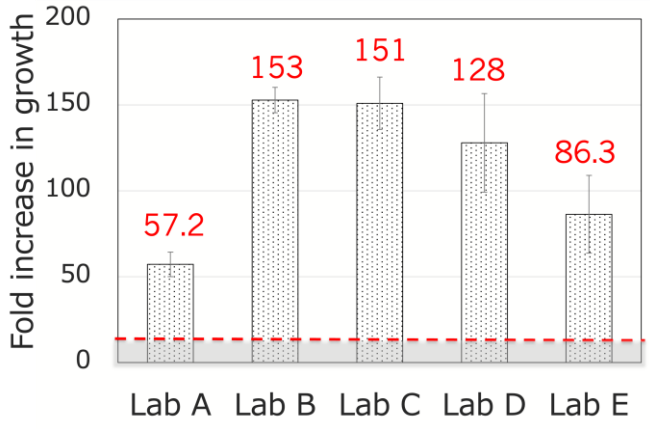


Figure 3-5
The fold increase in growth within 72 hours in the control results from the 3,5-DCP tests
Bars indicate means ± SD (n=6). Dotted line indicates a 16-fold growth.

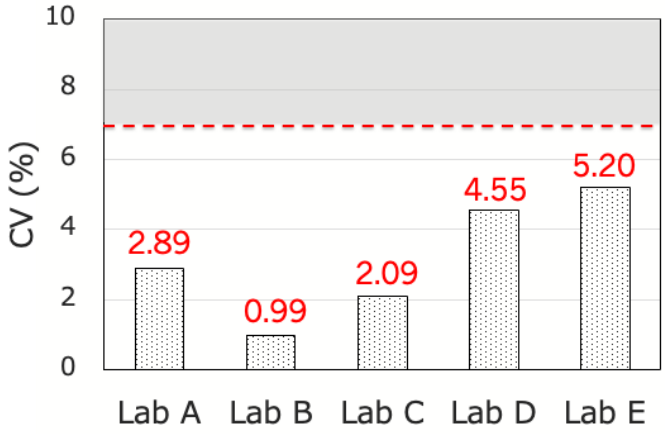


Figure 3-6
CV of the mean specific growth rates in the control results from the 3,5-DCP tests
Dotted line indicates a 7% CV in the mean specific growth rate.

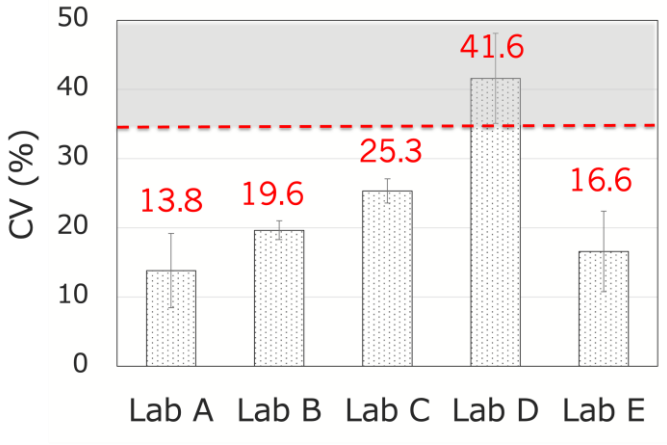


Figure 3-7
Mean CV for section-by-section growth rates in the control results from the 3,5-DCP tests
Bars indicate means ± SD (n=6). Dotted line indicates a 35% mean CV for the section-by-section growth rates.

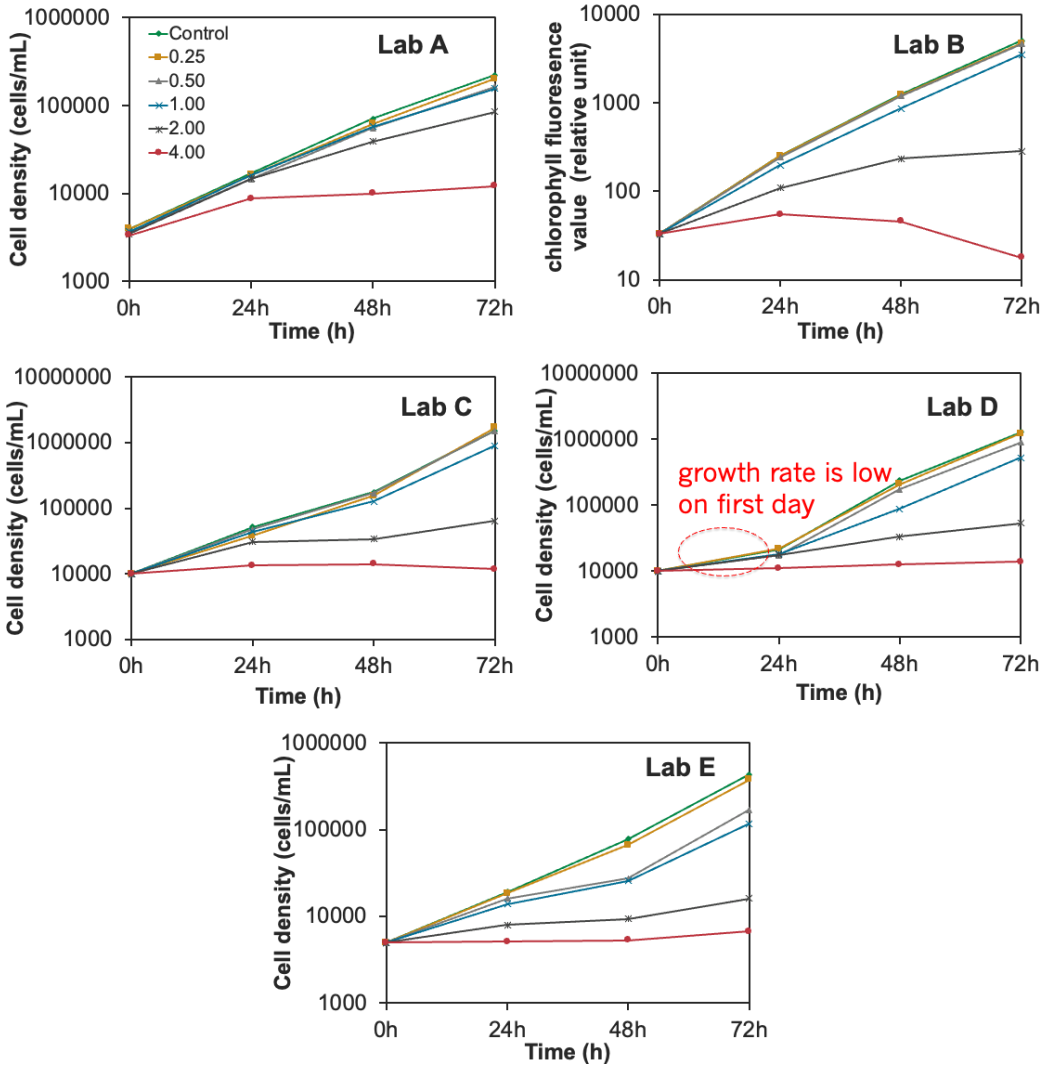


Figure 3-8 Cell density during testing (3,5-DCP)

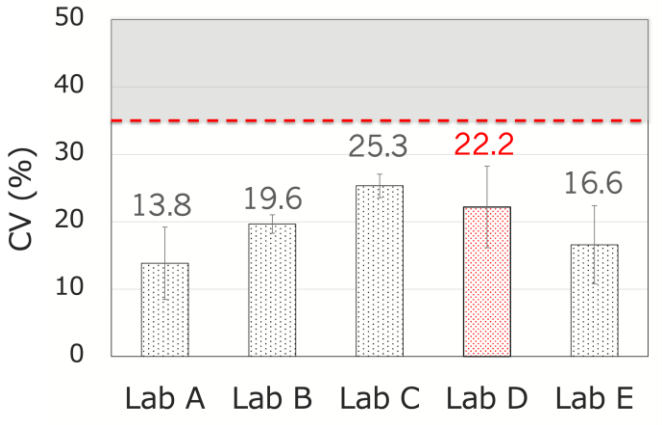


Figure 3-9
Mean CV for section-by-section growth rates in the control results from the 3,5-DCP tests
Bars indicate means ± SD (n=6). Dotted line indicates a 35% mean CV for the section-by-section growth rates.

Comparison of ECs values among the participants

Table 3-2 showed a comparison of EC values obtained using 3,5-DCP across testing laboratories. The EC values calculated with measured concentration of 3,5-DCP were similar among the five testing laboratories, with a variation of approximately twofold (a factor of 1.87 at maximum), including the two results from Lab D, one of which did not meet the criterion for the mean CV of section-by-section growth rates.

Table 3-2 Comparison of EC value across testing laboratories (3,5-DCP) (mg/L)

Lab code	EC50	EC10	EC5	NOEC
Lab A nominal	2.76 (2.57-2.94)	1.31 (1.09-1.52)	1.01 (0.80-1.23)	0.25
Lab A measured	2.43 (2.27-2.59)	1.15 (0.97-1.34)	0.89 (0.71-1.08)	0.22
Lab B nominal	1.93 (1.69-2.17)	1.49 (0.20-2.78)	1.37 (-0.16-2.89)	0.5
Lab B measured	1.77 (1.65-1.89)	1.27 (0.90-1.64)	1.13 (0.71-1.56)	0.49
Lab C nominal	1.74 (1.71-1.78)	1.00 (0.94-1.05)	0.83 (0.77-0.88)	0.5
Lab C measured	1.64 (1.61-1.67)	0.98 (0.93-1.03)	0.82 (0.77-0.87)	0.47
Lab D* nominal	1.64 (1.56-1.71) 1.63 (1.56-1.72)	0.78 (0.70-0.87) 0.85 (0.77-0.97)	0.61 (0.53-0.70) 0.69 (0.60-0.80)	0.5
Lab D* measured	1.54 (1.47-1.62) 1.53 (1.46-1.61)	0.79 (0.69-0.89) 0.87 (0.77-0.98)	0.63 (0.53-0.73) 0.72 (0.61-0.83)	0.48
Lab E nominal	1.30 (1.14-1.46)	0.45 (0.31-0.59)	0.31 (0.19-0.44)	0.25
Lab E measured	1.43 (1.36-1.51)	0.65 (0.57-0.72)	0.49 (0.42-0.57)	0.21
Average	1.83 ± 0.50 (nominal) 1.72 ± 0.36 (measured)	0.98 ± 0.38 0.95 ± 0.23	0.81 ± 0.37 0.78 ± 0.22	0.4 ± 0.14 0.37 ± 0.15
CV (%)	27.1 (nominal) 21.2 (measured)	38.4 24.2	45.0 28.2	35.0 40.5

*Two independent tests were performed in Lab D. The two data sets from Lab D were included in the calculation of mean and CV for the presented EC values.
The number in parentheses: 95% CI, Average: mean EC50 among testing laboratories ± SD

CONCLUSION

The results of this ring test suggested that the EC values from the growth inhibition test using NIES-2724 were similar across testing laboratories, with a variation of approximately twofold, provided the test is conducted under established conditions. Interlaboratory tests based on the algal growth inhibition test specified in International Standard (ISO 8692)[6], which later became the basis for OECD TG 201 (1981), were carried out in 1980 and 1981. In this ring test, the coefficients of variation (CVs) of EC50 values obtained with $K_2Cr_2O_7$ and 3,5-DCP were 23 % and 38%, respectively, for the widely used green alga *Raphidocelis subcapitata*. In the present ring test, CVs of EC50 values obtained using $K_2Cr_2O_7$ and 3,5-DCP were 33.3% and 21.2%, respectively (ISO 8692), indicating a level of variability comparable to that observed in *R. subcapitata*. The sensitivity of NIES-2724 to $K_2Cr_2O_7$ and 3,5-DCP was significantly higher than that of green algae *Desmodesmus subspicatus* and *R. subcapitata*. In particular, for $K_2Cr_2O_7$, the sensitivity of NIES-2724 was 10-fold and 6-fold higher than that of *R. subcapitata* and *D. subspicatus*, respectively (Table 4-1).

On the other hand, one of the five participating laboratories (Lab D) did not meet the validity criterion for the mean CV of section-by-section growth rates due to a low growth rate on the first day of exposure. However, this issue is commonly observed even in growth inhibition tests using the green alga *R. subcapitata* when pre-culture is not enough. Therefore, it is well known that this issue can often be resolved by performing a second or third propagation step of the pre-culture. Lab A and C re-confirmed that additional pre-culture can restore the low growth rate on the first day of exposure, thereby bringing the mean CV of section-by-section growth rates within the acceptable range (Table 4-2). Although Lab D did not perform second propagation step, the result from a re-test, in which two pre-culture (3 days old for the first one and 4 days old for the second one) were mixed to initiate the test, suggest that a longer pre-culture period can also restore the low growth rate on the first day of exposure (Table 4-2).

Most diatom species, including *Mayamaea permitis*, have been reported to accumulate at the surface film due to lipid production (Suzuki et al. 2022)[7], leading to difficulties in accurately counting cells. This accumulation may also hinder exponential growth during testing or in inoculum cultures. In such cases, we recommended the use of a reciprocating shaker instead of a rotary shaker. If a reciprocating shaker is not available or extensive cell aggregation was observed, we recommended direct sonication treatment of inoculum culture flask for 3 to15 minutes at 40–50 kHz before sampling for the inoculation. This direct sonication treatment helped homogenize the cells; however, re-accumulated was observed within 72 hours after the inoculation in many participating laboratories (Lab B, Lab C, Lab E, and Lab F). In such cases, we recommended directly sonicating each test flask under same condition prior to sampling for cell counting every 24 hours. This sonication treatment every 24 hours allowed cultures to maintain stable exponential growth during both pre-culture and testing phases (Lab C, and E) (Table 4-2). Exposure to low-intensity ultrasonication (40 kHz) has been reported to promote diatom growth by disrupting cellular connections (Abate et al., 2020)[8]. In our previous study, we confirmed that this treatment does not harm diatom growth. Table 4-2 suggested that performing additional propagation step of the pre-culture and/or sonication treatment is recommended to reduce cell accumulation (Table 4-2).

Table 4-1 Comparison of Interlaboratory test results (EC50) among the recommended species

Test organism and test substaces	Number or lab	Mean value ± SD mg/L	Coefficient of variation %
*Desmodesmus subspicatus**			
Potassium dichromate	20	0.84 ± 0.12	14
3,5-Dichlorophenol	18	6.42 ± 2.38	37
*R. subcapitata**			
Potassium dichromate	9	1.19 ± 0.27	23
3,5-Dichlorophenol	9	3.38 ± 1.30	38
M. permitis			
Potassium dichromate	5	0.14 ± 0.05	33.3
3,5-Dichlorophenol	5	1.72 ± 0.36	21.2

*The results on the test specified in this International Standard carried out in 1980 and 1981.

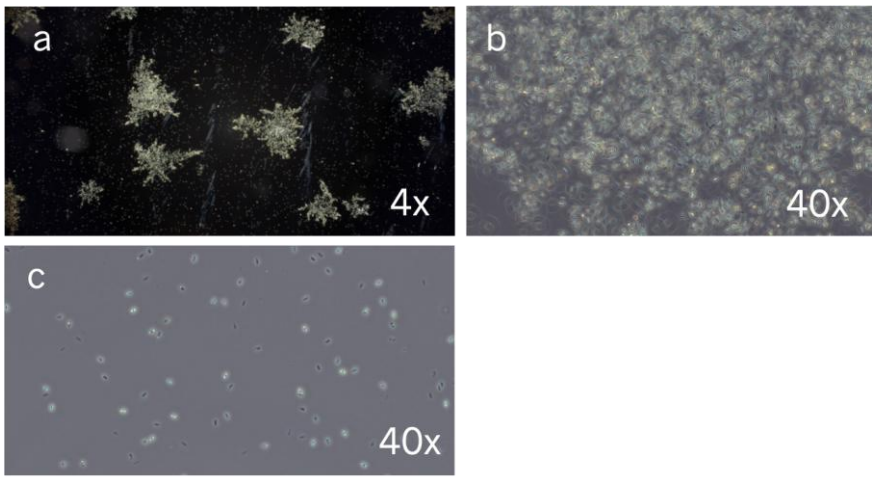


Figure 4-1 Images of NIES-2724 (provided from Lab F)
Culture before sonication 4x (a), 40x (b), and culture after sonication 40x (c)

Table 4-2 Summary of pre-culture frequency and sonication treatment

Lab	The number of pre-cultures	Sonication treatment every 24 hours during pre-culture and testing phase	Type of shaker (rpm)	Mean CV for section-by-section growth rates
A	2	–	Rotary (100)	< 35%
B	1	+	Rotary (100)	< 35%
C	2	–	Rotary (100)	< 35%
D	1	–	Rotary (100)	> 35%
	1	–	Rotary (100)	< 35%*
E	2	+	Rotary (100)	< 35%

*An additional pre-culture was not performed; instead, two pre-cultures (3 days old for the first one and 4 days old for the second one) were mixed to initiate the test.

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Annex 1.A. ANNEX 1

Details about the cell density at each concentration of Cr(VI)

Annex Table 1: Details about the cell density at each concentration of Cr(VI) (Lab A)

Concentration mg/L (as Chromium VI)	cells/mL Day 0 ($x10^4$)	cells/mL Day 1 ($x10^4$)	cells/mL Day 2 ($x10^4$)	cells/mL Day 3 ($x10^4$)	Growth rate D0 – D3	Inhibition
Control	0.5	2.47	9.74	28.3	1.36	
	0.5	2.19	8.74	28.4	1.36	
	0.5	2.16	9.11	31.1	1.40	
	0.5	2.28	9.58	27.7	1.34	
	0.5	2.45	9.08	35.6	1.44	
	0.5	2.31	10.8	39.3	1.44	
0.02	0.5	2.09	6.80	20.4	1.17	
	0.5	2.07	8.08	20.7	1.26	
	0.5	2.04	7.12	20.7	1.18	**13.4**
0.05	0.5	1.80	4.52	9.78	0.91	
	0.5	1.77	4.26	9.45	1.01	
	0.5	1.78	4.55	9.72	0.94	**31.4**
0.09	0.5	1.26	1.91	2.36	0.53	
	0.5	1.33	1.89	2.76	0.59	
	0.5	1.30	2.22	2.72	0.53	**60.3**
0.18	0.5	0.97	1.04	1.14	0.27	
	0.5	1.10	1.07	1.15	0.25	
	0.5	0.93	1.15	1.17	0.25	**81.7**
0.35	0.5	0.90	0.88	0.89	0.25	
	0.5	0.90	0.92	0.86	0.19	
	0.5	0.78	0.77	0.86	0.21	**84.5**

Validity criteria for control replicates

- Multiplication factor: **64.9**
- Coefficient of variation of average specific growth rates: **3.04 %**
- Mean coefficient of variation for section-by-section specific growth rates: **13.8 %**

Annex Table 2: Details about the cell density at each concentration of Cr(VI) (Lab B)

Concentration mg/L (as Chromium VI)	**R.U. Day 0** ($x10^3$)	**R.U. Day 1** ($x10^3$)	**R.U. Day 2** ($x10^3$)	**R.U. Day 3** ($x10^3$)	**Growth rate D0 – D3**	**Inhibition**
Control	0.049	0.26	1.32	5.70	1.59	
	0.049	0.25	1.37	5.79	1.59	
	0.049	0.27	1.40	5.30	1.56	
	0.049	0.25	1.20	5.79	1.59	
	0.049	0.26	1.37	5.92	1.60	
	0.049	0.27	1.37	6.03	1.60	
0.02	0.049	0.21	0.69	1.92	1.22	
	0.049	0.21	0.72	2.11	1.25	
	0.049	0.20	0.67	1.96	1.23	**22.2**
0.05	0.049	0.13	0.22	0.16	0.39	
	0.049	0.14	0.22	0.21	0.49	
	0.049	0.14	0.22	0.16	0.39	**73.3**
0.09	0.049	0.092	0.099	0.095	0.22	
	0.049	0.085	0.098	0.11	0.25	
	0.049	0.088	0.095	0.11	0.27	**84.4**
0.18	0.049	0.062	0.063	0.061	0.07	
	0.049	0.066	0.066	0.064	0.09	
	0.049	0.062	0.059	0.062	0.08	**95.0**
0.35	0.049	0.057	0.048	0.037	-0.10	
	0.049	0.051	0.053	0.037	-0.10	
	0.049	0.054	0.054	0.036	-0.10	**106**

R.U.: relative unit
Measurements were performed using chlorophyl fluorescence (ex: 430 nm, em: 670 nm)

Validity criteria for control replicates

- Multiplication factor: **117**
- Coefficient of variation of average specific growth rates: **0.93 %**
- Mean coefficient of variation for section-by-section specific growth rates: **7.35 %**

Annex Table 3: Details about the cell density at each concentration of Cr(VI) (Lab C)

Concentration mg/L (as Chromium VI)	cells/mL Day 0 ($x10^4$)	cells/mL Day 1 ($x10^4$)	cells/mL Day 2 ($x10^4$)	cells/mL Day 3 ($x10^4$)	Growth rate D0 – D3	Inhibition
Control	0.5	3.11	9.13	44.0	1.49	
	0.5	3.40	9.40	53.2	1.56	
	0.5	3.55	10.4	61.8	1.61	
	0.5	2.12	13.3	59.7	1.59	
	0.5	2.45	10.4	55.6	1.57	
	0.5	2.73	10.8	45.3	1.50	
0.004	0.5	2.35	11.1	47.5	1.52	
	0.5	2.93	12.4	46.0	1.51	
	0.5	2.83	9.64	43.1	1.49	**3.20**
0.009	0.5	2.46	14.1	51.2	1.54	
	0.5	3.30	12.0	49.3	1.53	
	0.5	2.24	9.75	44.0	1.49	**2.00**
0.017	0.5	2.26	6.45	29.3	1.36	
	0.5	2.93	7.26	25.2	1.31	
	0.5	1.95	5.87	29.4	1.36	**13.7**
0.035	0.5	2.08	4.08	13.3	1.09	
	0.5	2.13	4.57	11.4	1.04	
	0.5	2.25	4.77	12.1	1.06	**31.4**
0.07	0.5	1.38	2.30	3.53	0.65	
	0.5	1.65	2.51	4.24	0.71	
	0.5	1.26	2.20	3.22	0.62	**57.4**

Validity criteria for control replicates

- Multiplication factor: **107**
- Coefficient of variation of average specific growth rates: **3.02 %**
- Mean coefficient of variation for section-by-section specific growth rates: **13.3 %**

Annex Table 4: Details about the cell density at each concentration of Cr(VI) (Lab D) No.1

Concentration mg/L (as Chromium VI)	**cells/mL Day 0** ($x10^4$)	**cells/mL Day 1** ($x10^4$)	**cells/mL Day 2** ($x10^4$)	**cells/mL Day 3** ($x10^4$)	**Growth rate D0 – D3**	**Inhibition**
Control	1 .00	2.12	34.24	233.91	1.818	
	1.00	2.42	26.17	184.29	1.739	
	1.00	1.58	23.72	162.56	1.697	
	1.00	2.49	32.08	198.30	1.763	
	1.00	4.80	31.19	177.57	1.726	
	1.00	2.06	29.35	194.85	1.757	
0.02	1.00	3.60	11.79	57.25	1.349	
	1.00	1.90	13.95	61.93	1.375	
	1.00	2.21	10.44	52.11	1.318	**23.0**
0.05	1.00	1.27	5.22	9.96	0.766	
	1.00	1.26	5.45	12.82	0.850	
	1.00	1.37	4.79	10.00	0.768	**54.6**
0.09	1.00	0.98	1.51	1.94	0.221	
	1.00	1.40	2.53	2.70	0.331	
	1.00	1.04	2.22	5.24	0.552	**79.0**
0.18	1.00	1.02	1.62	1.55	0.146	
	1.00	0.86	1.41	1.41	0.115	
	1.00	0.77	1.40	1.77	0.190	**91.4**
0.35	1.00	0.81	1.22	1.19	0.058	
	1.00	1.20	0.95	1.30	0.087	
	1.00	0.68	1.48	1.57	0.150	**94.4**

Validity criteria for control replicates

- Multiplication factor: **192**
- Coefficient of variation of average specific growth rates: **2.34 %**
- Mean coefficient of variation for section-by-section specific growth rates: **37.9 %**

Annex Table 5: Details about the cell density at each concentration of Cr(VI) (Lab D) No. 2

Concentration mg/L (as Chromium VI)	cells/mL Day 0 ($x10^4$)	cells/mL Day 1 ($x10^4$)	cells/mL Day 2 ($x10^4$)	cells/mL Day 3 ($x10^4$)	Growth rate D0 – D3	Inhibition
Control	1 .00	2.01	18.40	111.58	1.572	
	1.00	3.99	22.20	126.40	1.613	
	1.00	2.66	22.01	141.92	1.652	
	1.00	2.72	27.26	151.58	1.674	
	1.00	2.26	20.59	146.47	1.662	
	1.00	1.81	16.90	113.22	1.576	
0.02	1.00	1.95	9.79	58.44	1.356	
	1.00	2.31	10.52	67.25	1.403	
	1.00	3.13	12.96	77.09	1.448	**13.7**
0.05	1.00	1.68	3.38	10.34	0.779	
	1.00	1.79	3.84	10.98	0.799	
	1.00	1.57	4.75	11.77	0.822	**50.8**
0.09	1.00	0.86	1.42	2.23	0.267	
	1.00	1.36	1.98	2.32	0.281	
	1.00	1.26	1.83	2.43	0.296	**82.7**
0.18	1.00	1.14	1.20	1.29	0.085	
	1.00	1.20	1.21	1.36	0.102	
	1.00	1.01	1.20	1.34	0.098	**94.2**
0.35	1.00	0.79	1.80	1.25	0.79	
	1.00	0.92	1.33	1.86	0.92	
	1.00	1.11	1.39	1.33	1.11	**92.3**

Validity criteria for control replicates

- Multiplication factor: **132**
- Coefficient of variation of average specific growth rates: **2.7 %**
- Mean coefficient of variation for section-by-section specific growth rates: **39.1 %**

Annex Table 6: Details about the cell density at each concentration of Cr(VI) (Lab E)

Concentration mg/L (as Chromium VI)	**cells/mL Day 0** ($x10^4$)	**cells/mL Day 1** ($x10^4$)	**cells/mL Day 2** ($x10^4$)	**cells/mL Day 3** ($x10^4$)	**Growth rate D0 – D3**	**Inhibition**
Control	0.5	1.72	5.69	17.0	1.175	
	0.5	2.35	8.68	23.1	1.278	
	0.5	1.69	6.61	27.7	1.338	
	0.5	2.02	8.18	31.2	1.378	
	0.5	1.71	9.20	30.0	1.365	
	0.5	1.24	4.52	21.1	1.249	
0.02	0.5	1.29	5.01	10.6	1.018	
	0.5	1.76	4.39	6.33	0.846	
	0.5	1.40	4.15	4.45	0.729	**33.4**
0.05	0.5	1.12	2.19	1.42	0.348	
	0.5	1.03	1.77	1.84	0.434	
	0.5	1.00	2.11	2.24	0.500	**67.1**
0.09	0.5	0.67	0.88	1.23	0.300	
	0.5	0.64	0.90	1.42	0.348	
	0.5	0.60	1.08	1.20	0.292	**75.8**
0.18	0.5	0.47	0.41	1.03	0.241	
	0.5	0.56	0.33	0.60	0.062	
	0.5	0.41	0.41	0.62	0.073	**90.3**
0.35	0.5	0.54	0.66	0.39	-0.080	
	0.5	0.42	0.62	0.51	0.005	
	0.5	0.39	0.59	0.43	-0.047	**103**

Validity criteria for control replicates

- Multiplication factor: **50.0**
- Coefficient of variation of average specific growth rates: **6.00 %**
- Mean coefficient of variation for section-by-section specific growth rates: **14.2 %**

Details about the cell density at each concentration of 3,5-DCP

Annex Table 5: Details about the cell density at each concentration of 3,5-DCP (Lab A)

Concentration mg/L	cells/mL Day 0 ($x10^4$)	cells/mL Day 1 ($x10^4$)	cells/mL Day 2 ($x10^4$)	cells/mL Day 3 ($x10^4$)	Growth rate D0 – D3	Inhibition
Control	0.5	1.65	6.18	20.7	1.33	
	0.5	1.52	7.24	20.8	1.38	
	0.5	1.58	6.58	19.2	1.31	
	0.5	1.72	7.32	21.6	1.33	
	0.5	1.63	7.73	24.3	1.41	
	0.5	2.06	7.30	26.7	1.32	
0.25	0.5	1.58	6.10	21.5	1.32	
	0.5	1.71	5.39	17.2	1.26	
	0.5	1.61	7.04	21.6	1.34	**3.10**
0.50	0.5	1.42	4.73	14.2	1.24	
	0.5	1.70	6.60	17.5	1.28	
	0.5	1.24	5.32	17.6	1.28	**6.10**
1.0	0.5	1.44	5.66	14.8	1.24	
	0.5	1.70	5.42	14.5	1.20	
	0.5	1.67	6.10	17.5	1.30	**7.51**
2.0	0.5	1.47	4.03	8,61	1.09	
	0.5	1.50	3.84	8.92	1.10	
	0.5	1.45	3.88	7.99	1.01	**20.9**
4.0	0.5	0.92	0.97	1.12	0.38	
	0.5	0.91	0.97	1.27	0.47	
	0.5	0.79	1.07	1.21	0.44	**68.2**

Validity criteria for control replicates

- Multiplication factor: **57.2**
- Coefficient of variation of average specific growth rates: **2.89 %**
- Mean coefficient of variation for section-by-section specific growth rates: **13.8 %**

Annex Table 6: Details about the cell density at each concentration of 3,5-DCP (Lab B)

Concentration mg/L	**R.U. Day** ($x10^3$)	**R.U. Day 1** ($x10^3$)	**R.U. Day 2** ($x10^3$)	**R.U. Day 3** ($x10^3$)	**Growth rate D0 – D3**	**Inhibition**
Control	0.033	0.26	1.27	5.24	1.69	
	0.033	0.26	1.27	5.23	1.69	
	0.033	0.25	1.26	4.58	1.65	
	0.033	0.25	1.27	5.04	1.68	
	0.033	0.26	1.24	5.11	1.68	
	0.033	0.24	1.21	4.99	1.67	
0.25	0.033	0.24	1.18	4.70	1.65	
	0.033	0.24	1.25	4.51	1.64	
	0.033	0.24	1.25	4.81	1.66	**1.44**
0.50	0.033	0.20	1.21	4.46	1.64	
	0.033	0.20	1.18	4.83	1.66	
	0.033	0.19	1.19	4.61	1.65	**1.63**
1.0	0.033	0.10	0.83	3.64	1.57	
	0.033	0.11	0.86	3.65	1.57	
	0.033	0.11	0.89	3.21	1.53	**7.22**
2.0	0.033	0.10	0.23	0.28	0.71	
	0.033	0.11	0.23	0.30	0.74	
	0.033	0.11	0.23	0.27	0.70	**57.2**
4.0	0.033	0.054	0.042	0.014	-0.28	
	0.033	0.058	0.047	0.018	-0.21	
	0.033	0.052	0.048	0.022	-0.14	**112**

R.U.: relative unit
Measurements were performed using chlorophyl fluorescence (ex: 430 nm, em: 670 nm)

Validity criteria for control replicates

- Multiplication factor: **153**
- Coefficient of variation of average specific growth rates: **0.99 %**
- Mean coefficient of variation for section-by-section specific growth rates: **19.6 %**

Annex Table 7: Details about the cell density at each concentration of 3,5-DCP (Lab C)

Concentration mg/L	cells/mL Day 0 ($x10^4$)	cells/mL Day 1 ($x10^4$)	cells/mL Day 2 ($x10^4$)	cells/mL Day 3 ($x10^4$)	Growth rate D0 – D3	Inhibition
Control	1 .00	5.66	18.8	159	1.69	
	1.00	5.39	20.2	166	1.70	
	1.00	4.74	17.5	149	1.67	
	1.00	5.28	17.1	126	1.61	
	1.00	5.23	16.2	164	1.70	
	1.00	4.71	14.8	142	1.65	
0.25	1.00	4.47	13.5	165	1.70	
	1.00	3.33	14.7	167	1.71	
	1.00	3.56	18.3	174	1.72	**-2.29**
0.50	1.00	5.64	19.3	157	1.69	
	1.00	4.98	14.0	154	1.68	
	1.00	3.77	17.5	138	1.64	**0.12**
1.0	1.00	4.42	11.8	88.7	1.50	
	1.00	4.25	13.0	92.0	1.51	
	1.00	4.57	13.0	87.6	1.49	**10.4**
2.0	1.00	2.83	3.71	6.84	0.64	
	1.00	2.65	3.33	5.86	0.59	
	1.00	3.72	3.20	6.48	0.62	**63.0**
4.0	1.00	1.15	1.23	1.31	0.09	
	1.00	1.71	1.34	1.15	0.05	
	1.00	1.15	1.64	1.08	0.03	**96.8**

Validity criteria for control replicates

- Multiplication factor: **151**
- Coefficient of variation of average specific growth rates: **2.09 %**
- Mean coefficient of variation for section-by-section specific growth rates: **25.3 %**

Annex Table 8: Details about the cell density at each concentration of 3,5-DCP (Lab D) No. 1

Concentration mg/L	**cells/mL Day 0** ($x10^4$)	**cells/mL Day 1** ($x10^4$)	**cells/mL Day 2** ($x10^4$)	**cells/mL Day 3** ($x10^4$)	**Growth rate D0 – D3**	**Inhibition**
Control	1 .00	2.31	25.64	167.95	1.708	
	1.00	1.99	19.43	118.61	1.592	
	1.00	2.16	30.09	120.98	1.599	
	1.00	1.95	22.81	106.15	1.555	
	1.00	1.98	22.07	157.99	1.688	
	1.00	2.48	22.23	96.05	1.522	
0.25	1.00	2.25	14.88	121.30	1.599	
	1.00	2.64	23.96	115.59	1.583	
	1.00	1.67	24.05	130.88	1.625	**0.6**
0.50	1.00	1.86	17.57	78.54	1.455	
	1.00	1.67	14.15	80.01	1.461	
	1.00	1.70	20.55	111.68	1.572	**7.1**
1.0	1.00	1.58	6.97	48.37	1.293	
	1.00	1.91	8.71	48.09	1.291	
	1.00	1.97	10.81	60.06	1.365	**18.3**
2.0	1.00	1.50	2.83	4.46	0.498	
	1.00	1.55	3.45	5.54	0.571	
	1.00	2.25	3.70	6.12	0.604	**65.4**
4.0	1.00	1.04	1.14	1.43	0.119	
	1.00	1.12	1.41	1.43	0.119	
	1.00	1.15	1.23	1.31	0.090	**93.2**

Validity criteria for control replicates

- Multiplication factor: **128**
- Coefficient of variation of average specific growth rates: **4.55 %**
- Mean coefficient of variation for section-by-section specific growth rates: **41.6 %**

Annex Table 8: Details about the cell density at each concentration of 3,5-DCP (Lab D) No. 2

Concentration mg/L	**cells/mL Day 0** ($x10^4$)	**cells/mL Day 1** ($x10^4$)	**cells/mL Day 2** ($x10^4$)	**cells/mL Day 3** ($x10^4$)	**Growth rate D0 – D3**	**Inhibition**
Control	1 .00	5.02	39.67	263.91	1.859	
	1.00	4.63	50.49	257.10	1.850	
	1.00	5.19	56.24	236.87	1.823	
	1.00	5.72	66.12	276.44	1.874	
	1.00	5.37	50.85	243.03	1.831	
	1.00	4.10	42.93	228.18	1.810	
0.25	1.00	6.95	39.36	238.55	1.825	
	1.00	4.37	38.57	189.34	1.748	
	1.00	5.59	39.89	244.69	1.833	**2.1**
0.50	1.00	3.69	27.66	165.90	1.704	
	1.00	3.98	33.55	194.54	1.757	
	1.00	4.16	33.42	237.86	1.824	**4.3**
1.0	1.00	3.47	19.88	105.58	1.553	
	1.00	3.33	17.98	93.48	1.513	
	1.00	2.88	16.52	130.67	1.624	**15.1**
2.0	1.00	1.97	3.65	5.32	0.557	
	1.00	1.92	4.08	5.51	0.569	
	1.00	2.16	3.54	6.36	0.617	**68.4**
4.0	1.00	1.14	1.02	1.45	0.124	
	1.00	1.30	1.06	2.37	0.288	
	1.00	1.29	1.27	1.07	0.023	**92.1**

Validity criteria for control replicates

- Multiplication factor: **251**
- Coefficient of variation of average specific growth rates: **1.30 %**
- Mean coefficient of variation for section-by-section specific growth rates: **22.2 %**

Annex Table 9: Details about the cell density at each concentration of 3,5-DCP (Lab E)

Concentration mg/L	**cells/mL Day 0** ($x10^4$)	**cells/mL Day 1** ($x10^4$)	**cells/mL Day 2** ($x10^4$)	**cells/mL Day 3** ($x10^4$)	**Growth rate D0 – D3**	**Inhibition**
Control	0.5	1.75	7.05	38.5	1.69	
	0.5	1.87	9.50	36.1	1.70	
	0.5	1.79	7.76	45.7	1.67	
	0.5	2.26	10.1	65.1	1.61	
	0.5	1.89	5.72	37.8	1.70	
	0.5	1.86	6.82	35.8	1.65	
0.25	0.5	1.42	5.01	24.2	1.70	
	0.5	1.85	5.34	31.7	1.71	
	0.5	2.26	9.67	57.9	1.72	**3.92**
0.50	0.5	1.52	2.62	24.0	1.69	
	0.5	1.59	3.10	12.2	1.68	
	0.5	1.73	2.47	14.9	1.64	**21.4**
1.0	0.5	1.33	2.22	11.1	1.50	
	0.5	1.43	3.15	14.3	1.51	
	0.5	1.40	2.35	0.99	1.49	**29.0**
2.0	0.5	0.90	1.25	1.56	0.64	
	0.5	0.73	0.85	1.62	0.59	
	0.5	0.76	0.72	1.65	0.62	**73.6**
4.0	0.5	0.56	0.48	0.56	0.09	
	0.5	0.48	0.67	0.78	0.05	
	0.5	0.50	0.44	0.66	0.03	**93.7**

Validity criteria for control replicates

- Multiplication factor: **86.3**
- Coefficient of variation of average specific growth rates: **5.20 %**
- Mean coefficient of variation for section-by-section specific growth rates: **16.6 %**

Annex 1.B. ANNEX 2

Results of analytical measurements of Cr(VI)

Five testing laboratories provided analytical results. Water samples were collected on days 0 and 3 for Cr(VI). Tables provided an overview of the resulting measured concentrations of days 0 and 3. The numbers in parentheses indicated the ratio of the measured concentration to the nominal concentrations.

Lab A:

Nominal concentrations $K_2Cr_2O_7$ (mg/L)	0.06	0.13	0.25	0.50	1.0
Measured concentrations T0 (mg/L)	0.06 (100)	0.15 (115)	0.23 (92.0)	0.47 (94.0)	1.09 (100)
Measured concentrations T72h (mg/L)	0.06 (100)	0.14 (108)	0.25 (100)	0.49 (98.0)	0.98 (98.0)
Geometric mean	0.06 (100)	0.15 (115)	0.24 (96.0)	0.48 (96.0)	1.03 (103)

Lab B:

Nominal concentrations $K_2Cr_2O_7$ (mg/L)	0.06	0.13	0.25	0.50	1.0
Measured concentrations T0 (mg/L)	0.06 (100)	0.15 (115)	0.26 (104)	0.52 (104)	1.12 (112)
Measured concentrations T72h (mg/L)	0.06 (100)	0.14 (108)	0.27 (108)	0.50 (100)	1.09 (109)
Geometric mean	0.06 (100)	0.14 (108)	0.26 (104)	0.51 (102)	1.10 (110)

Lab C:

Nominal concentrations $K_2Cr_2O_7$ (mg/L)	0.013	0.025	0.05	0.1	0.2
Measured concentrations T0 (mg/L)	0.013 (100)	0.024 (96.0)	0.048 (96.0)	0.098 (98.0)	0.21 (105)
Measured concentrations T72h (mg/L)	0.013 (100)	0.024 (96.0)	0.047 (96.0)	0.099 (99.0)	0.20 (100)
Geometric mean	0.013 (100)	0.024 (96.0)	0.048 (96.0)	0.099 (99.0)	0.21 (105)

Lab D (No. 1)

Nominal concentrations $K_2Cr_2O_7$ (mg/L)	0.06	0.13	0.25	0.50	1.0
Measured concentrations T0 (mg/L)	0.07 (117)	0.14 (108)	0.23 (92.0)	0.46 (92.0)	1.10 (110)
Measured concentrations T72h (mg/L)	0.06 (100)	0.13 (100)	0.29 (116)	0.48 (96.0)	0.98 (98.0)
Geometric mean	0.06 (100)	0.13 (100)	0.26 (104)	0.47 (94.0)	1.04 (104)

Lab D (No. 2)

Nominal concentrations $K_2Cr_2O_7$ (mg/L)	0.06	0.13	0.25	0.50	1.0
Measured concentrations T0 (mg/L) r	0.06 (100)	0.13 (100)	0.24 (96.0)	0.39 (78.0)	0.99 (99.0)
Measured concentrations T72h (mg/L)	0.05 (83.3)	0.13 (100)	0.23 (92.0)	0.54 (108)	0.99 (99.0)
Geometric mean	0.05 (83.3)	0.13 (100)	0.23 (92.0)	0.46 (92.0)	0.99 (99.0)

Lab E

Nominal concentrations $K_2Cr_2O_7$ (mg/L)	0.06	0.13	0.25	0.50	1.0
Measured concentrations T0 (mg/L)	0.06 (100)	0.14 (108)	0.23 (92.0)	0.43 (86.0)	0.99 (99.0)
Measured concentrations T72h (mg/L)	0.06 (100)	0.13 (100)	0.23 (92.0)	0.47 (94.0)	0.99 (99.0)
Geometric mean	0.06 (100)	0.13 (100)	0.23 (92.0)	0.45 (90.0)	0.99 (99.0)

These results showed that the nominal and measured concentration were consistent, and the concentrations of Cr(VI) remained stable during the exposure period.

Results of analytical measurements of 3,5-DCP

Five testing laboratories provided analytical results. Water samples were collected on days 0 and 3 for 3,5-DCP. Tables provided an overview of the resulting measured concentrations of days 0 and 3. The numbers in parentheses indicated the ratio of the measured concentration to the nominal concentrations.

Lab A

Nominal concentrations (mg/L)	0.25	0.5	1.0	2.0	4.0
Measured concentrations T0 (mg/L)	0.23 (92.0)	0.42 (84.0)	0.93 (93.0)	1.92 (96.0)	3.57 (89.3)
Measured concentrations T72h (mg/L)	0.21 (84.0)	0.44 (88.0)	0.88 (88.0)	1.61 (80.5)	3.50 (87.5)
Geometric mean	0.22 (88.0)	0.43 (86.0)	0.90 (90.0)	1.76 (88.0)	3.53 (88.3)

LabB

Nominal concentrations (mg/L)	0.25	0.5	1.0	2.0	4.0
Measured concentrations T0 (mg/L)	0.24 (96.0)	0.51 (102)	1.10 (110)	2.03 (102)	4.28 (107)
Measured concentrations T72h (mg/L)	0.22 (88.0)	0.48 (96.0)	0.89 (89.0)	1.70 (85.0)	3.23 (80.8)
Geometric mean	0.23 (92.0)	0.49 (98.0)	0.99 (99.0)	1.86 (93.0)	3.72 (93.0)

Lab C

Nominal concentrations (mg/L)	0.25	0.5	1.0	2.0	4.0
Measured concentrations T0 (mg/L)	0.24 (96.0)	0.49 (98.0)	1.11 (111)	1.99 (99.5)	3.98 (99.5)
Measured concentrations T72h (mg/L)	0.22 (88.0)	0.46 (92.0)	0.89 (89.0)	1.74 (87.0)	3.63 (90.8)
Geometric mean	0.23 (92.0)	0.47 (94.0)	0.99 (99.0)	1.86 (93.0)	3.80 (95.0)

Lab D (No. 1)

Nominal concentrations (mg/L)	0.25	0.5	1.0	2.0	4.0
Measured concentrations T0 (mg/L)	0.21 (84.0)	0.51 (102)	1.09 (109)	1.88 (94.0)	3.74 (93.5)
Measured concentrations T72h (mg/L)	0.22 (88.0)	0.45 (90.0)	0.95 (95.0)	1.81 (90.5)	3.85 (96.3)
Geometric mean	0.21 (84.0)	0.48 (96.0)	1.02 (102)	1.84 (92.0)	3.79 (94.8)

Lab D (No. 2)

Nominal concentrations (mg/L)	0.25	0.5	1.0	2.0	4.0
Measured concentrations T0 (mg/L)	0.25 (100)	0.51 (102)	0.89 (89.0)	1.79 (89.5)	3.23 (80.8)
Measured concentrations T72h (mg/L)	0.26 (104)	0.46 (92.0)	0.88 (88.0)	1.77 (88.5)	3.35 (83.8)
Geometric mean	0.25 (100)	0.48 (96.0)	0.88 (88.0)	1.78 (89.0)	3.29 (82.3)

Lab E

Nominal concentrations (mg/L)	0.25	0.5	1.0	2.0	4.0
Measured concentrations T0 (mg/L)	0.24 (96.0)	0.48 (96.0)	0.95 (95.0)	1.94 (97.0)	3.78 (94.5)
Measured concentrations T72h (mg/L)	0.18 (72.0)	0.39 (78.0)	0.78 (78.0)	1.66 (83.0)	3.50 (87.5)
Geometric mean	0.21 (84.0)	0.43 (86.0)	0.86 (86.0)	1.79 (89.5)	3.64 (91.0)

These results showed that the nominal and measured concentration were consistent, and the concentrations of 3,5-DCP remained stable during the exposure period.

Annex 1.C. ANNEX 3

Calibration curve for estimating the cell number (NIES-2724) based on chlorophyll fluorescence (ex: 430 nm, em: 670 nm) or absorbance measurements (680 nm and 440 nm).