Toxicity of 3 water samples tested with the Algae growth inhibition test using *Raphidocelis subcapitata*

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Wageningen University &
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Test report

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Contents

1 Introduction

A risk assessment concerning the potential environmental risk of highway run-off water is performed by WMR, under the assignment of TNO. To support the risk assessment, the potential toxicity of three samples from run-off and surface water from different highways in Europe, is tested by WMR. Fresh water WET tests are performed for species from multiple tropic levels, namely bacteria (*Vibrio fischeri*), algae (*Raphidocelis subcapitata*) and crustacean (*Daphnia magna*).

This report describes the results of the algae growth inhibition tests with the algae *Raphidocelis subcapitata*.

Reported are the 72h NOEC and/or EC_{10} and EC_{25} as indication for chronic effects and the 72h EC_{50} as indication for acute toxicity. Furthermore the TU_C is reported.

2 Materials and Methods

2.1 Abbreviations

Milli-Q	Ultra-pure water
EC_{xx}	Calculated concentration causing xx% (growth inhibition) effect
NOEC	No Observed effect concentration; i.e. the highest test concentration without significant negative effects
TUc	Chronic Toxicity Units ($100/EC_{25}$)

2.2 Test material

Sample 1	52019067-015 (E18)
Sample 2	52019067-001 (A61)
Sample 3	52019067-007 (A2)

Sample transport: The samples were delivered at WMR's laboratory in Den Helder on August 6th and 15th, 2019. At arrival the samples were stored at 7°C and gradually brought back to room temperature before used in the test.

2.3 Test method

The test was carried out according to the procedure detailed in SOP E_4_052 'Zoetwater algengroeiremmingstest', which is summarised below. The test procedure is based upon ISO 8692:2012 'Fresh water algal growth inhibition test with unicellular green algae', but adapted for use on a plate reader with small volumes.

Species	*Raphidocelis subcapitata*
Strain	Algae beads MicroBioTests™
Cultivation	Continuously shaking 250ml Erlenmeyer's
Growth phase	exponential
Growth medium	ISO, <12 months old

Test vessels	96-multiwell plates (polystyrene)
Dilution water	Milli-Q
pH dilution water+ media	8.1±1.0
pH test water	6-9
Water treatment	Prior to the test, the dilution water was aerated to ensure dissolved oxygen was >90%. The samples were homogenised 24 hours before the test to enable particles to settle out before using the water in the test. Furthermore the test water was 0.45µm filtered to remove indigenous organisms that might interfere with the test results.
Test concentrations	0% (blank), 31.6%, 42.2%, 56.2%, 75.0% and 100% These are nominal concentrations. For the test 10µl algae concentrate is added to 240 µl of the test concentration, which is 4% of the volume.
Start density	10,000 cells/ml
Test volume	0.25 ml
Replicates	8
Temperature	23±2 °C
Photoperiod	Continuous
Light intensity	6000-10000 lux with LED light
Test duration	72 h
Endpoint	Growth inhibition
Observations	Fluorescence (every 24h±2)
Water quality	In the test water pH is measured in 0% and 100% test concentrations at start and after 72h.

Quality parameters

Control growth	μ_c>1.4 r^{-1} (increase 16x)
Control growth variance	<7%, after T=72
Day to day growth variance	<35%, after T=72
Control pH	<1.5 unit increase
Reference toxicant	Several times per year, the reference $K_2Cr_2O_7$ is tested. The results are added to a control chart.
Calculation of results	The EC_{XX} are calculated using a 'sigmoidal dose-response curve' with variable slope and is based on inhibition of growth in the test concentrations relative to the 0% blank. The calculated effect concentrations are not corrected for dilution by algal stock. The NOEC is derived from the data noting that the effect at the NOEC should not exceed 10% (ECB, 2003). The TU_C is derived from the data by calculating $100/EC_{25}$.

3 Results

3.1 Water quality

Table 1 shows that the pH after 72 hours did not increase more than 1.5 units compared to the starting pH. So the test meets this test validity requirement.

Table 1 pH of control (C0) and 100% (C5) at start and end of the test

Sample	pH start	pH end
Sample 1 (C0)	8.46	8.74
Sample 1 (C5)	8.22	8.22
Sample 2 (C0)	8.46	8.85
Sample 2 (C5)	7.58	8.12
Sample 3 (C0)	8.46	9.06
Sample 3 (C5)	7.86	9.22

3.2 Control validity

Table 2 and Table 3 show that the mean growth rate of the control wells is always above the requirement of 1.5 μ^{d-1}. And the coefficient of this growth is <7% after 72 hours, which also meets the test requirement.

Table 2 Mean growth rate in μ^{d-1}, after 24, 48 and 72 hours in the control wells

Growth rate (μ d^{-1})	24 hours	48 hours	72 hours
Sample 1	1.85	1.88	1.76
Sample 2	1.63	1.74	1.68
Sample 3	1.90	1.86	1.71

Table 3 Coefficient of variation (in %) in the control wells after 72 hours

Coefficient of variation (%)	72 hours
Sample 1	2.3
Sample 2	4.4
Sample 3	3.9

Table 4 and Table 5 show that the day to day growth rate of the control wells does decrease a little bit in the last 24 hours of the test. However, the algae are still in exponential growth phase. Figure 1 shows that the growth of the algae is still linear, plotting it on a log scale. The coefficient of variation for the day to day growth should be <35%, which is met for all 3 samples.

Table 4 Day to day growth rate in the control wells for day 0-1, 1-2 and 2-3

Day to day growth rate	0-1	1-2	2-3
Sample 1	1.84	1.88	1.52
Sample 2	1.63	1.87	1.50
Sample 3	1.90	1.81	1.35

Table 5 Day to day growth rate variance in the control wells in % for day 0-1, 1-2 and 2-3

Coefficient of variation (%)	0-1	1-2	2-3
Sample 1	6.7	2.2	2.9
Sample 2	12.2	3.4	17.4
Sample 3	9.2	3.3	12.0

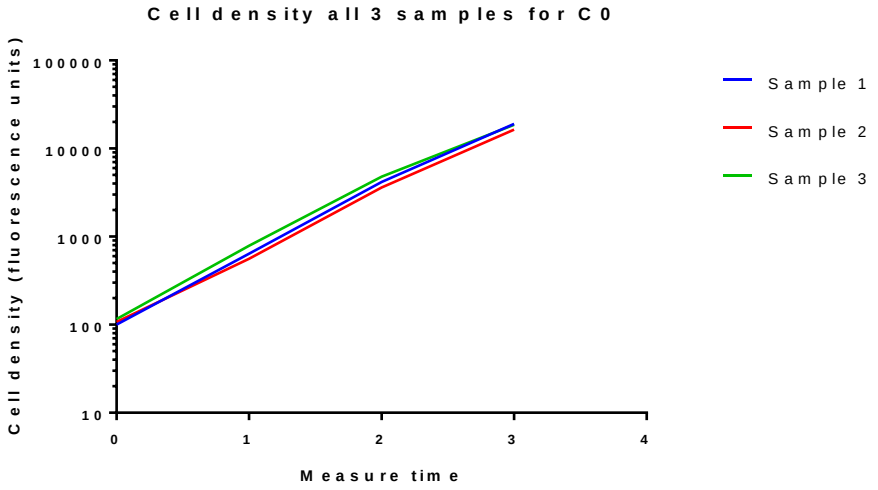

Figure 1 cell density of the control wells of all 3 samples plotted on log scale

3.3 Growth inhibition

Table 6, Table 7 and Table 8 show the day to day growth in measured fluorescence units for all 3 samples for all measurement times. Also the corresponding growth inhibition after 72h is shown. These tables show that both sample 1 and sample 2 show significant inhibition effects that are clearly dose-related. Sample 3 does not show growth inhibition effects.

Table 6 Day-to-day growth (fluorescence units) for sample 1 and mean % inhibition after 3 days

Concentration/Day	0	1	2	3	Inhibition
0.00	100	638	4174	19073	0%
30.3	100	606	4595	18200	0%
40.5	100	615	4609	18312	0%
54.0	100	522	2912	9458	12%
72.0	100	474	2202	6453	19%
96.0	100	344	1185	2940	35%

Table 7 Day-to-day growth (fluorescence units) for sample 2 and mean % inhibition after 3 days

Concentration/Day	0	1	2	3	Inhibition
0.00	108	560	3619	16369	0%
30.3	108	123	197	254	84%
40.5	108	109	73	81	99%
54.0	108	116	61	60	100%
72.0	108	108	48	44	100%
96.0	108	116	61	61	100%

Table 8 Day-to-day growth (fluorescence units) for sample 2 and mean % inhibition after 3 days

Concentration/Day	0	1	2	3	Inhibition
0.00	116	788	4787	18604	0%
30.3	116	1045	6795	22047	-5%
40.5	116	936	6253	19351	-2%
54.0	116	870	5845	16528	0%
72.0	116	767	5095	13525	4%
96.0	116	715	4823	11732	6%

4 Conclusion and recommendation

Table 9 shows the endpoints for the algae growth inhibition test with *Raphidocelis subcapitata*. Sample 1 shows significant dose-related growth inhibition with a NOEC at 42.2% sample, but an EC_{50} could not be calculated as the effect at the highest concentration was only 35%. Chronic effect concentrations could be calculated, however. Sample 2 shows clear growth inhibition and even at the lowest concentration of 30.3%, 84% effect was observed. A NOEC could not be established, and acute, as well as chronic effect parameters were too far below the lowest concentration tested to enable calculation. In order to be able to calculate an EC_{50}-value, the test should be repeated, using a further diluted concentration range. Furthermore, for both the samples 1 and 2, a TIE (Toxicity Identification Evaluation) assessment might be performed. This may help identifying the type of toxicant responsible for the observed effects. Sample 3 did not show significant growth inhibition.

Table 9 Endpoints for all 3 samples after 72 hours

72 hours	**EC_{50}**	**Effects**	**NOEC**	**EC_{10}**	**EC_{25}**	**TU_C**
Sample 1	>96%	35% effect at highest concentration	C2 (42.2%)	58%	84.5%	0.845
Sample 2	<30.3%	84% effect at lowest concentration	<31.6%	<31.6%	<31.6%	<0.316
Sample 3	>96%	6% effect at highest concentration	n.a.	>100%	>100%	>1

5 Quality Assurance

Wageningen Marine Research utilises an ISO 9001:2015 certified quality management system. This certificate is valid until 15 December 2021. The organisation has been certified since 27 February 2001. The certification was issued by DNV GL.

Justification

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The scientific quality of this report has been peer reviewed by a colleague scientist and a member of the Management Team of Wageningen Marine Research

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