Study No. J17000621-3

TEST REPORT

Impact assessment study for marine organisms
on a test plate coated with AQUATERRAS
—Saltwater Algal Toxicity Test—

October 6, 2017

WDB Environmental & Biological Research Institute Co., Ltd.

Sponsor	Nippon Paint Marine Coatings Co., Ltd.
Test substance	Test plate coated with AQUATERRAS
Study Title	Impact assessment study for marine organisms on a test plate coated with AQUATERRAS —Saltwater Algal Toxicity Test—
Study Number	J17000621-3

This study was conducted with the test substance provided to us on September 11, 2017.

I hereby certify that the reported results reflect accurately the raw data of testing, and that the test results are valid.

Date: October 6, 2017 *Approved by:* 中村 翔治

Laboratory & Study Manager

SUMMARY

Impact assessment study for marine organisms
on a test plate coated with AQUATERRAS
—Saltwater Algal Toxicity Test—

This study was conducted to evaluate the toxicity of the test substance provided to us by the sponsor with *Skeletonema costatum* under the test conditions described below.

<Test Conditions>

1) Test substance:	Test plate coated with AQUATERRAS
2) Test organism:	*Skeletonema costatum* (NRIA-0103)
3) Test duration:	96 hours
4) Test vessel:	200-mL Erlenmeyer flask
5) Test section:	Addition section and control section
6) Test concentration*:	31.4 cm^2/L by painted area
7) Number of replicates:	3 replicates/section
8) Test type:	Static, shaken by hand twice a day
9) Temperature:	20±1℃
10) Photoperiod:	14 hours light: 10 hours dark, 4,300 lx±10%
11) Observation:	24, 48, 72 and 96 hours

*The test concentration was set based on the assumption of seawater being in a stationary state within a distance of 30 cm from the coated object.

<Test Results>

No observed effect concentration (NOEC): 31.4 cm^2/L by painted area

No inhibition of growth in *Skeletonema costatum* was observed in the test section. Therefore, the test plate coated with AQUATERRAS showed no toxicity to the test organisms during the test period (P>0.05).

FINAL REPORT

1. Study Title

Impact assessment study for marine organisms on a test plate coated with AQUATERRAS —Saltwater Algal Toxicity Test—

2. Sponsor

Name: Nippon Paint Marine Coatings Co., Ltd.
Address: 1-26 Komagabayashi Minami-Cho, Nagata-Ku, Kobe Hyogo 653-0045, Japan

3. Testing Facility

Name: WDB Environmental and Biological Research Institute Co.,Ltd.
Address: 1-6 Tonomui, Aza, Yamagawauchi, Minami-Cho, Kaifu-Gun, Tokushima 779-2307, Japan

4. Purpose of the Study

This study was conducted to evaluate the acute toxicity of the test substance with saltwater algae.

5. Test Period

Start date: September 11, 2017
End date: September 15, 2017

6. Study manager: Tomoharu Nakamura (Laboratory Manager)

7. Experimental Staff: Chie Horita (Chief of Algal Culture Section)
Kensuke Iwamoto (Research Engineer)

8. Test Substance

1) Name: Test plate coated with AQUATERRAS
2) Date received: September 11, 2017
3) Storage conditions: Dark place at room temperature

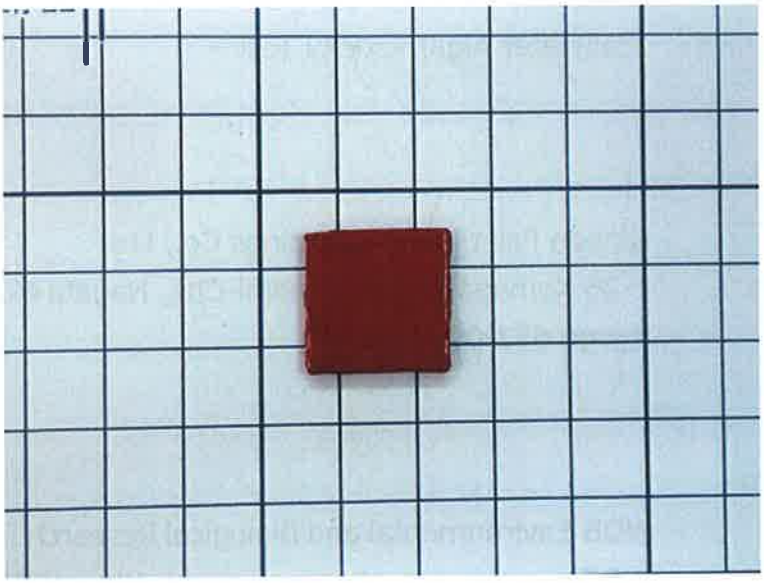

Fig. 1. Test plate coated with AQUATERRAS

9. Materials and Methods

1) Test organism

(1) Common name: -
(2) Scientific name: *Skeletonema costatum*
(3) Origin: National Research Institute of Aquaculture, Japan
(4) Source: In-house cultivation
(5) Acclimation: Subculture under the same conditions as testing
(6) Other: Logarithmically-growing stock culture

Fig. 2. *Skeletonema costatum*

2) Growth medium

The growth medium shown in U.S. EPA OCSPP 850.4500 was used for both the pre-culture and test. The composition of the medium was as follows (pH 7.5, 30±5‰).

● Filtered natural sea water	1L
● $NaHCO_3$	15 mg/L
● $NaNO_3$	25.5 mg/L
● $MgCl_2 \cdot 6(H_2O)$	12.16 mg/L
● $CaCl_2 \cdot 2(H_2O)$	4.41 mg/L
● $MgSO_4 \cdot 7(H_2O)$	14.6 mg/L
● K_2HPO_4	1.044 mg/L
● $FeCl_3 \cdot 6(H_2O)$	0.160 mg/L
● $Na_2EDTA \cdot 2(H_2O)$	0.300 mg/L
● H_3BO_3	0.186 mg/L
● $MnCl_2 \cdot 4(H_2O)$	0.415 mg/L
● $ZnCl_2$	3.27 μg/L
● $CoCl_2 \cdot 6(H_2O)$	1.43 μg/L
● $Na_2MoO_4 \cdot 2(H_2O)$	7.26 μg/L
● $CuCl_2 \cdot 2(H_2O)$	0.012 μg/L

3) Testing devices

Test vessel:	200-mL Erlenmeyer flask
Lighting system:	Cool-white fluorescent lighting
Temperature control:	Thermostatic room

4) Test conditions

Test type:	Static, shaken by hand twice a day
Test duration:	96 hours
Test water:	Filtered natural sea water (30±5‰)
Test volume:	100 mL
Test concentration*:	31.4 cm^2/L by painted area
Test section:	Addition section and control section
Number of replicates:	3 replicates/section
Temperature:	20±1°C
Photoperiod:	14 hours light: 10 hours dark, 4,300 lx±10%

*The test concentration was set based on the assumption of seawater being in a stationary state within a distance of 30 cm from the coated object.

5) Preparation of test substance and test solution

The test substance provided by the sponsor was washed lightly with the sterilized test medium and immersed in the test vessel containing the sterilized test medium to prepare the test solution.

6) Observations and measurements

(1) Algal observation

The state of the cells in each test vessel was observed using direct microscopic observation at 24, 48, 72, and 96 hours. The algal cell density was enumerated using a hemacytometer at the same time.

(2) Measurement of test conditions

The pH of the test solution was measured and adjusted at the time of preparation of the growth medium, and measured again at the end of the test. Temperature and light intensity of the culture environment were measured once a day during the test period.

7) Treatment of results

The average number of cells in the addition section and the control section were plotted against time to prepare a growth curve.

(1) Average specific growth rate

The average specific growth rate for a specific period was calculated from the following equation for each test vessel:

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i}$$

μ_{i-j} = average specific growth rate per day (day^{-1}) of the observed biomass (cell density) from time i to j

X_i = observed biomass (cell density) at the beginning of the observation interval, time i

X_j = observed biomass (cell density) at the end of the observation interval, time j

t_i = time of the i^{th} measurement after test initiation

t_j = time of the j^{th} measurement after test initiation

The percentage of growth rate inhibition for the addition section (I_μ) was calculated from the following equation for each test vessel:

$$I_\mu = \frac{\mu_c - \mu_T}{\mu_c} \times 100$$

μ_c = mean average specific growth rate in the control section
μ_T = average specific growth rate for the addition section

(2) Area under the growth curve

The area under the growth curve for each test vessel based on cell density was calculated from the following equation:

$$A = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_2 - 2N_0}{2} \times (t_2 - t_1) + \cdots + \frac{N_{n-1} + N_n - 2N_0}{2} \times (t_n - t_{n-1})$$

A = area under the growth curve for biomass (cell density)
N_0 = observed biomass (cell density) at test initiation
N_1 = observed biomass (cell density) at time t_1
N_n = observed biomass (cell·density) at time t_n
t_1 = time of the first measurement after test initiation
t_n = time of the n^{th} measurement after test initiation

The percentage of inhibition using the area under the growth curve for the addition section (I_A) was calculated from the following equation for each test vessel:

$$I_A = \frac{A_c - A_t}{A_c} \times 100$$

A_c = mean yield in the control section
A_t = yield for the addition section

(3) NOEC

In the case that no significant difference (5% level) was observed between the addition and control sections in the statistical analysis, the arbitrary addition amount was taken as the NOEC (NOEC ≥ addition amount) for the limit test.

10. Test Results

1) Cell density and growth curve

The cells in the control section grew exponentially during the test period. At the end of the exposure period, the number of cells increased to at least 32 times the number of initial cells in the control. This meets the validity of the test since cell growth in the control should have increased by a factor of at least 30 times at 96 hours after the start of the exposure.

Inhibition was not observed in the addition section. This means that growth in the addition section was almost the same as that in the control section (Tables 1, 2 and Fig. 3).

Based on the results of the statistical analysis (*F*-test and t-test), the NOEC based on growth was ≥31.4 cm^2/L (P>0.05).

Table 1. Cell densities of *Skeletonema costatum* at 24, 48, 72, and 96 hours

Section	No.	Cell density (cells/mL)				
		開始時	24 h	48 h	72 h	96 h
Test flasks	1	10,000	39,000	124,000	219,000	303,000
	2	10,000	43,000	126,000	233,000	323,000
	3	10,000	41,000	128,000	244,000	323,000
	Average	10,000	41,000	126,000	232,000	316,333
	SD	0	2,000	2,000	12,530	11,547
Control	1	10,000	42,000	126,000	230,000	320,000
	2	10,000	45,000	150,000	282,000	363,000
	3	10,000	43,000	130,000	240,000	333,000
	Average	10,000	43,333	135,333	250,667	338,667
	SD	0	1,528	12,858	27,592	22,053

SD, Standard deviation

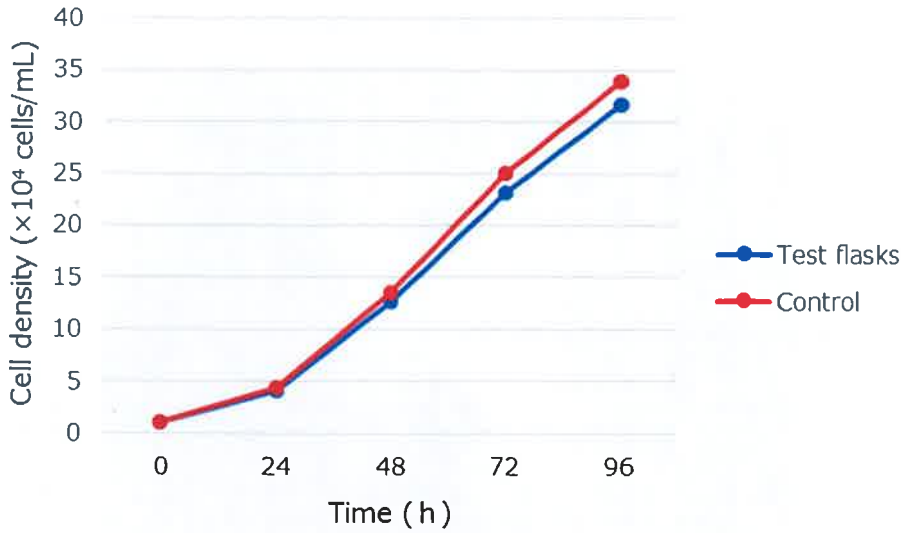

Fig. 3. Algal growth curve of *Skeletonema costatum*

Table 2. Percent growth inhibition of *Skeletonema costatum*

Section	No.	Area under the growth curve ($\times 10^4$)	Inhibition (%)	Growth rate (0-96 時間)	Inhibition (%)
Test flasks	1	1,196	1.91	0.0355	7.36
	2	1,268		0.0362	
	3	1,295		0.0362	
	Average	1,253		0.0360	
Control	1	1,255	-	0.0361	-
	2	1,496		0.0374	
	3	1,307		0.0365	
	Average	1,353		0.0367	

2) Appearance of test solution

The test solution was clear and colorless at the start of the exposure and the appearance of the test solution at end of the exposure was light brown due to algae growth.

3) Water quality and environmental conditions

The pH of the test solution was 7.5 at the start, and 9.1 to 9.2 at the end of the exposure (Table 3). Temperature ranged from 20.1 to 20.3°C and light intensity was 4,400 lx (Table 4).

Table 3. pH of the test solution

Section	At the start	At the end
Test flasks	7.5	9.1
Control	7.5	9.2

Table 4. Temperature and light intensity

Time	At the start	24 h	48 h	72 h	96 h
Temperature (°C)	20.1	20.1	20.3	20.2	20.2
Light intensity (lx)	4,400	4,400	4,400	4,400	4,400

4) Factors affecting the reliability of the test results

There were no factors that might have affected the reliability of the test results.

11. Validity of the Test

Cell growth in the control increased by a factor of at least 30 times at 96 hours after the start of the exposure, and the water quality and test conditions were good. Therefore, the validity of this test was confirmed.

12. References

- United States Environmental Protection Agency. Ecological Effects Test Guidelines. OCSPP 850.4500: Algal Toxicity (2012).

13. Images

Fig. 4. Overhead view of the test