Macrothink Institute™

Aquatic Science and Technology
ISSN 2168-9148
2015, Vol. 3, No. 2

Variations in the Toxic Effects of Petroleum Hydrocarbons to *Isochrysis galbana* under Different Environmental Factors

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Received: April 25, 2015 Accepted: May 30, 2015 Published: July 25, 2015

doi:10.5296/ast.v3i2.8051 URL: http://dx.doi.org/10.5296/ast.v3i2.8051

Abstract: In order to study the toxic effect of petroleum hydrocarbons on marine phytoplankton, *Isochrysis galbana* was investigated by using the index of 96 h-EC50 and population growth. The results showed that the 96 h-EC50 increased significantly ($P<0.05$) when temperature rising from 15℃ to 25℃. The intrinsic growth rates (r) increased but the time to enter into the stationary phase shortened from 15℃ to 25℃, and the carrying capability (K) reached highest at 20℃. In addition, the 96 h-EC50 in the salinity of 31 is higher than that in other salinities and the carrying capability reached the highest level. Moreover, the 96 h-EC50 increased when the pH changing from 6.5 to 8.1, but decreased

when the pH more than 8.1. The results also show that the maximum (K) and (r), and the shortest time into stationary phases in the natural pH of seawater. It shows the minimum (K) in the low pH of 6.5. There is no significant ($P > 0.05$) between pH7.5 and pH 8.5. In conclusion, temperature, pH and salinity did influence the toxicity of petroleum hydrocarbon to the microalgae (*I. galbana*).

Keywords: *Isochrysis galbana*, Environmental factors, Petroleum hydrocarbon, Toxic effect

1. Introduction

Nowadays, marine oil pollution has been accelerated by the current economic globalization and has become one of the key issues faced by the government and society. It comes from many ways such as ocean transportation, offshore oil production and the use as a kind of primary fuel. The deepwater horizon oil rig of ConocoPhillips oil spills in June, 2011 was described as an ecological disaster and the greatest oil spills in the history of China. Crude oil is comprised of a complex mixture of petroleum hydrocarbon and non-hydrocarbon compounds (Board, 2003). After the oil spill , the oil was floating on the surface of sea and typically each ton of oil in terms of oil film covering the sea surface range of 12km^2 (Tian, 1999). And it can also be transformed into small molecules dissolved in seawater, which began to spread with the ocean currents and waves push. Therefore, a large area of the barrier film formed and hindered normal air-sea exchange, impact on the physiological and biochemical phytoplankton, which led to the destruction of the ecological balance of oceans in climate anomalies. The main components of WAF were long-chain hydrocarbons, short chain hydrocarbons and few of aromatic hydrocarbons. Studies showed that the most poisonous oil component was small molecular weight PAHs in a short time, which became the primary source of persistent toxicity effects in the area of oil spill (Neff, 1995; Boehm & Page, 2007; Neff et al., 2000). Studies showed that WAF in seawater poses a significant threat to marine lives, as WAF toxic effects can cascade across the trophic levels. Ecologically, WAF can change the structure and function of both seawater and food webs.

Phytoplanktons are unicellular primary producers, which often referred to as algae—which collectively form the base for the most spatially extensive food webs in nature. Exposed in WAF long-term, physiological and biochemical process of phytoplankton can be altered, organelles damaged, and the growth and reproduction waken. Petroleum hydrocarbons exert toxic effects on marine lives, varying in different environment factors. The toxicity may vary in solubility and structure in water under different temperatures, salinities, pH values and illumination intensities.

There were various researches on acute toxicity effects of fuel oil and PAHs (Zhang, 2013; Bi, 2015), but the variation of toxic effect of WAF on microalgae under different environmental factors were less.

This article studied the variations of toxicity effects of *Isochrysis galbana* exposed to WAF in a period of 96h and long-term culture under different environmental factors, which will be the base of research on WAF toxicity and offshore oil contamination biological monitoring.

2. Materials and Methods

2.1 Chemicals

Water-accommodated fraction (WAF): the crude oil comes from Shengli oilfield was mixed with seawater according to the volume ratio 1:9 in a 5 L glass mixing chamber, stirring for 24h with a magnetic stirrer at room temperature. The mother liquor was separated from the mixture water in a separating funnel after 4h's standing, and kept in the brown jars in

refrigerator (4 ℃). The concentration of the mother liquor was determined by UV-spectrophotometer, and diluted it before using it.

2.2 Organisms

Isochrysis galbana, a species of marine phytoplankton, was used in this study. It was obtained from the Marine Microalgae Research Center, Ocean University of China.

2.3 Toxicity Experiments

To determine the toxicity of WAF on *Isochrysis galbana* under different environmental factors, we set the WAF concentration series as 0, 0.5, 2, 4, 8,16mg/L. The 0mg/L was control, each concentration have three repeats. The initial cell density of the algae was set to 20×10^4cells mL^{-1}. The algae were grown in closed Erlenmeyer flasks with modified f/2 media at 20±1℃ 80 μmol photon $m^{-2}s^{-1}$ with the light-dark cycle was 12h:12h, and waved the conical flask twice a day. The initial pH and salinity of the culture medium were 8.1± 0.02 and 31, respectively. The experimental volume was 250ml, and a 0.5ml sample was collected at 24h, 48h, 72h, and 96h to determine the cell density with a haemocytometer and an optional microscope (OlympusCX31, Japan).

Treatment1: Set the temperature of culture medium as 15℃, 20℃, 25℃, and the initial salinity and pH as natural seawater level that was 8.1±0.02 and 31,respectively.

Treatment2: Set the initial salinity of culture medium as 15, 25, 35, and the initial pH was 8.1 ±0.02 at 20±1℃.

Treatment3: Set the initial pH of culture medium as 6.5, 7.5, 8.5, and the initial salinity was 31 at 20±1℃.

2.4 Monoculture Exposed in WAF

Preculture the algae during the exponential growth phase. The initial cell density was set as 1 ××10^4cells mL^{-1} for *Isochrysis galbana*. Treatments of different environmental factors of the culture medium was same as in toxic experiments. The experimental volume was 100ml, and a 0.5ml sample was collected every two days to determine the cell density with a haemocytometer and an optional microscope (OlympusCX31, Japan).

2.5 Data Analysis and Statistics

The experiment data ware In-transformed, and the growth rate of algae fit to the following equation:

$$\mu_{0\text{-}L}= (\mathrm{In}X_L-\mathrm{In}X_0)/(t_L-t_0)$$

Where $\mu_{0\text{-}L}$ is the average specific rate of growth from 0 day to L day; X_L stand for the cell density in day L while X_0 means the initial cell density.

The growth inhibition rate was fit to the following equation:

$$I\%= (\mu_C-\mu_T)/\mu_C\times100\%$$

I stand for the average of growth inhibition rate; μ_C stand for the average specific rate of growth of the control grop; μ_T stand for the average specific rate of growth of the treatment group.

The linear relationship of growth inhibition and concentration of the natural logarithm was established and the 50% inhibition concentration was calculated by nonlinear regression with the software called Spss for windows 22.0, which called 96h EC_{50}.

To investigate the effect of biomass ratio on carrying capacity, we involved the logistic growth model suit for the population growth curve of *Isochrysis galbana*. The equation as following:

$$N_t=K/(1+e^{a-rt})$$

N_t stands for the cell density at time t ($\times10^4$ cells mL^{-1}); K stands for the carrying capability of the population ($\times10^4$ cells mL^{-1}) that defines as maximum sustainable population density (biomass) in a given ecosystem; t stands for the sampling time (d); r stands for the specific growth rate (d^{-1}); a stands for the constant determining the initial cell density (N_0).

Estimated values of growth parameters were obtained by the nonlinear regression in SPSS for windows 22.0. It was also conducted to analyze the different in both of 96h EC_{50} and growth parameters among different environmental factors, with the significance level of $P<0.05$ and extremely significance level of $P<0.01$.

3. Results

3.1 Variations of the 96h EC50 of WAF for I. galbana under Different Environmental Factors

The figure 1 shows that the 96h EC_{50} of WAF for *Isochrysis galbana* switching with the temperature from 15 to 25 were 6.674±1.096mg/L, 47.097±2.224mg/L and 78.369±2.518mg/L respectively. It meant that the WAF toxic effect on *I. galbana* decreased significantly with the increase of the temperature.

In addition, the results also indicates that the 96h EC_{50} value of WAF were at a low level and have no significant variation during the salinity of 15 to 25, which was 24.416±1.845mg/L in the salinity of 15 and 21.437±1.770mg/L in the salinity of 25. In the salinity of 31, the 96h EC_{50} of WAF value was 47.097±2.224mg/L. It went up significantly ($P<0.05$) with the salinity increasing during the salinity of 25 to 31, but dropped with the continuous increase of the salinity. In the salinity of 35, the 96h EC_{50} value of WAF was 35.437±2.060mg/L.

Moreover, the results suggests that the 96h EC_{50} value of WAF mounted with the pH rising from 6.5 to 8.1 but decreased with the pH continuing rising. When the initial pH of the culture mediums was 6.5, 7.5 and 8.5 respectively, the 96h EC50 values of WAF were 18.433±2.91mg/L, 21.916±3.087mg/L and 33.124±3.500mg/L respectively. At the natural seawater pH level (8.1), the 96h EC_{50} values of WAF reached 47.097±2.224mg/L. Both of low and high level of the initial pH decreased the 96h EC_{50} values of WAF.

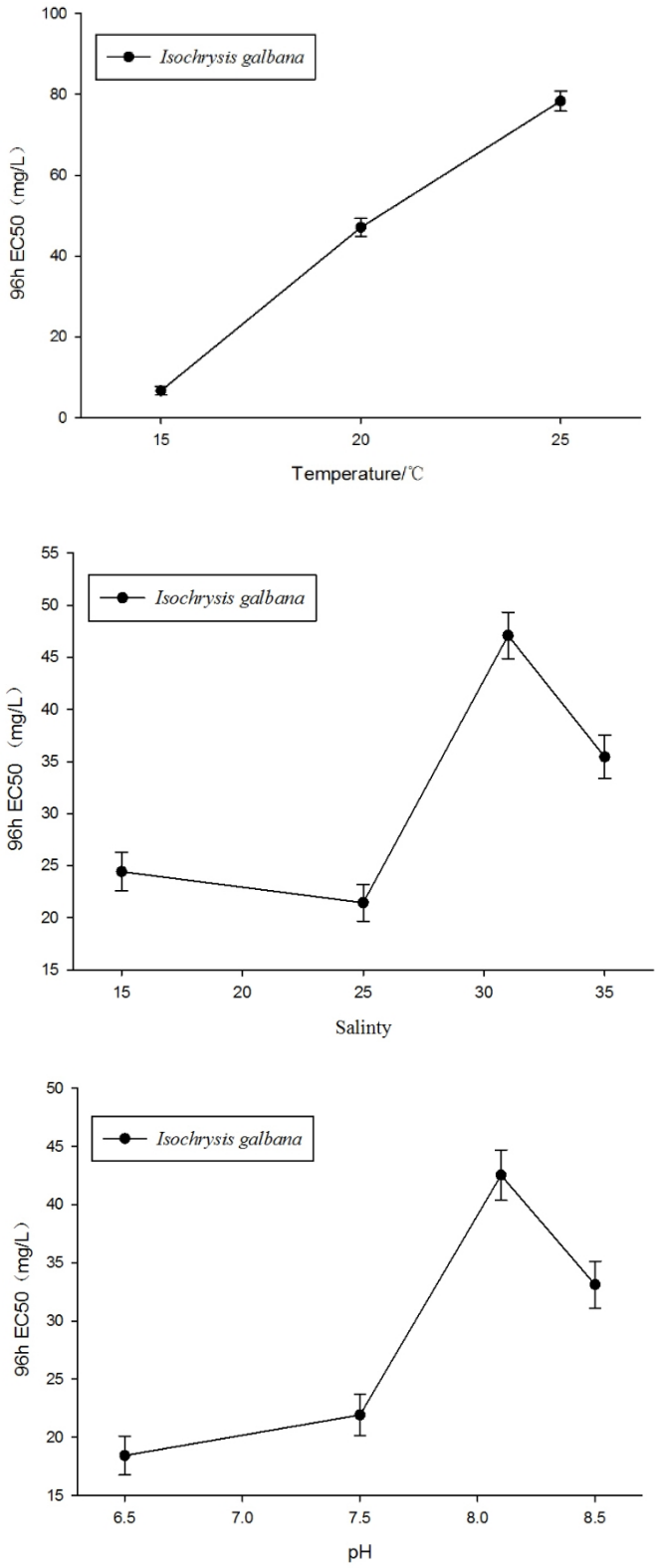

Figure 1. The 96h WAF EC_{50} on *Isochrysis galbana* under different environmental factors

3.2 Variations of the Population Growth of I. galbana Exposed to WAF under Different Environmental Factors

Table1, table 2 and table 3 listed Variations of the population growth of *I. galbana* exposed to WAF at different temperature, salinity and pH respectively.

Firstly, from table 1,it is clear that the maximum of sustainable population density（K） reached to the highest value in 20℃ at each concentration of WAF and there is a sharp drop at low temperature of 15℃ ($P<0.05$). The intrinsic rate of population growth was increased significantly with the temperature increasing ($P<0.05$). For the time to reach the platform, it showed shorter at a higher temperature expect the control and 0.5mg/L groups.

Table 1. The parameters, regression coefficient (R^2) and the reflection point of Logistic equation of *I. galbana* cultured in different density of WAF under different temperatures

WAF(mg/L)	T (℃)	K	a	r/d^{-1}	R^2	Tp(d)
0	15	766.840±21.005	5.002±0.198	0.518±0.038	0.997	9.7
	20	1096.990±81.523	4.385±0.166	0.444±0.036	0.996	9.9
	25	1065.856±19.122	5.227±0.091	0.598±0.025	0.991	8.7
0.5	15	724.758±34.968	4.670±0.130	0.491±0.034	0.994	9.5
	20	1058.975±46.692	4.564±0.090	0.468±0.021	0.996	9.8
	25	1016.128±19.433	5.283±0.132	0.629±0.014	0.995	8.4
2	15	703.682±12.505	4.617±0.072	0.472±0.018	0.992	9.8
	20	949.166±30.315	4.904±0.299	0.518±0.038	0.996	9.5
	25	931.717±32.435	5.774±0.451	0.700±0.068	1.000	8.2
4	15	676.638±45.669	4.674±0.127	0.454±0.038	0.995	10.3
	20	884.940±19.204	5.095±0.250	0.533±0.034	0.989	9.6
	25	863.270±18.554	5.978±0.133	0.727±0.021	0.999	8.2
8	15	623.354±12.874	4.873±0.077	0.434±0.022	0.993	11.2
	20	838.556±23.786	5.322±0.093	0.547±0.006	0.998	9.7
	25	814.460±24.500	6.102±0.267	0.726±0.031	0.997	8.4
16	15	558.027±38.839	5.199±0.198	0.445±0.037	0.996	11.7
	20	792.283±24.163	5.314±0.406	0.520±0.044	0.999	10.2
	25	759.908±7.737	6.229±0.033	0.713±0.005	0.998	8.7

Secondly, As is shown in the table 2, the maximum of sustainable population density (K) was highest in the salinity of 31, either higher or lower salinities resulted the lower value. The results show the different carrying capabilities (K), intrinsic growth rates (r) and the timings into stationary phases in different salinities. It shows the maximum K and r, and the shortest time into stationary phases of *I. galbana* exposed in WAF in the natural salinity of seawater. Both of the lower and the higher salinity make the opposite results.

Table 2. The parameters, regression coefficient (R^2) and the reflection point of Logistic equation of *I. galbana* cultured in different density of WAF under different salinities

WAF (mg/L)	salinity	K	a	r	R^2	Tp(d)
0	15	906.353 ± 22.106	5.332 ± 0.186	0.595 ± 0.03	0.9960	9.0
	25	1064.048 ± 50.606	5.025 ± 0.162	0.604 ± 0.041	0.9968	8.3
	31	1096.99 ± 81.523	4.385 ± 0.166	0.444 ± 0.036	0.9961	9.9
	35	1043.025 ± 41.021	5.459 ± 0.057	0.619 ± 0.018	0.9991	8.8
0.5	15	891.641 ± 20.567	5.666 ± 0.315	0.636 ± 0.045	0.9960	8.9
	25	1021.386 ± 8.923	5.341 ± 0.031	0.662 ± 0.008	0.9985	8.1
	31	1058.976 ± 46.692	4.564 ± 0.090	0.468 ± 0.021	0.9974	9.8
	35	1008.042 ± 17.716	5.940 ± 0.154	0.682 ± 0.025	0.9979	8.7
2	15	892.977 ± 17.788	5.439 ± 0.409	0.586 ± 0.048	0.9960	9.3
	25	950.275 ± 12.87	5.781 ± 0.242	0.726 ± 0.029	0.9979	8.0
	31	949.166 ± 30.315	4.904 ± 0.299	0.518 ± 0.038	0.9977	9.5
	35	934.537 ± 12.712	6.368 ± 0.288	0.739 ± 0.03	0.9982	8.6
4	15	818.435 ± 22.158	5.644 ± 0.145	0.608 ± 0.018	0.9890	9.3
	25	873.21 ± 20.325	6.296 ± 0.231	0.794 ± 0.045	0.9988	7.9
	31	884.94 ± 19.204	5.095 ± 0.250	0.533 ± 0.034	0.9981	9.6
	35	878.205 ± 19.602	6.988 ± 0.235	0.807 ± 0.04	0.9980	8.7
8	15	772.444 ± 9.678	5.839 ± 0.162	0.616 ± 0.014	0.9980	9.5
	25	799.086 ± 10.155	6.474 ± 0.327	0.814 ± 0.053	0.9978	8.0
	31	838.556 ± 23.786	5.322 ± 0.093	0.547 ± 0.006	0.9974	9.7
	35	808.156 ± 3.512	7.05 ± 0.151	0.807 ± 0.025	0.9978	8.7
16	15	690.917 ± 8.096	5.983 ± 0.437	0.589 ± 0.052	0.999	10.2
	25	652.567 ± 16.833	6.25 ± 0.191	0.715 ± 0.02	0.9924	8.7
	31	792.283 ± 24.163	5.314 ± 0.406	0.52 ± 0.044	0.9832	10.2
	35	656.346 ± 19.982	5.938 ± 0.287	0.658 ± 0.025	0.9993	9.0

Finally, the table 3 shows that the maximum of sustainable population density (K) was highest in the initial pH of 8.1, either higher or lower pH resulted in the lower value of K.It is apparent that the K in groups of the initial pH of 6.5 declined sharply than other levels of pH groups. The results show the maximum (K) and (r), and the shortest time into stationary phases in the natural pH of seawater. It is clear that the minimum (K) in the low pH of 6.5.

From above data, it can be seen that the environmental factors change the toxic effect of WAF on *Isochysis galbana* in population growth.

Table 3. The parameters, regression coefficient (R^2) and the reflection point of Logistic equation of *I. galbana* cultured in different density of WAF under different pH

WAF (mg/L)	pH	K	a	r/d^{-1}	R^2	Tp (d)
0	6.5	783.281±6.495	5.321±0.39	0.537±0.043	0.9963	9.9
	7.5	1012.252±69.098	5.063±0.213	0.507±0.036	0.9948	10
	8.1	1096.99±81.523	4.385±0.166	0.444±0.036	0.9899	9.9
	8.5	1021.532±70.694	5.053±0.338	0.529±0.056	0.9882	9.6
0.5	6.5	760.208±12.977	5.158±0.153	0.519±0.013	0.9958	9.9
	7.5	984.997±45.685	5.286±0.377	0.538±0.05	0.9954	9.8
	8.1	1058.976±46.692	4.564±0.09	0.468±0.021	0.9889	9.8
	8.5	987.2±38.648	5.298±0.169	0.561±0.029	0.9866	9.4
2	6.5	726.737±26.229	5.154±0.284	0.504±0.013	0.9934	10.2
	7.5	894.59±26.073	5.778±0.262	0.602±0.034	0.9987	9.6
	8.1	949.166±30.315	4.904±0.299	0.518±0.038	0.9939	9.5
	8.5	920.307±26.959	5.602±0.407	0.598±0.049	0.9957	9.4
4	6.5	660.445±11.119	5.02±0.322	0.491±0.031	0.9981	10.2
	7.5	833.387±17.88	6.092±0.473	0.629±0.051	0.9981	9.7
	8.1	884.94±19.204	5.095±0.25	0.533±0.034	0.998	9.6
	8.5	835.432±22.853	5.334±0.3	0.569±0.042	0.997	9.4
8	6.5	583.975±14.716	4.97±0.151	0.456±0.026	0.9943	10.9
	7.5	793.547±23.966	6.696±0.05	0.678±0.009	0.9993	9.9
	8.1	838.556±23.786	5.322±0.093	0.547±0.006	0.9979	9.7
	8.5	790.06±20.869	5.211±0.139	0.543±0.011	0.9971	9.6
16	6.5	527.259±12.869	5.365±0.566	0.47±0.054	0.9957	11.4
	7.5	765.929±25.601	6.309±0.637	0.602±0.067	0.9991	10.5
	8.1	792.283±24.163	5.314±0.406	0.52±0.044	0.999	10.2
	8.5	762.425±28.963	5.088±0.447	0.489±0.049	0.9985	10.4

4. Discussion

It has been well known that WAF effects on the growth and reproduction of algae (Gaur et al., 1981; Pérez et al., 2010; Jiang et al., 2012). In this study, the WAF 96h EC_{50} values on *I.*

galbana varied under different environmental factors, which phenomenon meant the variations of WAF toxic effect. The result showed a high toxicity of WAF in Fig. 1 at low temperature of 15℃.

At present, studies have shown that the effect of temperature fluctuation in the growth, physiological and biochemical characteristics of marine microalgae. The most important parts of the cell membrane damage effects, the primary reaction injury at low temperature during phase transition lipid molecules on biofilm systems (Gong et al., 2001). Another research shows that the active cryogenic oxygen plants metabolic balance is broken, keeping up with the rate of degradation of the active oxygen species generated. Therefore, some researchers pointed out that the damage caused by low temperature membrane system may related with free radicals and reactive oxygen on membrane lipid peroxidation and protein oxidative damage. The cell electrolyte leakage and membrane peroxidation increased and membrane permeability-increasing at low temperature. Therefore, the WAF components may enter into the cell easier at low temperature. It showed in Tab.1 that the population growth of *I. galbana* exposed in WAF was also influenced by the variation of temperature.

Salinity is one of the most important environmental factors. In this study, both the low and high levels of salinities lead to higher WAF toxicity, but showed a low toxicity in the salinity of natural seawater. Salt stress can lead to excessive production of ROS, causing oxidative damage to cells (Choo et al. 2004). ROS cause lipid peroxidation, the increase of MDA concentration peroxide generated content may reflect the rise of ROS (Tang et al., 2007). Thus, both low and high salinities make WAF poison the algae easier than in natural salinity.

The pH of offshore seawater was unsustainable result in human behavior and absolutely lower than in ocean. In this study, results showed a significant increase of WAF toxicity in low pH value. Studies indicated that may severely affect the photosynthesis of algae once pH value beyond the certain range (Ansari et al., 2015). Some studies have concluded that higher hydrogen ion content by algae cell membrane penetration to the interior of the cell in low pH value environment, so that a number of intracellular enzymes can be lowered the activity inactivation. Therefore, *I. galbana* has a lower tolerance to WAF in low pH value environment, and strengthens the toxicity of WAF.

5. Conclusions

From the analysis above, we can conclude that the WAF toxicity to *I. galbana* varied with the variations of the environmental factors. In the range of experiment, while the toxicity of WAF was weakened with the rising of temperature, both high and low levels of salinities and pH enhanced the toxicity of WAF.

Acknowledgements

Funding was provided by “Opening fund of the State Oceanic Administration Key Laboratory of Marine Spill Oil Identification and Damage Assessment Technology (201108).”

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