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Optimal chlorophyll fluorescence parameter selection for rapid and sensitive detection of lead toxicity to marine microalgae *Nitzschia closterium* based on chlorophyll fluorescence technology

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ABSTRACT

Heavy metal pollution as one of the most serious pollution problems of marine environment, seriously threatens the safety of marine organism and human health, and will lead to potential risks for the marine ecological environment. In order to develop a rapid and sensitive toxicity detection method for marine heavy metals, in this study, marine diatom *Nitzschia closterium* was used as the test organism, and the effects of different concentrations of lead (Pb) on the five chlorophyll fluorescence parameters of *N. closterium* including the maximum photochemical quantum yield of PSII (Fv/Fm), the effective quantum yield of PSII photochemical energy conversion (Φ_{PSII}), the effective absorption cross section of PSII photochemistry (σ_{PSII}'), the relative electron transfer rate of PSII (rP), and the PSII electron flux per unit volume (JVPII) at different exposure times were investigated based on chlorophyll fluorescence technology. By comparing with the photosynthetic activity fluorescence parameter Fv/Fm which is commonly used for toxicity analysis of pollutants using algae as test organisms, the optimal chlorophyll fluorescence parameter that could rapidly and sensitively determine Pb toxicity to *N. closterium* was selected. The results indicate that all the five chlorophyll fluorescence parameters of Fv/Fm, Φ_{PSII}, σ_{PSII}', rP and JVPII showed good dose-response relationships with Pb within 8 h exposure time, and they all could be used as endpoints to rapidly determine Pb toxicity to *N. closterium*. Among the five chlorophyll fluorescence parameters, JVPII was the most sensitive fluorescence parameter for detecting the toxicity of Pb to *N. closterium* within 6 h exposure. And for JVPII, the median effective concentration (EC_{50}) values of Pb at 2, 4 and 6 h were 0.329, 0.068 and 0.040 mmol L^{-1}, respectively. However, when the exposure time was 8 h, Φ_{PSII} was the most sensitive fluorescence parameter for the toxicity detection of Pb, and the EC_{50} value of Pb at 8 h was 0.038 mmol L^{-1}. This study will provide an important basis for the development of a rapid and sensitive detection method for the biological toxicity of marine heavy metals, and those results will be helpful for ecological risk assessment in marine environment.

1. Introduction

In recent years, with the rapid development of economy and industry in coastal areas, the problem of marine environmental pollution is becoming more and more serious, and the types and quantities of pollutants in the marine environment are increasing day by day. So the development of rapid and effective marine environmental biological monitoring technology is of great significance for the protection of marine environment. Due to the mining of ore and marine oil wells, as well as the discharge of industrial wastewater and agricultural sewage, the problem of heavy metal pollution in the ocean has become increasingly serious. Because of the characteristics of wide source, difficulty in degradability, bioaccumulation and long residual time [1,2], heavy metals will have toxic effects on organisms, and damage various tissues and functions of organisms once accumulated in living organisms. Therefore, marine heavy metal pollution seriously threatens the safety of marine organism and human health, and will lead to potential risks for the marine ecological environment. However, for the detection

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of heavy metals, though traditional physical and chemical analysis methods such as inductively coupled plasma-mass spectrometry [3] and atomic absorption spectrometry [4] can accurately detect the composition and content of heavy metal pollutants, they can't reflect the toxic effects of heavy metals on aquatic organisms, and can't predict the potential environmental risks of heavy metal pollution. Therefore, the development of biological toxicity detection methods for heavy metal pollutants has important practical significance for the assessment of marine environmental quality. At present, the aquatic toxicity of pollutants is mostly tested by luminescent bacteria [5–8], fleas [9–12], fish [13–15] and algae [16,17]. Compared with other organisms, marine microalgae, as a primary producer of marine ecosystems, is an important link in the material cycle and energy flow of marine ecosystems, and directly affects the structure and function of marine ecosystems [18]. Moreover, they also have the characteristics such as small size, rapid reproduction rate and strong sensitivity for environmental influences. So marine microalgae have become an important class of indicator organism for monitoring and evaluating the quality of marine environment. And they have attracted much attention of researchers in the study of toxicity analysis and detection of water pollutants. But for the toxicity test of heavy metals and other water pollutants using algae as a test organism, the algae are usually cultured for a long time in a medium containing toxic substances, and then the toxic effects of toxicant on the growth, reproduction and cell structure of algae are determined by measuring the changes of algal biomass [19,20], catalase activity [21], growth inhibition rate [22], chlorophyll concentration [23], and ultrastructural morphology of algal cells [24]. However, the experimental processes for obtaining those toxicity indexes are usually time-consuming and labor-intensive. The long experimental period (usually ≥ 24 h) makes these methods unable to achieve rapid analysis and detection of pollutant toxicity, and greatly reduces the timeliness of environmental risk assessment. Moreover, because living cells and dead cells of algae are often difficult to distinguish, so the above methods are also difficult to ensure the accuracy and reliability of the measurement results. Consequently, it is very necessary and urgent to find a method which is rapid, simple, sensitive and harmless to the test organism sample for assessing the toxicity of heavy metals.

As we all know, Chlorophyll as one of the main photosynthetic pigments, is the foundation of algae photosynthesis. Moreover, Chlorophyll is also an important indicator for reflecting the ability of marine microalgae to convert inorganic matter into organic matter. Photosynthesis is the most basic physiological process of microalgae. Most of the light energy absorbed by chlorophyll is used for photosynthesis, and the part that cannot be utilized will be emitted in the form of heat and fluorescence. Due to the mutual competition among these three ways for energy, the change of photosynthesis can cause the corresponding change of fluorescence emission. Based on this, the chlorophyll fluorescence analysis technology developed in recent years has become a new method for the measurement and diagnostic of plant living body based on the theory of photosynthesis. And this technology uses chlorophyll in vivo as a natural probe to research and detect the photosynthetic physiology and the effects of various external factors on plant. Due to the advantages of sensitivity, simplicity, rapidity and no damage, this technology has been widely used in the study of plant photosynthesis and stress physiology [25]. When contaminants interact with algal cells, the toxic effects of contaminants on algae can be expressed through photosynthesis , and then cause the change of chlorophyll fluorescence. Therefore, using chlorophyll fluorescence as a probe for biological toxicity analysis has become a new approach for the comprehensive assessment of water pollution.

A series of chlorophyll fluorescence parameters obtained by chlorophyll fluorescence technology are closely related to various reactions of photosynthesis. They not only can reflect the activity of photosystem II (PSII) and photosystem I (PSI), but also can fully display the important information of algal photosynthesis process, and comprehensively reflect the photosynthesis physiological status and photosynthesis ability of algae, such as absorption, transmission, dissipation and distribution of light energy by microalgae. Therefore, photosynthetic chlorophyll fluorescence parameters are ideal internal probes for studying the relationships between photosynthesis and stress factors. At present, the maximum photochemical quantum yield of PSII (Fv/Fm) as the photosynthesis activity fluorescence parameter is a widely used chlorophyll fluorescence parameter in the toxicity analysis of pollutants [26,27]. However, the other chlorophyll fluorescent parameters are not better utilized to evaluate the toxic effects of toxicants on algae.

In marine ecosystems, *Nitzschia closterium* is one of the most common marine microalgae, belonging to marine diatoms. It is also a common bait in coastal areas of China and has the characteristics of high sensitivity to toxic substances, rapid reproduction speed, small volume, wide distribution and easy to obtain. So *N. closterium* is a very suitable test organism for the study of marine environmental pollutant toxicity. In this paper, marine diatom *N. closterium* was used as the test organism, and chlorophyll fluorescence technology was adopted to study the toxic effects of different concentrations of Pb at different exposure times on various chlorophyll fluorescence parameters such as the maximum photochemical quantum yield of PSII (Fv/Fm), the effective quantum yield of PSII photochemical energy conversion (Φ_{PSII}), the effective absorption cross section of PSII photochemistry (σ_{PSII}'), the relative electron transfer rate of PSII (rP), and PSII electron flux per unit volume (JVPII), and then assess the toxic effect of Pb on *N. closterium*. By comparing and analyzing the sensitivity of different chlorophyll fluorescence parameters to Pb toxicity, the optimal chlorophyll fluorescence parameter which can sensitively and rapidly determine the toxicity of Pb was selected. And this study will provide an important basis for the development of a rapid and sensitive detection method for the biological toxicity of marine heavy metals, and will be helpful for ecological risk assessment and risk management of heavy metals pollution in marine environment.

2. Materials and Methods

2.1. Algal Culture

The marine diatom *N. closterium* used in this experiment were obtained from the Microalgae Germplasm Bank of Ocean University of China. The f/2 + Si medium containing $NaNO_3$, vitamins, Na_2SiO_3, and trace metals (e.g., $FeCl_3$, Na_2EDTA, $CuSO_4$, $ZnSO_4$, $CoCl_2$, $MnCl_2$, and $NaMoO_4$) for the inoculation and culture of *N. closterium* was prepared with filtered seawater and the pH was adjusted to 8.2 with 1 mol L^{-1} NaOH. Prior to use, the medium and all the glass flasks used in the experiment were sterilized by autoclaving in an autoclave at 121 °C for 30 min. *N. closterium* were aseptically inoculated in 250 mL glass flasks containing 100 mL f/2 + Si medium in a SW-CJ-1D ultra-clean workbench (Shangyu Aike Instrument Equipment Co., Ltd., China) and then were cultured in a HP400G intelligent light incubator (Wuhan Ruihua Instrument and Equipment Co., Ltd., China). And the culture temperature was 25 °C with a light illumination of 60 μmol m^{-2} s^{-1} in a 12 h: 12 h light: dark cycle. All flasks were shaken 3 times daily. And healthy diatom at exponential growth phase were used for Pb exposure experiment.

2.2. Pb Exposure Experiment

Lead nitrate ($Pb(NO_3)_2$) used in the exposure experiment was of analytical grade and purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Pb stock solution with a concentration of 0.01 mol L^{-1} was prepared using sterile f/2 + Si medium, and then diluted using sterile f/2 + Si medium to prepare a series of Pb work solutions with concentrations of 0.04, 0.10, 0.20, 0.40, 0.80 and 1.60 mmol L^{-1}. Then the same volume of Pb work solutions and *N. closterium* culture solution were added and mixed thoroughly in a 50 mL

glass flasks as Pb treatments, so that the final Pb concentrations in each of diatom suspensions were 0.02, 0.05, 0.1, 0.2, 0.4 and 0.8 mmol L^{-1}, respectively. And a blank sample as a control was prepared in the same way by mixing the same volume of sterile f/2 + Si medium and *N. closterium* culture solution in a 50 mL glass flasks. The initial chlorophyll concentrations of all test diatom samples were 400 μg L^{-1} which were measured using a FluoroProbe (BBE Moldaenke GmbH, Germany). All the test diatom samples were placed in an incubator and cultured under the same conditions as culture described above. Then chlorophyll fluorescence parameters of *N. closterium* were measured when the exposure time reached 2, 4, 6, and 8 h, respectively. All samples were measured in triplicate.

2.3. Chlorophyll Fluorescence Parameters Measurement

Chlorophyll fluorescence parameters were measured using a Fast Repetition Rate fluorometer (FRRf) (serial no. 16-0497-003; Chelsea Technologies Group Ltd., England) equipped with an optic glass cuvette and Act2Run system software. And 450 nm LEDs in FRRf were selected as the excitation light source for generating excitation pulses and the light intensity was set as 3.32×10^4 μmol m^{-2} s^{-1}. The voltage of the photomultiplier tube (PMT) was set as 300 V. And a series of intensities of photosynthetic active radiation (PAR) were set as 0, 22, 46, 72, 101, 132, 167, 205, 247, 294, 344 and 400 μmol m^{-2} s^{-1}. The irradiation time of each PAR intensity was 15, 30, 15, 15, 15, 15, 15, 15, 15, 15, 15 and 15 s, respectively. The FRRf was operated as described in References [28, 29]. First of all, 2 mL test sample solution was added into the optic glass cuvette. Then chlorophyll fluorescence induction curves under different PAR intensities were measured with a default flash sequence which comprises a saturation phase of 100 flash on a 2 μs pitch and a relaxation phase of 40 flash on a 60 μs pitch. For a chlorophyll fluorescence induction curve, the flash sequence was repeated 20 times. And the five chlorophyll fluorescence parameters of Fv/Fm, Φ_{PSII}, σ_{PSII}', rP and JVPII were automatically calculated and obtained from the measured chlorophyll fluorescence induction curves by algorithms in FRRf system software Act2Run. The chlorophyll fluorescence parameter Fv/Fm is the maximum photochemical quantum yield of PSII and it is usually calculated according to the following Eq. (1):

$$F_v/F_m = (F_m - F_0)/F_m \quad (1)$$

where F_0 and F_m are minimal fluorescence and maximum fluorescence measured when the test algal samples have been dark acclimated, respectively [30]. The chlorophyll fluorescence parameter Φ_{PSII} is the effective quantum yield of PSII photochemical energy conversion and it usually can be calculated as Eq. (2):

$$\Phi_{PSII} = (F_m' - F_s)/F_m' \quad (2)$$

where Fm' and Fs are maximum fluorescence and steady-state fluorescence measured under ambient light [31]. The chlorophyll fluorescence parameter rP is the relative electron transfer rate of PSII, and it is calculated according to Eq. (3) in FRRf:

$$rP = E \times F_v'/F_m' \quad (3)$$

where E is the PAR intensity, Fv′ and Fm′ are variable fluorescence and maximum fluorescence measured under ambient light [32]. The chlorophyll fluorescence parameters σ_{PSII}' and JVPII are the effective absorption cross section of PSII photochemistry and the PSII electron flux per unit volume, respectively [33,34].

2.4. Data Processing and Statistical Analysis

Statistical analyses were carried out using SPSS 19.0 software. Significant differences were determined by one-way ANOVA analysis of variance with post-hoc multiple test. And $p < 0.05$ was considered statistically significant. To determine the 10, 20, 30, 40 and 50% effective concentration (EC_{10}, EC_{20}, EC_{30}, EC_{40}, and EC_{50}) of Pb for the different chlorophyll fluorescence parameters at different exposure times, the inhibition degree or inhibition rate of Pb on the different chlorophyll fluorescence parameters of *N. closterium* was calculated according to following Eq. (4):

$$I_t(\%) = [(P_{ck-t} - P_t)/P_{ck-t}] \times 100\% \quad (4)$$

where P_{ck-t} and P_t were the chlorophyll fluorescence parameters of *N. closterium* in the absence and presence of Pb at exposure time of *t*, respectively. And I_t (%) was the inhibition rate of Pb on the chlorophyll fluorescence parameter of *N. closterium* at exposure time of *t*. All the dose-response relationships between the chlorophyll fluorescence parameters and Pb were fitted using logistic curve. And the EC_{10}, EC_{20}, EC_{30}, EC_{40}, and EC_{50} of different chlorophyll fluorescence parameters at different exposure times were calculated according the dose-response curve.

3. Results

3.1. The Effect of Pb on Chlorophyll Fluorescence Parameter Fv/Fm

Fv/Fm is an important parameter for evaluating photosynthesis activity of plants. And this chlorophyll fluorescence parameter usually can reflect the maximum photosynthetic capacity of algal cells. When the photosynthetic activity of algae is affected by any environmental factors, the value of Fv/Fm will also change and will be lower than that of the normal photosynthetic activity. So the decrease of Fv/Fm value is an important information indicating that the photosynthetic activity of algal cells is affected. At present, Fv/Fm has become a widely used chlorophyll fluorescence parameter for assessing the toxic effects of toxicants on algae cells [26,27]. In this study, the effect of different concentrations of Pb on the chlorophyll fluorescence parameter Fv/Fm of *N. closterium* at different exposure times (2 h, 4 h, 6 h, and 8 h) was first investigated. And the Fv/Fm values of *N. closterium* exposed to different concentrations of Pb during 8 h were shown in Fig. 1. As can been seen from Fig. 1 that for the control, the photosynthetic activity fluorescence parameter Fv/Fm showed a small fluctuation in the range of 0.375–0.422 within 8 h, and the relative standard deviation calculated from the Fv/Fm values at different exposure times was 3.94%. This indicates that for the control without Pb, the photosynthetic activity of *N. closterium* within 8 h was relatively stable. But for the treatments, we can see from Fig.1 that Fv/Fm value decreased rapidly

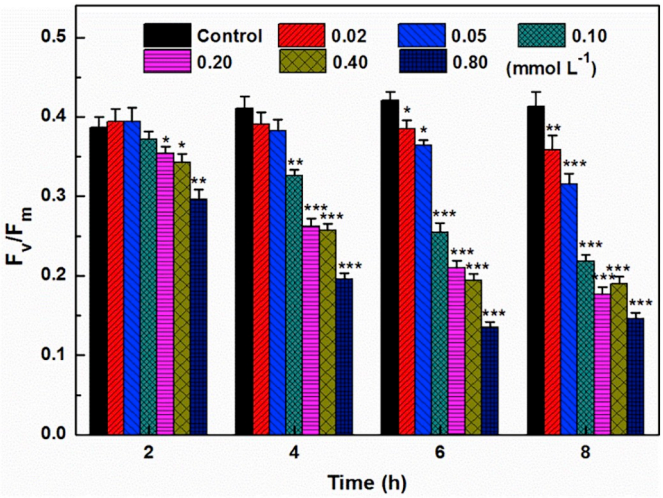

Fig. 1. Fv/Fm values of *N. closterium* exposed to different concentrations of Pb during 8 h. *Indicates there was a significant difference between the treatment and the control (*0.01 < p < 0.05; **0.001 ≤ p ≤0.01; ***p < 0.001, post-hoc multiple test).

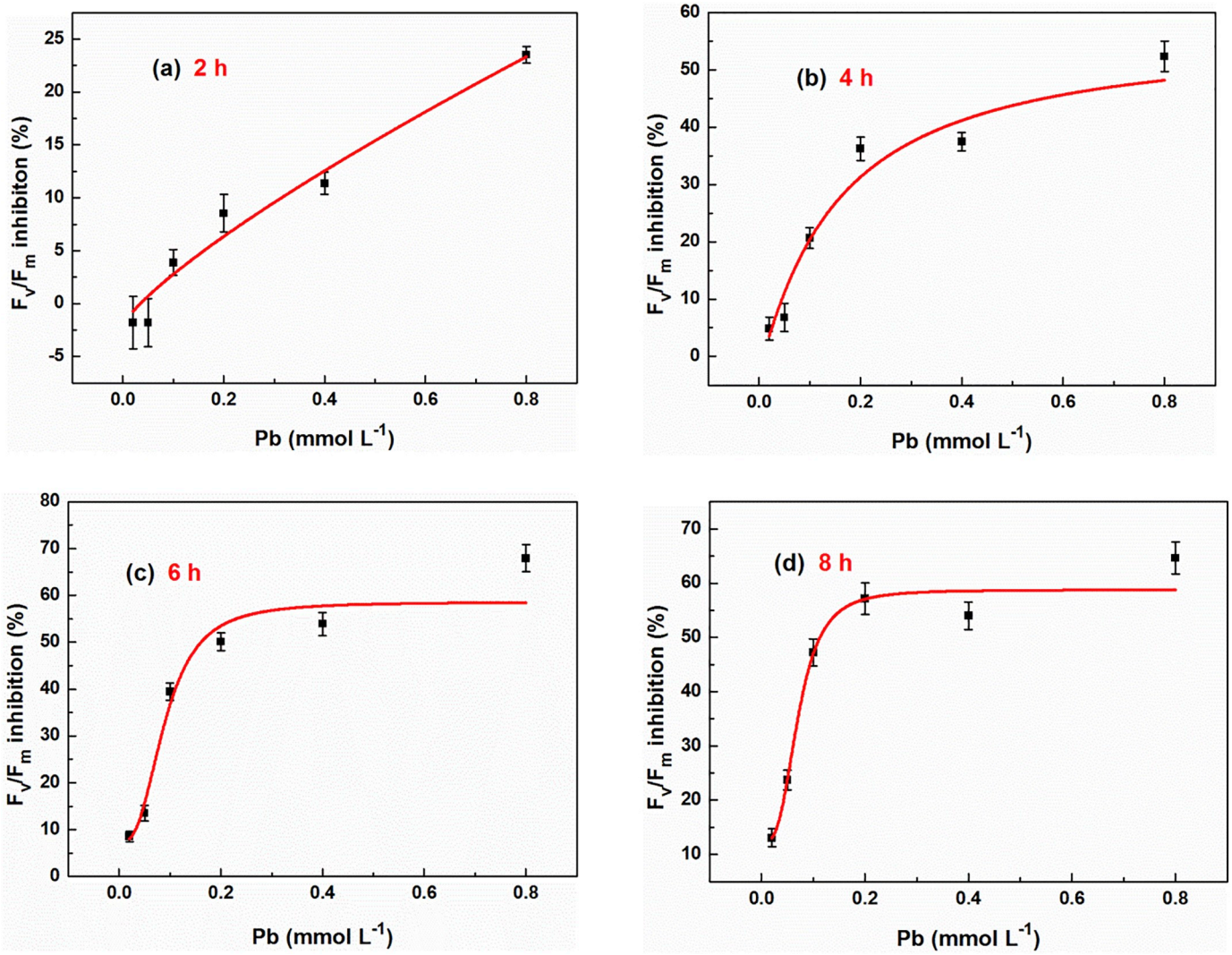

Fig. 2. Dose-response relationships between Fv/Fm and Pb concentration at different exposure times. (a) 2 h, (b) 4 h, (c) 6 h, and (d) 8 h.

with the increase of exposure time and Pb concentration. When *N. closterium* was exposed for 2 h, there had been a significant difference between the 0.2 mmol L^{-1} Pb treatment and control ($p < 0.05$). When the concentration of Pb was > 0.1 mmol L^{-1}, the treatments and the control had extremely significant differences at the exposure time of 4 h ($p < 0.01$). After a 6 h exposure or 8 h exposure, 0.02 mmol L^{-1} Pb treatment had been a significant difference with the control ($p < 0.05$ and $p < 0.01$), respectively. This indicates that chlorophyll fluorescence parameter Fv/Fm had a significant dose-dependence and time-dependence with Pb. And the photosynthesis activity of *N. closterium* was significantly affected by Pb.

When the exposure time was 2, 4, 6, and 8 h, the changes of inhibition rate of chlorophyll fluorescence parameter Fv/Fm of *N. closterium* with the concentration of Pb were shown in Fig. 2, respectively. As can been seen from Fig. 2 that when the concentration of Pb was < 0.2 mmol L^{-1}, the inhibition rate of Fv/Fm increased rapidly with the increase of Pb concentration. But for the concentration of Pb > 0.2 mmol L^{-1}, the increase of inhibition rate of Fv/Fm was slow, and the longer the exposure time was, the slower the increase rate was. When *N. closterium* were exposed to Pb for 2, 4, 6, and 8 h, the dose-response relationships between Fv/Fm inhibition rate and Pb concentration were fitted by Logistic curve, and correlation coefficient R^2 were 0.9605, 0.8804, 0.9388, and 0.9597, respectively. According to the dose-response curves, the median effective concentration (EC_{50}) values of Pb to *N. closterium* at different exposure times were calculated as follows: 4 h-EC_{50} was 0.767 mmol L^{-1}, 6 h-EC_{50} was 0.224 mmol L^{-1}, and 8 h-EC_{50} was 0.109 mmol L^{-1}, respectively. However, from Fig. 2(a) we can observe that the upper limit of inhibition rate of Fv/Fm was 23.52% for the exposure time of 2 h, so 2 h-EC_{50} was not calculated, and 2 h-EC_{10} of 0.292 mmol L^{-1} and 2 h-EC_{20} of 0.677 mmol L^{-1} were calculated according to the dose-response curve of 2 h as shown in Table 1, respectively. Those results indicate that chlorophyll fluorescence parameter Fv/Fm can been used as the endpoint to rapidly detect the toxicity of Pb on *N. closterium*.

3.2. The Effects of Pb on Chlorophyll Fluorescence Parameter Φ_{PSII}, σ_{PSII}', rP and JVPII

In addition to the photosynthesis activity fluorescence parameter Fv/Fm, FRRf also can obtain a variety of other chlorophyll fluorescence parameters which also can display the important information of photosynthesis process in algal cells. According to the above analysis, we know that Pb had effect on chlorophyll fluorescence parameter Fv/Fm of *N. closterium*. Therefore, in order to confirm the optimal chlorophyll fluorescence parameters for determination of Pb toxicity to *N. closterium* based on chlorophyll fluorescence method, the toxic effects of Pb on the four chlorophyll fluorescence parameters including the effective quantum yield of PSII photochemical energy conversion (Φ_{PSII}), the effective absorption cross section of PSII photochemistry (σ_{PSII}'), the relative electron transfer rate of PSII (rP), and PSII electron flux per unit

Table 1
Values of ECx of Pb for different chlorophyll fluorescence parameters at different exposure times.

Time	Parameter	EC_{10}	EC_{20}	EC_{30}	EC_{40}	EC_{50}
		(mmol L^{-1})				
2 h	Fv/Fm	0.292	0.677	–	–	–
	Φ_{PSII}	0.044	0.072	0.110	0.177	0.520
	σ_{PSII}'	0.017	0.041	0.106	0.265	–
	rP	0.070	0.116	0.186	0.539	–
	JVPII	0.022	0.039	0.065	0.112	0.329
4 h	Fv/Fm	0.047	0.095	0.180	0.397	0.767
	Φ_{PSII}	–	–	–	0.062	0.084
	σ_{PSII}'	–	0.022	0.060	0.157	0.804
	rP	–	–	0.058	0.084	0.098
	JVPII	–	–	0.008	0.054	0.068
6 h	Fv/Fm	0.028	0.049	0.079	0.125	0.224
	Φ_{PSII}	–	–	–	–	0.052
	σ_{PSII}'	–	–	0.032	0.059	–
	rP	–	–	–	0.037	0.071
	JVPII	–	–	–	0.013	0.040
8 h	Fv/Fm	–	0.041	0.063	0.083	0.109
	Φ_{PSII}	–	–	0.001	0.020	0.038
	σ_{PSII}'	0.016	0.018	0.023	0.057	0.210
	rP	–	–	0.008	0.037	0.055
	JVPII	0.007	0.014	0.022	0.034	0.052

– indicates that the value can't be available.

volume (JVPII) were also investigated in this study.

Since all the four fluorescence parameters of Φ_{PSII}, σ_{PSII}', rP and JVPII can be measured when the algae are irradiated by ambient light, these four fluorescence parameters are related to the PAR intensity. In order to study the toxic effects of Pb on *N. closterium* using Φ_{PSII}, σ_{PSII}', rP and JVPII measured under the optimal ambient light intensity as the endpoints, the influences of different PAR intensities on fluorescence parameter Φ_{PSII}, σ_{PSII}', rP and JVPII were first studied. When *N. closterium* was exposed to different concentrations of Pb for 2 h, the changes of chlorophyll fluorescence parameter Φ_{PSII}, σ_{PSII}', rP and JVPII with PAR intensity were shown in Fig. 3. We can see from Fig. 3 that for the treatments, all the four fluorescence parameters of Φ_{PSII}, σ_{PSII}', rP and JVPII were obviously different from those of the control. Fig. 3(a) and (b) show that for *N. closterium* exposed to different concentrations of Pb, the fluorescence parameter Φ_{PSII} and σ_{PSII}' gradually decreased with the increase of PAR intensity. And when the intensity of PAR was the same, the higher the Pb concentration was, the smaller the values of Φ_{PSII} and σ_{PSII}' were. Fig. 3(c) and (d) show that with the increase of PAR intensity, the fluorescence parameter rP and JVPII of *N. closterium* exposed to different concentrations of Pb increased rapidly first, then remained stable or even decreased. And for the same PAR intensity, the higher the Pb concentration was, the smaller the values of rP and JVPII were.

According to Fig. 3, for *N. closterium* exposed 2 h by different concentrations of Pb, the changes of the inhibition rates of Φ_{PSII}, σ_{PSII}', rP and JVPII with the intensity of PAR were shown in Fig. 4. It can be seen from Fig. 4 that with the increase of PAR intensity, all inhibition rates of Φ_{PSII}, σ_{PSII}', rP and JVPII increased first and then decreased. Fig. 4(a) and (d) show that when the PAR intensity was 167 μmol m^{-2} s^{-1}, the inhibition rates of Φ_{PSII} and JVPII were generally the maximum, and they all showed a obvious dependence on the concentration of Pb. But as shown in Fig. 4(b) and (c) that when the PAR intensity was 205 μmol m^{-2} s^{-1}, Pb had the greatest inhibition on the chlorophyll fluorescent parameter σ_{PSII}' and rP. At the same time the inhibition rates of σ_{PSII}' and rP also showed a obvious dependence relationship with Pb concentration. Therefore, chlorophyll fluorescence parameter Φ_{PSII} and JVPII measured under a PAR intensity of 167 μmol m^{-2} s^{-1} and σ_{PSII}' and rP measured under a PAR intensity of 205 μmol m^{-2} s^{-1} were more beneficial to improve the sensitivity of toxicity analysis of Pb to *N. closterium*.

Based on the above analysis, the chlorophyll fluorescence parameter Φ_{PSII} and JVPII measured under a PAR intensity of 167 μmol m^{-2} s^{-1} and σ_{PSII}' and rP measured under a PAR intensity of 205 μmol m^{-2} s^{-1} were selected and used to analyze the toxic effects of Pb on *N. closterium*. When the exposure time was 2, 4, 6, and 8 h, the values of chlorophyll fluorescence parameter Φ_{PSII}, σ_{PSII}', rP and JVPII of *N. closterium* exposed to different concentrations of Pb were shown in Fig. 5. As can be seen from Fig. 5 that for the control, the values of chlorophyll fluorescence parameter Φ_{PSII}, σ_{PSII}', rP and JVPII remained basically unchanged within 8 h, and the relative standard deviations from the values of Φ_{PSII}, σ_{PSII}', rP and JVPII measured at different times within 8 h were 7.49%, 0.75%, 9.29% and 3.33%, respectively. However for the treatments, the values of Φ_{PSII}, σ_{PSII}', rP and JVPII of *N. closterium* were significantly lower than those of the control except for the rP value of *N. closterium* exposed to 0.02 mmol L^{-1} Pb for 2 h. For the chlorophyll fluorescence parameter Φ_{PSII}, When the exposure time was 2 h, there had been a significant difference between the 0.1 mmol L^{-1} Pb treatment and the control ($p < 0.01$). And when the concentration of Pb was > 0.02 mmol L^{-1}, there had been very significant differences between the treatments and the control at the exposure time of 4 h ($p < 0.01$). Moreover, after a 6 h exposure, 0.02 mmol L^{-1} Pb treatment had been an extremely significant difference with the control ($p < 0.001$). For the chlorophyll fluorescence parameter σ_{PSII}', rP, and JVPII, When the exposure time was 2 h, there had been an extremely significant difference between the 0.02 mmol L^{-1} Pb treatment and the control ($p < 0.001$). This indicates that similar to the photosynthetic activity fluorescence parameter Fv/Fm, the four fluorescence parameters of Φ_{PSII}, σ_{PSII}', rP and JVPII can also rapidly determine the toxic effects of Pb on *N. closterium*.

All the relationships between the inhibition rates of Φ_{PSII}, σ_{PSII}', rP and JVPII and Pb concentration at the exposure time of 2, 4, 6, and 8 h could be fitted by logistic curve. This indicates that when the exposure time was within 8 h, there were good S-shaped curve relationships between the inhibition rates of fluorescence parameter Φ_{PSII}, σ_{PSII}', rP and JVPII of *N. closterium* and Pb concentration. And the correlation coefficients R^2 were all > 0.983. Those results indicate that there was a good dose-response relationship between the fluorescence parameter Φ_{PSII}, σ_{PSII}', rP or JVPII and Pb within 8 h, respectively. According to the dose-response curves and the concentration range of Pb used in the exposure experiment, for different chlorophyll fluorescence parameters, the values of x% effective concentration (ECx) at the exposure time of 2, 4, 6, and 8 h were calculated and shown in Table 1. From Table 1 we can see that when *N. closterium* was exposed for 2 h, the EC_{10}, EC_{20}, EC_{30}, EC_{40}, and EC_{50} values of fluorescence parameter Φ_{PSII}, and JVPII, and the EC_{10}, EC_{20}, EC_{30}, and EC_{40} values of fluorescence parameter σ_{PSII}', rP could be obtained. This indicates that these four fluorescence parameters could rapidly predict the toxic effects of Pb on *N. closterium*. By comparing and analyzing the ECx values of different parameters with the same inhibition degree, we can see that when the exposure time was 2, 4, and 6 h, respectively, the ECx values of chlorophyll fluorescence parameter JVPII were the lowest. This indicates that JVPII was the most sensitive chlorophyll fluorescence parameter for determining Pb toxicity to *N. closterium*. However, when *N. closterium* were exposed for 8 h, the ECx values of chlorophyll fluorescence parameter Φ_{PSII} were the lowest. And this indicates that Φ_{PSII} was the most sensitive chlorophyll fluorescence parameter for determining the toxicity of Pb to *N. closterium*.

4. Discussion

Pb is one of the most toxic heavy metal and poses a serious threat to the aquatic environment. So environmental toxic effects of Pb in the aquatic ecosystem have been attracted significant attention. And many researchers have carried out the study on the toxic effects of Pb on different algae species. For example, Bibiana Debelius et al. have investigated the toxic effects of Pb on growth rate of five marine

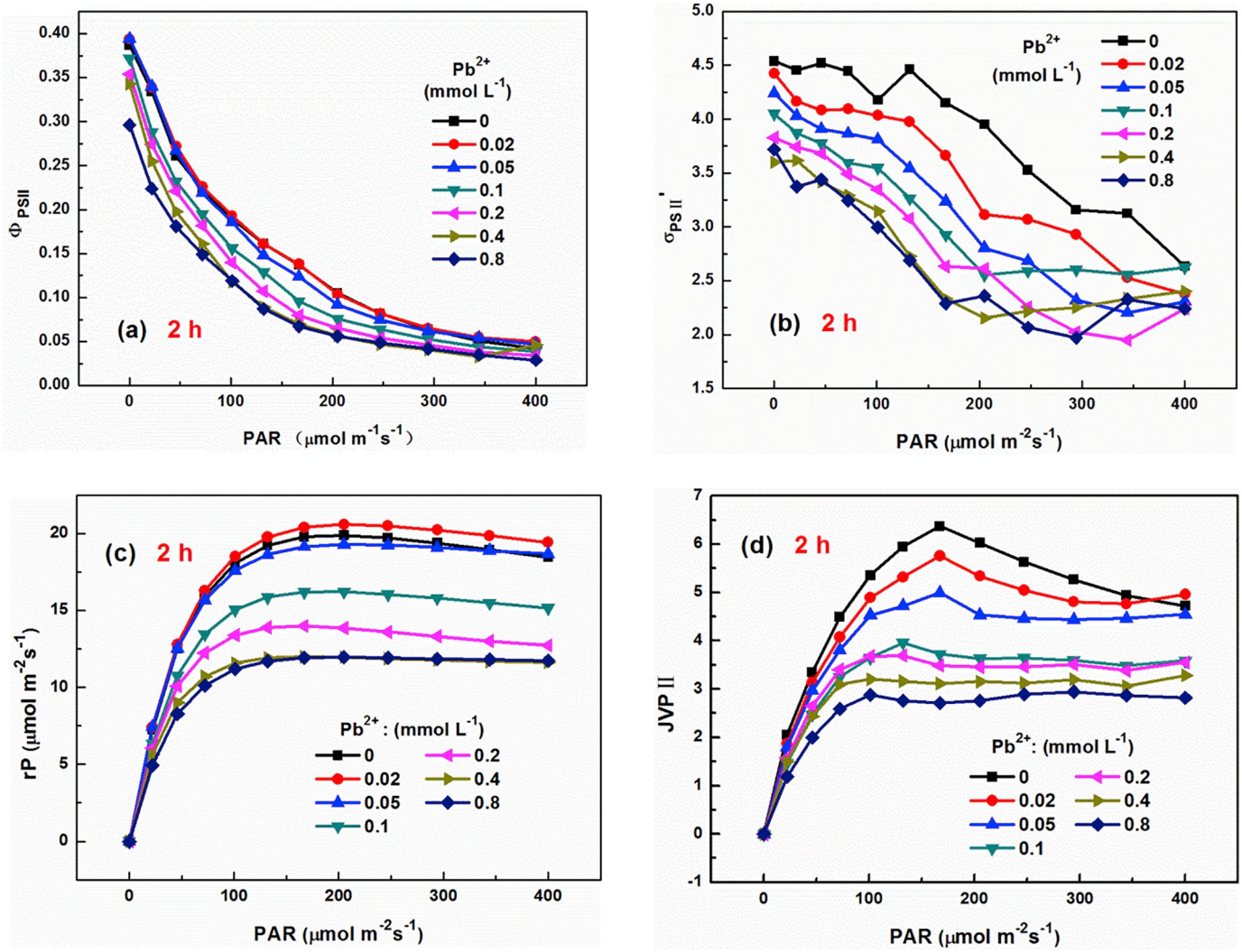

Fig. 3. The changes of fluorescence parameter (a) Φ_{PSII}, (b) σ_{PSII}', (c) rP and (d) JVPII of *N. closterium* exposed to different concentrations of Pb for 2 h with PAR intensity.

microalgae including *T. chuii*, *R. salina*, *Chaetoceros* sp., *I. galbana*, and *N. gaditana* [35]. Guangxu Liu et al. have investigated the toxic effects of Pb on the motility of two marine microalgae *I. galbana* and *T. chui* [36]. And the motility included motile percentage, curvilinear velocity, average path velocity, straight line velocity, linearity, straightness, and wobble. Vinitha Ebenezer et al. have assessed the toxicity of Pb by observing the change of cell counts and chlorophyll *a* levels of *C. polykrikoides* exposed to Pb [37]. Wei Zhang et al. and Weijie Mu et al. have carried out the study of toxicity assessment of Pb to *C. vulgaris*, *C. protothecoides*, *H. veneta*, and *S. crumena* by observing the changes in growth rate, chlorophyll *a* content, and superoxide dismutase (SOD) and catalase (CAT) activities, respectively [38,39]. All these results indicate that Pb can alter the chlorophyll *a* content, and motility of algae, and the toxicity of Pb can inhibit the growth and enzyme activities of algal cells. Consequently, growth rate, cell density, chlorophyll *a* content, SOD and CAT activities are usually used as indicators to assess Pb toxicity. And growth inhibition test has been as the Organization for Economic Co-operation and Decelopment (OECD) guidelines for testing of chemicals using freshwater alga and cyanobacteria (2006). However, for growth inhibition test, the exposure time usually has to reach > 24 h to achieve the 50% inhibition effect. Moreover, chlorophyll *a* content also needs to be extracted with 90% acetone for 24 h then measured using absorption spectrum after algal cells are exposed to Pb for > 24 h. Similarly, the activities of SOD and CAT also need to be detected after algal cells exposed to Pb for > 24 h. Consequently, growth rate, chlorophyll *a* content, SOD and CAT activities as the endpoints can't be used to rapidly assess the toxicity of Pb on algae.

Photosynthesis is an important physiological process of phytoplankton. And any factors that inhibit the growth of algae can directly inhibit the photosynthesis of algae cells. Therefore, the photosynthesis process can be used to rapidly assess the toxic effects of pollutants on algae. The research results of Ly H.T. Dao et al. also showed that Pb has significant effect on photosynthesis of *Chlorella*, and can significantly inhibit their PSII performance [30]. FRRf used in our study is adopted chlorophyll fluorescence induction kinetics technology and Fast Repetition Rate technology. A series of chlorophyll fluorescence parameters of tested algae samples can be measured by FRRf. And the measurement process has the advantages of simple operation, fast measurement, stable performance, non-destructive, and multiple fluorescence parameters and so on. Because these chlorophyll fluorescence parameters can display the important information of algal photosynthesis process, and comprehensively reflect the photosynthesis physiological status and photosynthesis ability of algae, they can be used as the endpoints to characterize the toxic effects of pollutants on algae.

In this study, the five chlorophyll fluorescence parameters including Fv/Fm, Φ_{PSII}, σ_{PSII}', rP, and JVPII were chosen to investigate the toxic effects of Pb on *N. closterium*. The chlorophyll fluorescence parameter

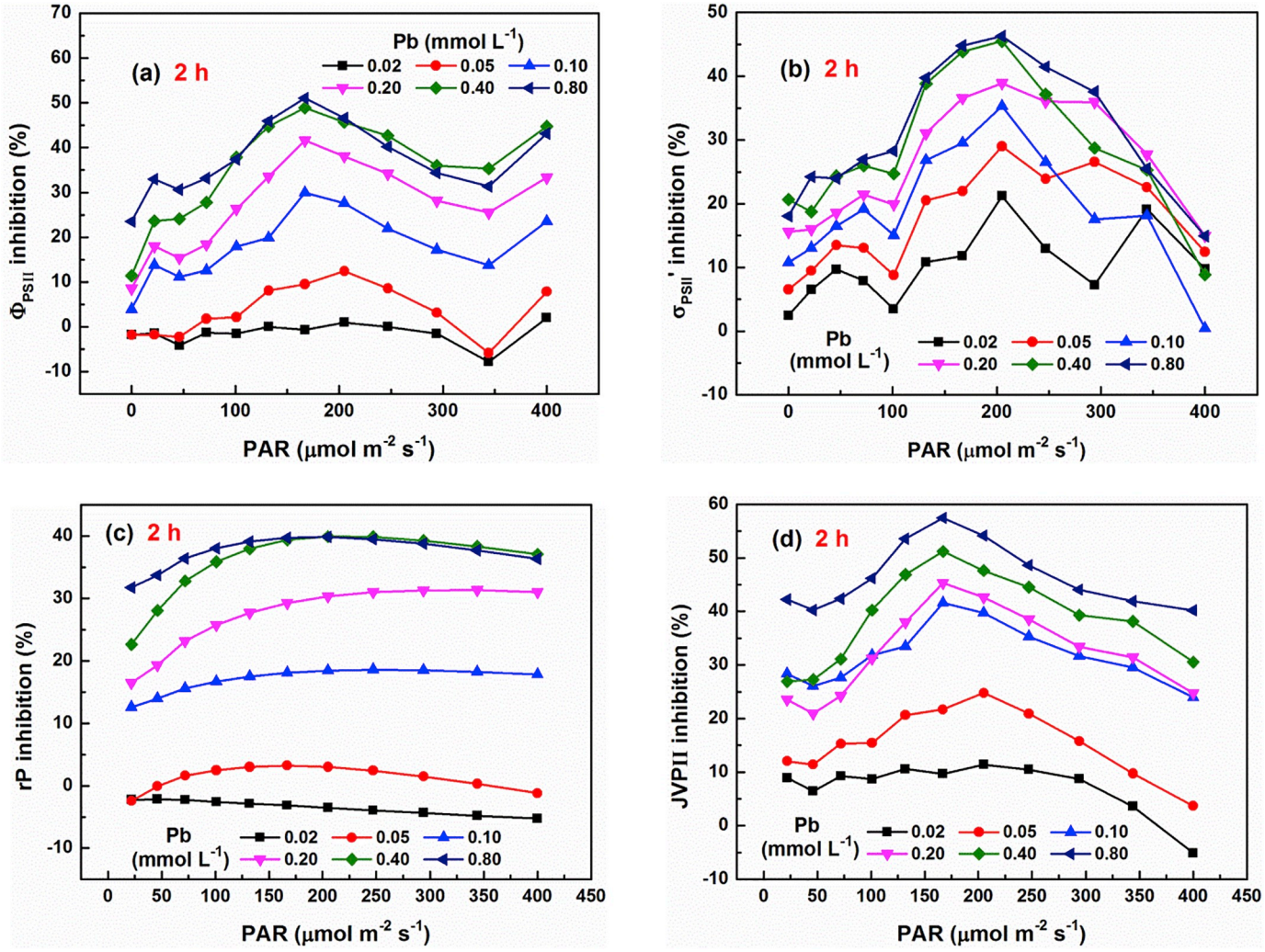

Fig. 4. Effects of PAR intensity on (a) Φ_{PSII}, (b) σ_{PSII}', (c) rP and (d) JVPII of *N. closterium* exposed to different concentrations of Pb for 2 h.

Fv/Fm, Φ_{PSII}, σ_{PSII}', rP, and JVPII represent the maximum photochemical quantum yield of PSII, the effective quantum yield of PSII photochemical energy conversion, the effective absorption cross section of PSII photochemistry, the relative electron transfer rate of PSII, and the PSII electron flux per unit volume, respectively. By analyzing the degree of inhibition of the five chlorophyll fluorescence parameters of *N. closterium* exposed to different concentrations of Pb for different times, the results indicate that Fv/Fm, Φ_{PSII}, σ_{PSII}', rP, and JVPII all showed significant dose-dependent and time-dependent relationships with Pb. And when the exposure time was 2 h, the concentrations of Pb which had an inhibitory effect on Fv/Fm, Φ_{PSII}, σ_{PSII}', rP, and JVPII were 0.10, 0.05, 0.02, 0.05, and 0.02 mmol L^{-1}, respectively. Moreover at this time, Pb had caused a 50% inhibition effect on both Φ_{PSII} and JVPII. And 2 h-EC_{50} values of Φ_{PSII} and JVPII were 0.52 mmol L^{-1} and 0.329 mmol L^{-1}, respectively. Those results indicate that compared with growth rate, chlorophyll *a* content, SOD and CAT activities, all the five chlorophyll fluorescence parameters of Fv/Fm, Φ_{PSII}, σ_{PSII}', rP, and JVPII can be used as endpoints to rapidly assess Pb toxicity, and the exposure time of 2 h is much faster than the exposure time of 24 h required for growth inhibition tests. When *N. closterium* were exposed to Pb for 4 h, all the five fluorescence parameters had achieved 50% inhibition degree, and the 4 h-EC_{50} values of Fv/Fm, Φ_{PSII}, σ_{PSII}', rP, and JVPII were 0.767, 0.084, 0.804, 0.098, and 0.068 mmol L^{-1}, respectively (see Table 1). By comparing and analyzing the ECx values of different parameters with the same inhibition degree, the results indicate that when the exposure time was 2, 4 and 6 h, the ECx values of fluorescence parameter JVPII were the lowest. And when the exposure time was 8 h, the ECx values of fluorescence parameter Φ_{PSII} were the lowest. This indicates that JVPII was the most sensitive chlorophyll fluorescence parameter for determining Pb toxicity to *N. closterium* within 6 h, and Φ_{PSII} was the most sensitive chlorophyll fluorescence parameter for determining the toxicity of Pb to *N. closterium* when *N. closterium* was exposed for 8 h.

In summary, the five chlorophyll fluorescence parameters of Fv/Fm, Φ_{PSII}, σ_{PSII}', rP and JVPII all can be used to rapidly determine the biological toxicity of Pb to *N. closterium*, and they all showed good dose-response relationships with Pb within 8 h. Among the five chlorophyll fluorescence parameters, When the exposure time was within 6 h, JVPII was the most sensitive chlorophyll fluorescence parameter to determine the toxicity of Pb to *N. closterium*. However, Φ_{PSII} was the most sensitive chlorophyll fluorescence parameter to determine the toxicity of Pb to *N. closterium* for the exposure time of 8 h.

5. Conclusions

In this paper, marine microalgae *N. closterium* was used as the test organism, and chlorophyll fluorescence technology was adopted to study the effects of different concentrations of Pb on the five chlorophyll fluorescence parameters such as Fv/Fm, Φ_{PSII}, σ_{PSII}', rP, and JVPII at different exposure times. By comparison and analysis, it is determined that all the five chlorophyll fluorescence parameters of Fv/Fm, Φ_{PSII}, σ_{PSII}', rP and JVPII can be used as endpoints to rapidly predict

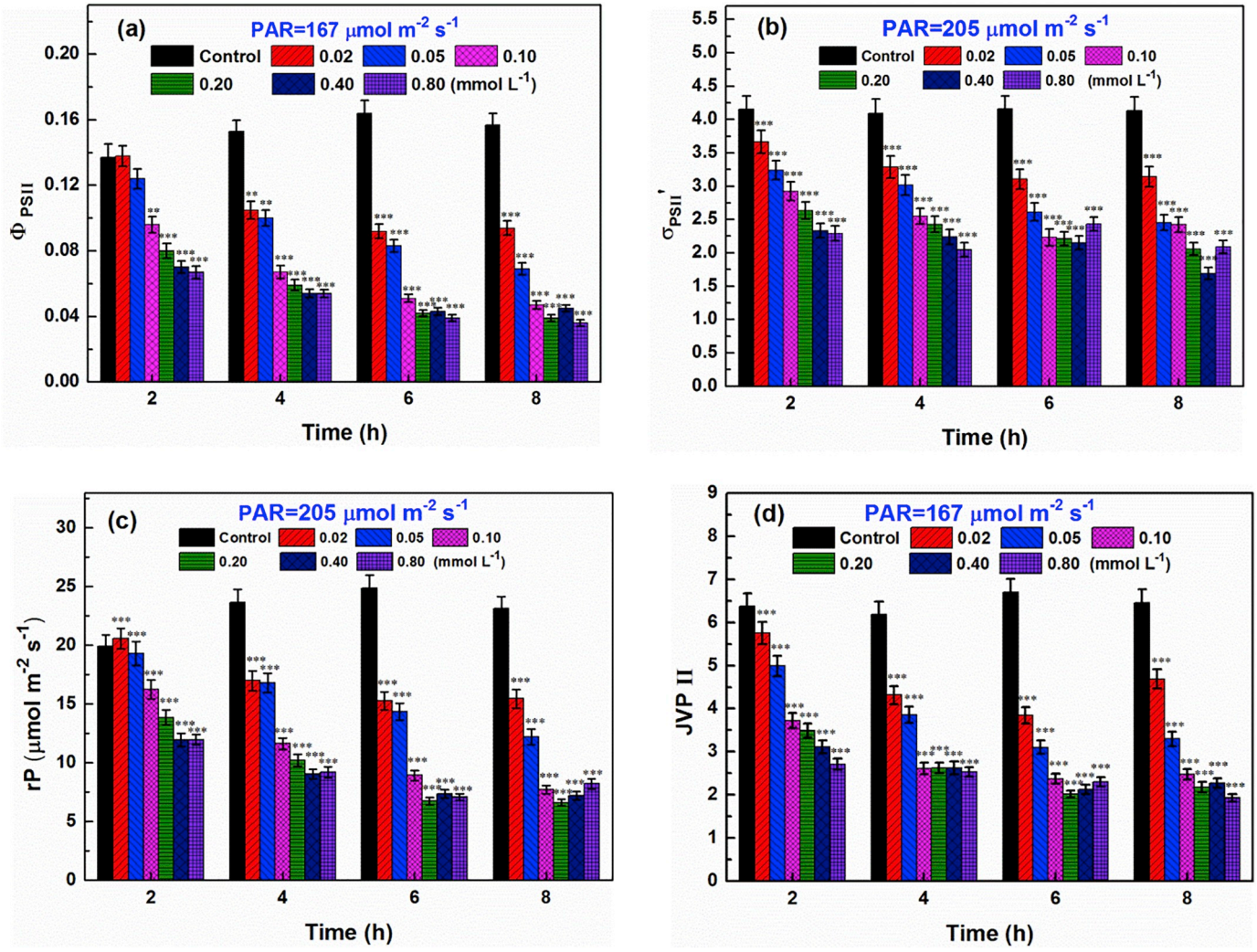

Fig. 5. (a) Φ_{PSII}, (b) σ_{PSII}', (c) rP and (d) JVPII values of *N. closterium* exposed to different concentrations of Pb for 2, 4, 6, and 8 h. *Indicates there was a significant difference between the treatment and the control (*0.01 < p < 0.05; **0.001 ≤ p ≤ 0.01; ***p < 0.001, post-hoc multiple test).

the biological toxicity of Pb to *N. closterium*, and they all showed good dose-response relationships with Pb within the exposure time of 8 h. Moreover, JVPII was the most sensitive chlorophyll fluorescence parameter for determining the biological toxicity of Pb to *N. closterium* when the exposure time was within 6 h. However, Φ_{PSII} was the most sensitive parameter to determine the biological toxicity of Pb to *N. closterium* for the exposure time of 8 h. This study will provide an important basis for the development of a rapid and sensitive detection method for the biological toxicity of marine heavy metals, and will be helpful for ecological risk assessment of heavy metal pollution in China's marine environment.

Declaration of Competing Interest

None.

Acknowledgements

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References

[1] I. Doyi, D. Essumang, G. Gbeddy, S. Dampare, E. Kumassah, D. Saka, Spatial distribution, accumulation and human health risk assessment of heavy metals in soil and groundwater of the Tano Basin, Ghana[J], Ecotoxicol. Environ. Saf. 165 (2018) 540–546.

[2] E. Nicole Dover, Naishal Y. Patel, Miroslav Styblo. Impact of *in vitro* heavy metal exposure on pancreatic β-cell function[J], Toxicol. Lett. 299 (2018) 137–144.

[3] J. Teran-Baamonde, S. Bouchet, E. Tessier, D. Amouroux, Development of a large volume injection method using a programmed temperature vaporization injector – gas chromatography hyphenated to ICP-MS for the simultaneous determination of mercury, tin and lead species at ultra-trace levels in natural waters[J], J. Chromatogr. A 1547 (2018) 77–85.

[4] V.S. Souza, L.S.G. Teixeira, M.A. Bezerra, Application of multivariate designs in the development of a method for vanadium determination in natural waters by HR-CS GF AAS after cloud-point extraction[J], Microchem. J. 129 (2016) 324–381.

[5] C.J. Kelly, N. Tumsaroj, C.A. Lajoie, Assessing wastewater metal toxicity with bacterial bioluminescence in a bench-scale wastewater treatment system[J], Water Res. 38 (2004) 423–431.

[6] P. Masner, B. Javurkova, L. Blaha, Rapid in situ toxicity testing with luminescent bacteria *Photorhabdus luminescens* and *Vibrio fischeri* adapted to a small portable luminometer[J], Environ. Sci. Pollut. Res. 24 (2017) 3748–3758.

[7] N.A. Qambrani, J.-H. Hwang, O. Sang-Eun, Comparison of chromium III and VI toxicities in water using sulfur-oxidizing bacterial bioassays[J], Chemosphere 160 (2016) 342–348.

[8] S.H.A. Hassan, S.E. Oh, Improved detection of toxic chemical by Photobacterium phosphoreum using modified Boss medium[J], J. Photochem. Photobiol. B Biol. 101 (1) (2010) 16–21.

[9] Reza Aalizadeh, Peter C. von der Ohe, Nikolaos S. Thomaidis, Prediction of acute toxicity of emerging contaminants on the water flea *Daphnia magna* by Ant Colony Optimization-Support Vector Machine QSTR models[J], Environ. Sci. Process Impacts 19 (2017) 438–448.

[10] L.A. Golding, K. McKnight, M. Binet, M. Adams, S.C. Apte, Toxicity of dissolved and precipitated forms of barium to a freshwater alga (*Chlorella* sp. 12) and water flea (*Ceriodaphnia dubia*)[J], Environ. Toxicol. Chem. 37 (6) (2018) 1632–1642.
[11] K. Toyota, N.A. McNabb, D.D. Spyropoulos, T. Iguchi, S. Kohno, Toxic effects of chemical dispersant Corexit 9500 on water flea *Daphnia magna*[J], J. Appl. Toxicol. 37 (2017) 201–206.
[12] R. Cui, J.I. Kwak, Y.-J. An, A novel method for preventing surface film entrapment of water fleas and its application for toxicity testing with heavy metals[J], Environ. Sci. Pollut. Res. 24 (2017) 4210–4219.
[13] K.A. Hossain, K. Roy, Chemometric modeling of aquatic toxicity of contaminants of emerging concern (CECs) in Dugesia japonica and its interspecies correlation with daphnia and fish: QSTR and QSTTR approaches[J], Ecotoxicol. Environ. Saf. 166 (2018) 92–101.
[14] M.P. Castro, F.R. de Moraes, R.Y. Fujimoto, C. da Cruz, M.A. de Andrade Belo, J.R.E. de Moraes, Acute toxicity by water containing hexavalent or trivalent chromium in native Brazilian fish, *Piaractus mesopotamicus*:Anatomopathological alterations and mortality[J], Bull. Environ. Contam. Toxicol. 92 (2014) 213–219.
[15] P.Y. Robidoux, B. Virginie, L. Judith, D. Marc, Assessment of acute and chronic toxicity of unweathered and weathered diluted bitumen to freshwater fish and invertebrates[J], Ecotoxicol. Environ. Saf. 164 (2018) 331–343.
[16] Hai Yan, Gang Pan, Toxicity and bioaccumulation of copper in three green microalgal species[J], Chemosphere 49 (2002) 471–476.
[17] J. Mofeed, Y.Y. Mosleh, Toxic responses and antioxidative enzymes activity of *Scenedesmus obliquus* exposed to fenhexamid and atrazine, alone and in mixture[J], Ecotoxicol. Environ. Saf. 95 (2013) 234–240.
[18] P.L.G. Martins, L.G. Marques, P. Colepicolo, Antioxidant enzymes are induced by phenol in the marine microalga *Lingulodinium polyedrum*[J], Ecotoxicol. Environ. Saf. 116 (2015) 84–89.
[19] W. Yang, Z. Tang, F. Zhou, W. Zhang, L. Song, Toxicity studies of tetracycline on *Microcystis aeruginosa* and *Selenastrum capricornutum*[J], Environ. Toxicol. Pharmacol. 35 (2013) 320–324.
[20] V. Ebenezer, J.-S. Ki, Quantification of toxic effects of the herbicide metolachlor on marine microalgae *Ditylum brightwellii* (Bacillariophyceae), *Prorocentrum minimum* (Dinophyceae), and *Tetraselmis suecica* (Chlorophyceae)[J], J. Microbiol. 51 (1) (2013) 136–139.
[21] X. Nie, B. Liu, H. Yu, W. Liu, Y. Yang, Toxic effects of erythromycin, ciprofloxacin and sulfamethoxazole exposure to the antioxidant system in *Pseudokirchneriella subcapitata*[J], Environ. Pollut. 172 (2013) 23–32.
[22] Q.T. Gao, N.F.Y. Tam, Growth, photosynthesis and antioxidant responses of two microalgal species, *Chlorella vulgaris* and *Selenastrum capricornutum*, to nonylphenol stress[J], Chemosphere 82 (2011) 346–354.
[23] Sha Yan, Qixing Zhou, Toxic effects of Hydrilla verticillata exposed to toluene, ethylbenzene and xylene and safety assessment for protecting aquatic macrophytes [J], Chemosphere 85 (2011) 1088–1094.
[24] H. Liua, M. Xiong, Comparative toxicity of racemic metolachlor and S-metolachlor to *Chlorella pyrenoidosa*[J], Aquat. Toxicol. 93 (2009) 100–106.
[25] J.M. Banks, Chlorophyll fluorescence as a tool to identify drought stress in Acer genotypes[J], Environ. Exp. Bot. 155 (2018) 118–127.
[26] K. Suresh Kumar, H.-U. Dahms, J.-S. Lee, H.C. Kim, W.C. Lee, K.-H. Shin, Algal photosynthetic responses to toxic metals and herbicides assessed by chlorophyll a fluorescence[J], Ecotoxicol. Environ. Saf. 104 (2014) 51–71.
[27] H. Huang, X. Xiao, A. Ghadouani, J. Wu, Z. Nie, P. Cheng, X. Xu, J. Shi, Effects of natural flavonoids on photosynthetic activity and cell integrity in *Microcystis aeruginosa*[J], Toxins 7 (2015) 66–80.
[28] P. Pérez, P. Estévez-Blanco, R. Beiras, E. Fernández, Effect of copper on the photochemical efficiency, growth, and chlorophyll a biomass of natural phytoplankton assemblages[J], Environ. Toxicol. Chem. 25 (1) (2006) 137–143.
[29] P. Pérez, E. Fernández, R. Beiras, Use of fast repetiton rate fluorometry on detection and assessment of PAH toxicity on microalgae[J], Water Air Soil Pollut. 209 (2010) 345–356.
[30] H.T. Ly, Dao, John Beardall. Effects of lead on two green microalgae *Chlorella* and *Scenedesmus*: photosystem II activity and heterogeneity[J], Algal Res. 16 (2016) 150–159.
[31] T. Ogawa, M. Misumi, K. Sonoike, Estimation of photosynthesis in cyanobacteria by pulse-amplitude modulation chlorophyll fluorescence: problems and solutions[J], Photosynth. Res. 133 (2017) 63–73.
[32] Ly H.T. Dao, John Beardall, Effects of lead on growth, photosynthetic characteristics and production of reactive oxygen species of two freshwater green algae[J], Chemosphere 147 (2016) 420–429.
[33] D.J. Suggett, C.M. Moore, A.E. Hickman, R.J. Geider, Interpretation of fast repetition rate (FRR) fluorescence: signatures of phytoplankton community structure versus physiological state[J], Mar. Ecol. Prog. Ser. 376 (2009) 1–19.
[34] K. Oxborough, C.M. Moore, D.J. Suggett, T. Lawson, H.G. Chan, R.J. Geider, Direct estimation of functional PSII reaction center concentration and PSII electron flux on a volume basis: a new approach to the analysis of Fast Repetition Rate fluorometry (FRRf) data[J], Limnol. Oceanogr. Methods 10 (2012) 142–154.
[35] B. Debelius, J.M. Forja, Á. DelValls, L.M. Lubián, Toxicity and bioaccumulation of copper and lead in five marine microalgae[J], Ecotoxicol. Environ. Saf. 72 (2009) 1503–1513.
[36] G. Liu, X. Chai, Y. Shao, L. Hu, Q. Xie, W. Hongxi, Toxicity of copper, lead, and cadmium on the motility of two marine microalgae *Isochrysis galbana* and *Tetraselmis chui* [J], J. Environ. Sci. 23 (2) (2011) 330–335.
[37] Vinitha Ebenezer, Jang-Seu Ki, Evaluation of the sub-lethal toxicity of Cu, Pb, bisphenol A and polychlorinated biphenyl to the marine dinoflagellate *Cochlodinium polykrikoides*[J], Algae 27 (1) (2012) 63–70.
[38] W. Zhang, B. Xiong, C. Lin, K. Lin, X. Cui, H. Bi, M. Guo, W. Wang, Toxicity assessment of *Chlorella vulgaris* and *Chlorella protothecoides* following exposure to Pb (II)[J], Environ. Toxicol. Pharmacol. 36 (2013) 51–57.
[39] M. Weijie, Y. Chen, Y. Liu, X. Pan, Y. Fan, Toxicological effects of cadmium and lead on two freshwater diatioms[J], Environ. Toxicol. Pharmacol. 59 (2018) 152–162.