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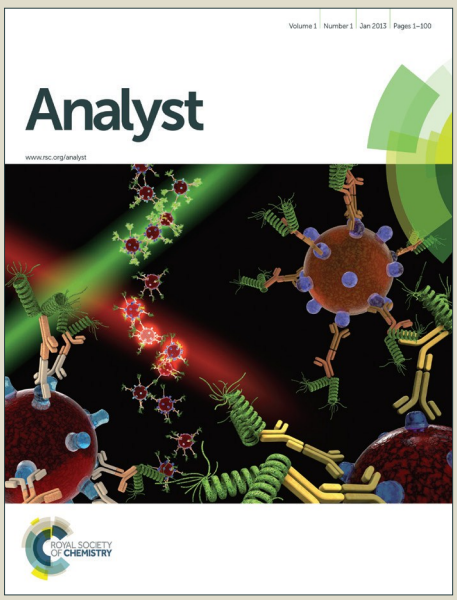

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An on-chip pollutant toxicity determination based on marine microalgal swimming inhibition

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Abstract

We report the microalgal swimming behavior as a sensor signal integrated into microfluidics for a rapid and high-throughput determination of pollutant toxicity. There are two types of chip. A poly (dimethylsiloxane) (PDMS) 12-well chip, used for optimization of experimental conditions (i.e. light level, temperature, cellular initial density and exposure time) can perform twelve parallel tests simultaneously. In concentration gradient generator (CGG) chip, a CGG connected with diffusible chambers enable large scale of dose-response bioassays to be performed in a simple way. The microalgal swimming was set as microfluidic bioassay signal and was evaluated as swimming manner, motile percentage (%MOT), curvilinear velocity (VCL), average path velocity (VAP) and straight line velocity (VSL).Under optimized physical conditions, toxicities of Cu, Pb, Phenol and Nonylphenol on four mobile marine microalgae *Platymonas subcordiformis*, *Platymonas helgolandica var. tsingtaoensis, Isochrysis galbana* and *Isochrysis zhanjiangensis sp. nov.,* were investigated. In all cases, a toxic response (i.e. a dose-related inhibition of swimming) was detected and a time of only 2 h was needed to predict EC_{50} values. 2h-EC_{50}s showed *I. galbana* as the most tolerant and *P. subcordiformis* as one of the most sensitive. Based on relative motile percentage data, EC_{50} of Cu of *I. galbana* and *P. subcordiformis* was 6.04 and 1.67 μM, respectively, while for Pb it was 15.30 and 3.87 μM, for phenol it was 8.69 and 6.08 mM, and for NP it was 29.65 and 14.47 μM, respectively. Taking into account all the swimming inhibition parameters, MOT provides move sensitive EC results.

The sensitivity difference between velocity parameters (VCL, VAP and VSL) ascribed to the difference in swimming manner under press of different classes of microalgae.

Keywords Toxicity Microfluidics Marine microalgae Swimming behavior

1 Introduction

Estuarine and costal environments are often exposed to various contaminants from anthropogenic origins. To maintain marine environments, environmental protection strategies, including effective monitoring and regulation need to be developed. Therefore, many bioassay methods, possible to analyze all potential toxicants in samples and their aggregated toxic effects as well as biological effects, have been established. Toxicity test using a range of microorganisms, algae, invertebrates and fish are being used increasingly.

Marine microalgae are the primary producers at the base of the marine food chain. They are the first group affected by contamination, and therefore provide important information for predicting the environmental impact of pollution. In addition, some authors, [1,2] established that microbial tests, using bacteria and single-celled algae, in particular, are very sensitive to toxic compounds and that they do not suffer from the animal-ethics constraints associated with using higher organisms such as invertebrates and fish. Standardized toxicity studies have conventionally focused on assessing median effect concentration (EC_{50}) on reproductive endpoints, since these techniques provide results that can be directly related to microalgal health.[3,4,5] However, depending on the life history and reproductive characteristics of microalgae, assessing endpoints requires a substantial amount of time and costs. With the wide range of environmental contaminants finding their way into marine environments, there is a growing need for monitoring tools that are fast and sensitive to a wide range of compounds, but also indicative of potential effects on survival, growth and health. Behavior analyses show promise for satisfying these requirements, and are often commended for their rapidity and sensitivity compared to traditional toxicological methods assessing reproductive effects.[6,7] Swimming is a widespread behavioral character among marine microalgae in euphotic zone.[8,9] Marine planktonic microalgae have complex and sophisticated sensory and behavioral

adaptations to their surroundings.[10] Their swimming behavior has been typically in relation to a range of important ecological phenomena including photic migration, [11] patchiness of resources, [12] physical stratification,[13] avoidance or sex.[14, 15] Microalgae swimming behavior can be a sensor to environmental signal and a linkage between biochemical processes on sub-cell level and ecological consequence on ecosystem level. Although few would argue that swimming responses offer comparatively fast and sensitive assessment of environmental perturbations, [16, 17]it has received less attention than algal test using growth, photosynthetic and metabolic assessments. It is largely due to the absence of user-friendly tools facilitating tests operation and signal acquisition.

The microfluidic approach offers several advantages over macro-scale systems for toxicity screening including low cost and flexibility in design. This design flexibility means the chips can be integrated with multiple functional units, as has been demonstrated with micro-total analysis (μTAS) and lab-on-a-chip (LOC) systems. [18] Thus, lab-intensive toxicity testing procedures such as microalgal culturing, stimulation and detection of physiological cellular endpoints can be streamlined and integrated into one single assay. A high-density patterning using channels or chambers array would make it possible to simultaneously conduct high-throughput screening. Micron-scale channel or chamber matching microalgal cell size can achieve on-line cell behavior observation easily. Additionally, miniaturized versions of microfludics reduced consumption of solvents, reagents and cells, as well as shrank physical footprint of current equipment.[19]

Besides experimental apparatus, attempts to improve a toxicity test with microalgae for regulatory purposes have revealed a number of methodological problems. Inter-laboratory comparisons carried out demonstrate that, to date, most of the test procedures traditionally used do not give comparable results.[4] For example, growth inhibition in microalgae is largely variable and depends on the species used, cell density, composition of medium or physical culture conditions. [20, 21, 22, 23] Luoma and Rainbow established that the toxicity effect is not only a function of exposure to contaminants, [24] but also of internal biological sequestration. Consequently, the data on algal sensitivity reported in the literature are only of value or use if the method employed is described in detail.

Herein, we propose to develop on-chip pollutant toxicity determination using marine

microalgal swimming measurement in order to serve as an easy and cost effective way to effectuate a high throughput screen test in lab or on-site. To do so, we have developed two types of chip. One is poly (dimethylsiloxane) (PDMS) 12-well chip, used for pre-screen and standardizing test conditions, i.e. light level, temperature, cellular initial density and exposure time. It can perform twelve parallel experiments simultaneously. A CGG chip integrated with a CGG and diffusible chambers enables large scale of dose-response bioassays to be performed in a simple way. Several parameters measured by computer assisted movement tracking including swimming manner, motile percentage (%MOT), curvilinear velocity (VCL), average path velocity (VAP), and straight line velocity (VSL) have been used to characterize the marine microalgal swimming behavior and to investigate the toxicity effects of typical anthropogenic contaminants (heavy metal and phenol compounds).

This study has several objectives. The first is to standardize the experiment conditions containing irradiance, temperature, initial cellular density and exposure time for motility test. The second objective is to assess the toxicity of typical environmental pollutants (copper, lead, phenol and NP) in use of microalgal swimming pattern and velocity, investigating the suitability and sensitivity of these end points to demonstrate microalgae behavior response, comparing sensitivities for four marine microalgae *P. subcordiformis*, *P. helgolandica var. tsingtaoensis, I. galbana* and *I. zhanjiangensis sp. nov.* These four marine microalgae were chosen because they represent different classes and species of marine microalgae and have significantly swimming ability. The third is to develop integrated microfludic device applying microalgal swimming as the response signal towards simple and rapid microalgae-based bioassay.

2 Experimental

2.1 Microfluidic chip device

The microfluidic device used in our study integrated a microfabricated Poly (dimethylsiloxane) (PDMS) chip with a temperature controlled container (Fig.1). The microfluidic chip is composed of two PDMS layers. Two negative master molds for the PDMS layers was fabricated with SU8-2035 negative photoresist (Microchem Corp, Newton, MA) by

conventional contact lithography.[18] Positive replicas with embossed channels were fabricated by molding PDMS against the master. An irreversible seal was formed between the PDMS layers, this assembly produced the required systems of microfluidic channels. The chip was then kept in a transparent PMMA container with a controlled temperature. 10 ml F/2 medium was stored in the container to maintain a 100% relative humidity environment. Polyethylene tubing was inserted into the hole of the chip to make the fluidic connections. The pieces of tubing were then connected to a syringe pump to complete the fluidic device.

Two types of PDMS chip were used for the experiment. PDMS 12-well cell culture chip to make a wet layer with accurate height of 50 μm were used for the tests of the influences of temperature, illmuniance, cellular density and exposure time on microalgal motility (Fig.1. A). Motility inhibition was determined using a concentration gradient generation (CGG) chip (Fig.1 B and C). When investigating temperature effect on microalgal motility, the temperature on the chip was set and stabilized to within 0.5 ℃ by flows of water through flow channels that surrounds the test cell wells. The surrounding channels and test cell well are apart of 30μm from each other without connection. The CGG chip mainly consists of an upstream CGG and a downstream diffusible culture module (Fig.1. B). For the current study, if one solution (concentration 0) and another one (concentration C) were introduced into CGG, the concentrations in the eight outlets are, respectively, 0, 1/7C, 2/7C, 3/7C, 4/7C, 5/7C, 6/7C and C, according to the equation described by Jeon et al.[25] The diffusible culture module is composed of an array of cell culture chambers designed to confine mobile microalgae that were integrated with the CGG unit. An array of torque-actuated valves remaining in a closed position can construct bath culture condition inside diffusible chambers. [26] Three cell culture chambers with the accurate depth of 50 μm were connected between two parallel channels of each outlet of CGG. The channels and chambers are apart of 30 μm from each other and connected by diffusion channels, which have a depth of ~2μm. The flat depth of the diffusions channel makes them impenetrable to microalgae but penetrable to substance by diffusion (Fig.1 B).

When observation, the integrated device was placed under the motorized stage of a microscope (AFT-ZML 1000). Thirty second video clips (25 frames per second) of motile microalgae in the microfluidic chip were captured using Microvision CCD camera (MV-EM120C) at 100× magnification (Fig.1 D). The experimental information was quantified

by a connected computer coupled with Image-J software. Percentage of motile cells was calculated as the number of motile algae divided by the total number of algae in the field of view. Movement tracking was performed using the manual tracking function of Image-J (Daniel Marsh, http://rsb.info.nih.gov/ij/plugins/ avi-reader.html). Path for each microalga present throughout all frames were then further analyzed by the plugin of commercial CASA systems to describe the motion characteristics for later determination of percent motility and of the average motion characteristics for all cells in the sample. Microalgal cell that could not be tracked throughout all frames due to movement out of view or plane of focus were not analyzed by the plugin. The microalgal motility characteristics analyzed and their abbreviations are given in Table S1 (see in Electronic Supplementary Information, ESI). A tracking algorithm from Mtrack2 (distributed as open source software for academic use by Nico Stuurman, http://valelab. ucsf.edu/_nico/IJplugins/MTrack2.html) was also used within the plugin to generate x, y coordinates for the centroid of each microalga present throughout the frames analyzed and assemble these coordinates into tracks for individual microalga.

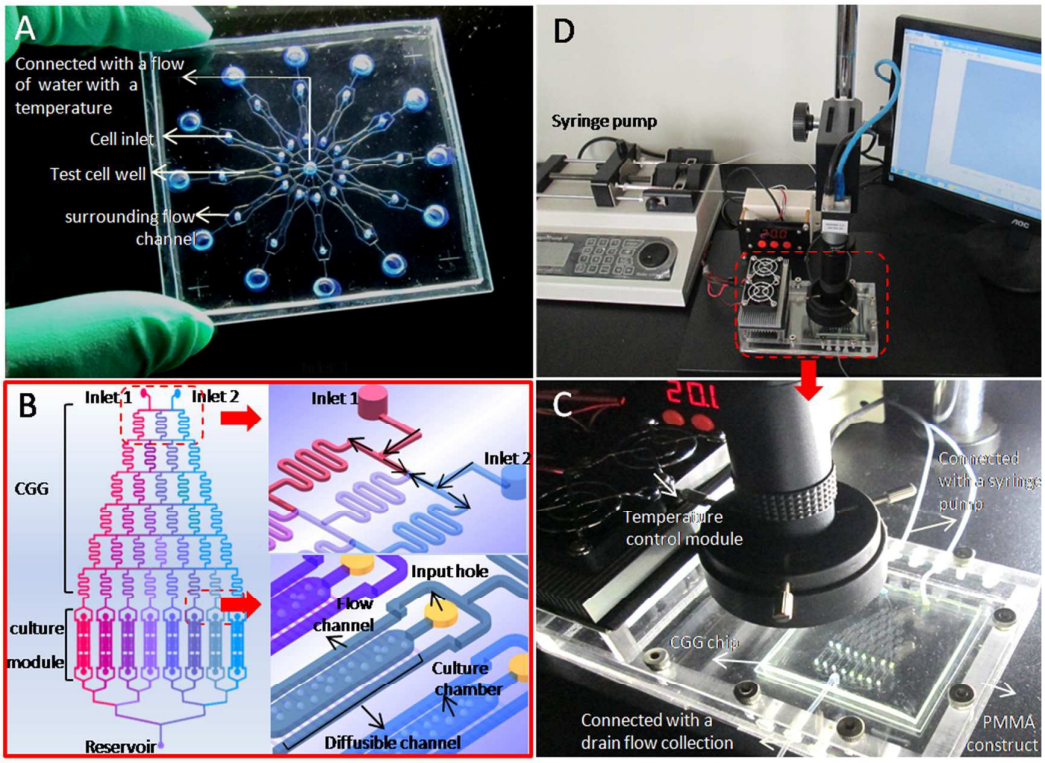


Fig. 1. Schematic and photograph of the microfluidic device for marine microalgal motility investigation. (A) Photograph of the PDMS 12-well cell culture chip. When investigating temperature effect on microalgal motility, the temperature on the chip was set by flows of water with a temperature through flow channels that surrounds the test cell wells. (B)Layout of the CGG chip that is composed of an upstream CGG and a downstream microalgal culturing module. When two solutions, represented by two colors, are introduced into a chip through inlet 1 and inlet 2, they were gradually diluted and form a gradient. Microalgae could be seeded into a chamber through an input hole. (C) A CGG chip was sealed into a temperature-controlled container to make up of a microfluidic device. A syringe pump is connected to a chip by polyethylene tubes. It drives the medium into the chip, meanwhile, waste liquid is drained and collected. When observation, the integrated device was placed under the motorized stage of a microscope. (D) Photograph of the whole experimental apparatus for microalgal motility studies

2.2 Test condition optimization

This experiment is designed to explore the influence of temperature, irradiance, initial cellular density and exposure time on microalgal swimming ability. *P. subcordiformis*, *P. helgolandica var. tsingtaoensis, I. zhanjiangensis sp.nov,* and *I. galbana* were inoculated from cultures in a log phase growth to obtain the initial cell densities of 0.9, 1.5, 11.3 and 12.4 ($\times 10^4$) cell ml^{-1}. Based on the volume of each species measured with a Coulter Counter, species were brought to the same biovolume quantity $821\times10^4 \mu m^3$ ml^{-1}. In the first series of assays, the four strains were exposed to different temperature of 0, 5, 10, 15, 20, 25, 30 and 35℃ under the same light condition of 200 μmol photons m^{-2} s^{-1}. For the illuminance influence test, all the four microgalae were exposed to different irradiance level of 0, 65, 130, 195, 260 and 325μmol photons m^{-2} s^{-1}under the same temperature of 20±1 □. Twelve replicates were used for each cell density and for each exposure time. A two hours treatment time was applied to all trials.

In order to study the influence of cell density and exposure time on toxicity tests, concentrations of Cu and NP were set at the EC_{50} values obtained from MOT inhibition for each strain. Tests with five different initial cell concentrations and exposure time were then analyzed, respectively. The established initial cell concentrations for each microalgal strain were the result of multiplying the above mentioned cellular concentrations, by the factors 0.1, 0.5, 1, 2 and 5. The established exposure time for each microalgal strain were the result of multiplying the exposure time of 2 h used for the toxicity tests, 0.5h, 1h, 2h, 4h, 6h, 8h and 12h. Twelve replicates were used for each cell density and each exposure time.

2.3 Swimming inhibition

P. subcordiformis, *P. helgolandica var. tsingtaoensis, I. zhanjiangensis sp.nov,* and *I. galbana*

were inoculated from cultures in a log phase growth to obtain the initial cell densities of 0.9, 1.5, 11.3 and 12.4 ($\times 10^4$) cell ml^{-1}. Based on the volume of each species measured with a Coulter Counter, species were brought to the same biovolume quantity 821×10^4 μm^3 ml^{-1}. All toxicity tests were kept at the same conditions of 20±1 □ and under light at 200 μmol photons m^{-2} s^{-1}. Experiments were performed using the natural seawater, obtained from the Bay of Baishi, Dalian. The background copper, lead, phenol and NP concentrations were under the limit detection values of voltammetry and HPLC, respectively. The sea water was filtered by GF/F Whatman, sterilized by autoclaving, and then added the following analytical grade salts: $CuSO4_2 \cdot 5H_2O$, $Pb(CH_3COO)_2 \cdot 3\ H_2O$, phenol (99 % purity) and Nonylphenol(99 % purity). Stock solutions were prepared in deionized water and acetone (1/10000) for heavy metal and phenols. All four microalgae were exposed to different concentrations of the four tested toxicants. Control tests were conducted with 0 μM of heavy metals and 1/10000 of acetone, respectively. Three replicates were used for each concentration and for the control tests.

2.4 Data analysis

For each toxicity test with motility inhibition as endpoint, the relationship between the different variables was explored using the Hill equation. Relative values of all the parameters including MOT, VCL, VAP and VSL incubated at different toxicant concentrations were obtained by dividing their values by that of the control tests.

$$f(x) = f_0 - (f_{inf} - f_0)\frac{x^H}{x^H + EC_{50}^H} \qquad (1)$$

where f(x) is the percentage of motility response (MOT, VCL, VAP and VSL (%)), f_0 and f_{inf} are the boundaries for 0 and infinite toxicant concentration. x the pollutant concentration (μmol l^{-1}or mmol l^{-1}). Hill number H and EC_{50} are the characteristics of the motility response f(x). Toxicant toxicity was expressed as different EC_{50} values, i.e., the concentrations of copper, lead, phenol or NP which reduce MOT, VCL, VAP and VSL by 50%, when compare to control. Other values of interest, such as the lowest observable effect concentration, EC_{10} and EC_{90} were computed from estimated values EC_{50} and H by solving Eq.(1).

$$EC_{100-A\%} = EC_{50}(100A\%^{-1} - 1)^{1/H} \qquad (2)$$

This model was not applicable to biological responses such as the Lead toxicity test based on microalgal swimming speed, for which a slight decrease in the swimming speed followed by a

sudden inhibition was observed. In these cases, a more suitable Logistic model was used:

$$f(x) = \frac{f_0}{1+e^{a(x-EC_{50})}} \quad (3)$$

in which f(x) is the percentage of swimming speed including VCL, VAP and VSL (%) , a is the slope parameter.

Data are presented as mean ± SD. Subsequently, analysis of variance and least-significant difference (LSD) post hoc testing were performed with SPSS for Windows version 14.0 professional. A p value < 0.05 was accepted as statistically significant for all tests.

3 Results and discussion

Along with physiological traits such as growth, metabolism, and photosynthesis, swimming is also a key behavioral characteristic of marine planktonic microalgae.[8,9,26] These microalgae propel themselves through liquid in nearly straight paths (‘runs’) by rotating flagella and achieve speeds of tens to hundreds of micrometers per second. Recent advances in technology and computer analysis have enabled this motility to be measured by computer assisted movement tracking.[27] Computer assisted sperm analysis (CASA) has, for example, been applied extensively to sperm mobility studies.[28, 29, 30] Several parameters, including motile percentage (%MOT), curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL) and linearity (LIN), have been developed to characterize cell motility.[31] Moreover, a tracking algorithm from Mtrack2 by Nico Stuurman was used within the plugin to generate x, y coordinates for the centroid of each cell present throughout the frames analyzed and assembles these coordinates into tracks for individual cell. For our analysis, 25 frames, representing 1 s of motion (verified by dividing the number of images saved by the length of time for which images were written), were imported into ImageJ, with the aid of these two plugins, motility and swimming manner for the four test strains haven been successfully characterized.

3.1 Test conditions optimization

Attempts to improve a toxicity test with microalgae for regulatory purposes have revealed a number of methodological problems. Several authors suggest that direct comparisons of EC_{50} values are difficult because of the use of different species, initial cell densities and laboratory

set-ups in respect of light illumination, temperature, composition of culture media and exposure time. An therefore, before investigating the toxicity effect as a function of exposure to contaminants, we demonstrated the key factors that might influence microalgal swimming and standardized toxicity test conditions.

The movement velocities of the four tested strains which belong to the same family were comparable. *Platymonas* （*Tetraselmis*）cells swim at (180.8±19.66) μm sec^{-1} for *P. subcordiformis* and (155.2 ±17.04) μm sec^{-1} for *P. helgolandica var. tsingtaoensis*. *Isochrysis* algae swim at (117.8±13.43) μm sec^{-1} for *I. galbana* and (97.1±7.43) μm sec^{-1} for *I. zhanjiangensis sp.nov*. These velocities are significantly affected by physical conditions such as light level and temperature. The relationship between motility and temperature was best described by a hyperbolic function with the highest motile percentage and fasted swimming found at about 20□ for the four strains (Fig. 2 I). Motile percentage and swimming velocity were generally reduced with significance at other temperature. It should attribute to photosynthetically produced energy. Photosynthetic organisms can drive photosynthetic processes efficiently in a suitable temperature range. Enzyme in photosynthesis can be inhibited of their activity or completely destroyed under relatively low or high temperature, and thus photosynthetically produced energy can be decreased. [32] Irradiance also exerted strong influences on the motility of the four marine microalgae (Fig. 2 II). Although the four marine microalgae were generally motile at all tested light levels, significant difference in swimming speed occurred. All irradiance-swimming velocity including VCL, VAP and VSL showed a general hyperbolic tangent shape in the four strains with an initial increase followed by a slower swimming velocity at high irradiance levels (Fig. 2 II. B-D). The optimal irradiance levels were generally from 195μmol photons m^{-2} s^{-1} to 260 μmol photons m^{-2} s^{-1} for the four mobile microalgae. The light-induced increase in velocity should be attributed to photosynthetically produced energy. [33] Since the microalgae in our experiment were exposed in a temperature-controlled container at 20 ℃, overheating cannot be the reason for the inhibition. The motility inhibition in high irradiance can be due to the saturating of light amount for the photosynthetic process. Any further increase in the amount of light will not cause an increase in the rate of photosynthesis, even cause a decrease or inhibition.[33] These results suggest that temperature and irradiance need to be controlled during microalgal toxicity test using motility

in order to optimize microalgal mobile activity and standardized physical conditions in toxicity studies.

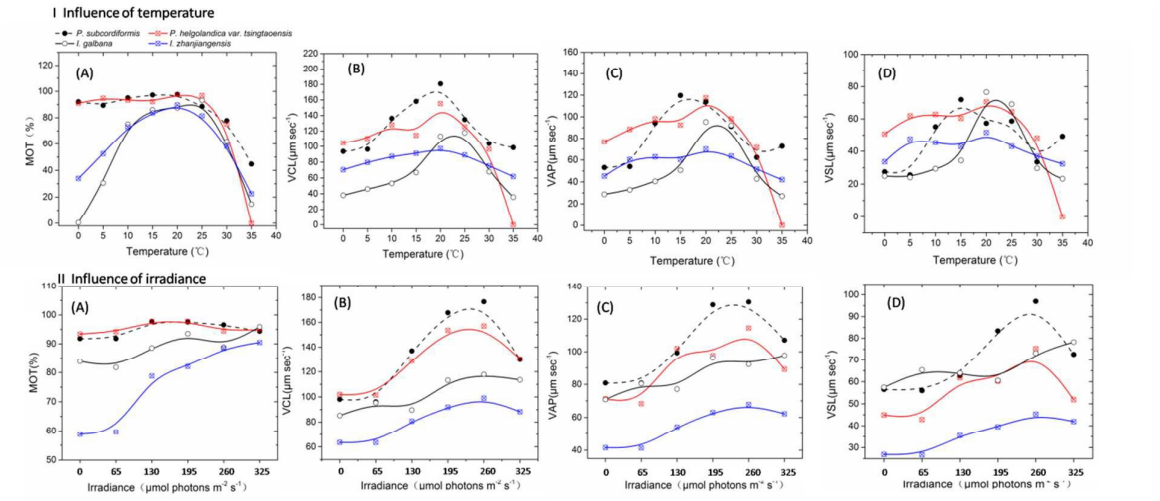


Fig.2. Influence of I temperature and II irradiance on microalgal motility parameters including (A) MOT (B)VCL (C)VAP and (D)VSL for *P. subcordiformis, P. helgolandica var. tsingtaoensis, I. galbana and I. zhanjiangensis sp.nov*

As to cellular density, there is established evidence that the sensitivity of toxicity tests increases with a decrease in initial cell density.[46, 47, 48, 49] It was due, in part, to adsorption onto the algal biomass. As cell density increases, less toxicant is bound at the cell surface, leading to less toxicant uptake into the cell and consequently less disruption of cell division. This trend was not found in our experiment, which with increasing initial cell density, the toxicity was generally consistent (P>0.05) (Fig. 3 I). Although the number of cells increases, equal contaminant is bioavailable to each cell, because that contaminant should be saturating during the short period of test time. Exposure time is another important factor that can affect toxicity drastically. The copper and NP toxicity to both species may cause a sequence of events in relation to exposure time: after 30min-4h exposure, MOT inhibition is noticed followed after 4-12h exposure by partial recovering of motility, which can be attributed to the physiological adaptations of microalgae to toxicant (Fig. 3 II). When observing optimal up-effect time of two contaminants, the time of copper was shorter than that of NP in the four strains. It can ascribe that small molecular is more permeable than large molecular through biomembrane. These results illustrated that when evaluating toxicity values using motility studies of microalgae,

exposure time should be carefully considered and not initial cellular density.

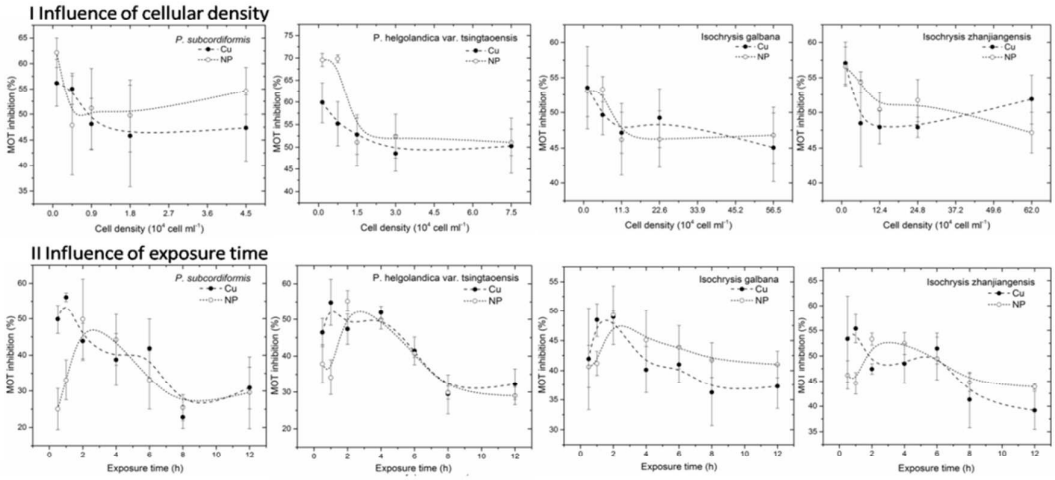


Fig.3. Data plot of I. cellular density and II. exposure time vs. EC_{50} copper and NP values calculated from MOT for the four strains studied

3.2 Toxicity on microalgal swimming

Under optimized experimental conditions, the impact of copper, lead, phenol and NP on microalgal motility parameter appeared at Fig. S1 (see in ESI). When the four strains were exposed to increasing toxicant concentrations for two hours, the relative values of all motility parameters showed a significant decrease. This indicates that increasing copper, lead, phenol and NP concentrations all affected microalgal motility in a dose-dependent manner. However, the degree of motility inhibition was affected in deferent ways, depending on the species of microalga and toxicant concentration added in solution. EC_{50} values shown in Table 1 were calculated from fitted sigmoid equations of Hill model with r^2 values ranging from0.901 to 0.999 or Logistic model with r^2 values ranging from 0.896 to 0.973.

The effects of the four pollutants on the motility (EC_{10}, EC_{50}, and EC_{90}) of *P. subcordiformis*, *P. helgolandica var. tsingtaoensis*, *I. galbana* and *I. zhanjiangensis sp.nov* are summarised in Table 1. The contaminant concentrations required to reduce the motility by 10%, 50% and 90% were generally lower for *P. subcordiformis*, and higher for *I. galbana* than for other three microalgae. For example EC_{50} values calculated from VCL for *P. subcordiformis*, *I. zhanjiangensis sp.nov, P. helgolandica var. tsingtaoensis* and *I. galbana* are 2.36, 2.89, 4.97 and

7.34μM of copper and EC_{90} values are 10.92, 44.08, 22.17 and 130.15μM of phenol. *P. subcordiformis* motility appeared to be the most sensitive to the tested pollutants among the four marine microalgae. In most cases, *I. zhanjiangensis sp.nov* motility showed less sensitive to toxicant exposure than *P. helgolandica var. tsingtaoensis* since the EC values for *I. zhanjiangensis sp.nov* were higher than those for *P. helgolandica var. tsingtaoensis*. However, in the case of EC_{10} values of lead, phenol and NP, the order of increasing sensitivity is *I. zhanjiangensis sp.nov* followed by *P. helgolandica var.* and *I. galbana.*

Table 1 The effects of the four pollutants on motility (EC_{10}, EC_{50}, and EC_{90}) of four strains

species	*P. subcordiformis*	*P. helgolandica var. tsingtaoensis*	*I. galbana*	*I. zhanjiangensis sp.nov*
Copper toxicity estimates from motility parameters including MOT, VCL, VAP and VSL				
EC_{10} (μmol l^{-1})	0.65,0.56,0.79, 0.90	1.66, 2.87, 1.87, 1.97	1.61, 0.62, 0.84, 2.02	0.80, 0.94, 1.55, 0.45
EC_{50} (μmol l^{-1})	1.67, 2.36, 1.99, 2.15	2.52, 4.97, 3.27, 3.97	6.04, 7.34, 6.27, 6.88	2.57, 2.89, 2.84, 1.96
EC_{90} (μmol l^{-1})	4.29, 9.93, 5.01, 5.10	3.81, 10.82, 5.72, 8.01	22.69, 86.90, 46.85, 23.49	8.24, 8.91, 5.16, 8.47
Lead toxicity estimates from motility parameter including MOT, VCL, VAP and VSL				
EC_{10} (μmol l^{-1})	2.36	3.19	5.11	2.94, 4.70, 5.32, 6.67
EC_{50} (μmol l^{-1})	3.87, 5.19*, 4.06*,3.34*	5.86,8.51*, 7.00*, 7.05*	15.30, 44.53*, 29.69*, 26.81*	8.11, 9.39, 9.94, 10.64
EC_{90} (μmol l^{-1})	6.34	10.75	45.82	22.33, 18.81, 18.56, 16.96
Phenol toxicity estimates from motility parameter including MOT, VCL, VAP and VSL				
EC_{10} (mmol l^{-1})	3.46, 3.93, 3.66, 3.47	4.53, 4.03, 3.23,4.28	2.55, 3.47, 1.34, 1.80	2.51, 3.07, 2.96, 4.38
EC_{50} (mmol l^{-1})	6.08, 6.54, 6.86, 5.47	6.85, 9.45, 8.45, 7.40	8.69, 14.84, 11.82, 14.45	10.41, 11.64, 13.96, 15.4
EC_{90} (mmol l^{-1})	10.69, 10.92, 12.83, 8.61	10.36, 22.17, 22.08, 12.78	29.64, 130.15, 104.17, 115.71	43.06, 44.08, 65.92, 54.18
NP toxicity estimates from motility parameter including MOT, VCL, VAP and VSL				

EC_{10} (μmol l^{-1})	12.69, 12.34, 12.08, 12.35	13.32, 12.39, 12.03, 11.39	19.87, 52.8, 32.42, 46.44	8.06
EC_{50} (μmol l^{-1})	14.47, 14.62, 14.43, 14.15	18.87, 19.34, 18.10, 18.25	29.65, 84.36, 48.35, 59.83	20.81, 60.39*, 62.32*, 66.62*
EC_{90} (μmol l^{-1})	16.51, 17.31, 17.21, 16.23	26.73, 30.18, 27.21, 29.25	44.21, 134.77, 72.11, 77.08	80.81

*EC_{50} values calculated from fitted sigmoid equations of Logistic model and others from Hill model

From the EC_{50} values calculated from MOT for each microalga, it is found that the contaminant effect of copper results in lower EC_{50} concentrations than that of lead. NP results in lower EC_{50} concentrations than that of phenol. The literature toxicity data gained from classic acute toxicity tests including microalgal growth inhibition test, Daphnia immobility test and fish mortality test were consistent with this trend (Table 2). From an ecological point of view, microalgal motility inhibition can be considered as a relevant endpoint. Also, considering reliability, it is important to note that the proposed protocol showed equal sensitivity in comparison with the 72h microalgal growth inhibition test for all the tested toxicants (Table 2). In the case of the metal tested, the novel protocol also showed comparable EC_{50} values to those of the Daphnia immobility test and 96h fish mortality test. Toxicity of phenol and NP determined by MOT showed comparable EC_{50} values to those determined by the 72h microalgal growth inhibition, 96h macroalgal growth inhibition and bacterium bioluminescent inhibition.

Table 2 EC_{50} values of Cu, Pb, phenol and NP calculated from MOT in this study and corresponding EC_{50} values form the literature for the same toxicants measured with a variety of other assay systems.

Toxicant	EC_{50} Calculated from MOT (μM)	Organism/Test used	EC_{50} Literature (μM)	Organism/Test used	Reference
Copper	1.67,2.52, 6.04, 2.57	*P. subcordiformis*, *P. helgolandica var. tsingtaoensis, I. galbana, I. zhanjiangensis sp.nov/* 2h, swimming inhibition	2.2, 5.2	*Nannochloropsis gaditana, Tetraselmis chuii*(microalga)/72h, growth inhibition	(5)
			6.28, 1.30	*D.magna* (Daphnia)/24h, 48h immobility test	(34)
			[1]4.72, [2]3.57-5.21	*Cyprinus carpio, Lepomis macrochirus* (fish)/ 96h, mortality LC_{50}	[1](35), [2](36)
Lead	3.87, 5.86,15.3, 8.11	*P. subcordiformis*, *P. helgolandica var. tsingtaoensis, I. galbana, I. zhanjiangensis sp.nov/* 2h, swimming inhibition	3.6, 6.5, 13	*Nannochloropsis gaditana, Tetraselmis chuii*(microalga)/72h, growth inhibition	(5)
			2.17	*D.magna* (Daphnia)/48h, immobility test	(36)
			2.12-6.42	*Cyprinus carpio* (fish)/ 96h, mortality LC_{50}	(35)
phenol	6.08, 6.85, 8.69, 10.41(mM)	*P. subcordiformis*, *P. helgolandica var. tsingtaoensis, I. galbana, I. zhanjiangensis sp.nov/* 2h, swimming inhibition	4.62, 4.13 (mM)	*Chlorella pyrenoidosa, Chlamydomonas reinhardtii* (microalga)/72h, growth inhibition	(37)
			0.44, 0.04 (mM)	*D.magna* (Daphnia)/24h,48h immobility test	(38)
			0.72(mM)	*Pimephales promelas* (fish)/ 96h, mortality	(39)

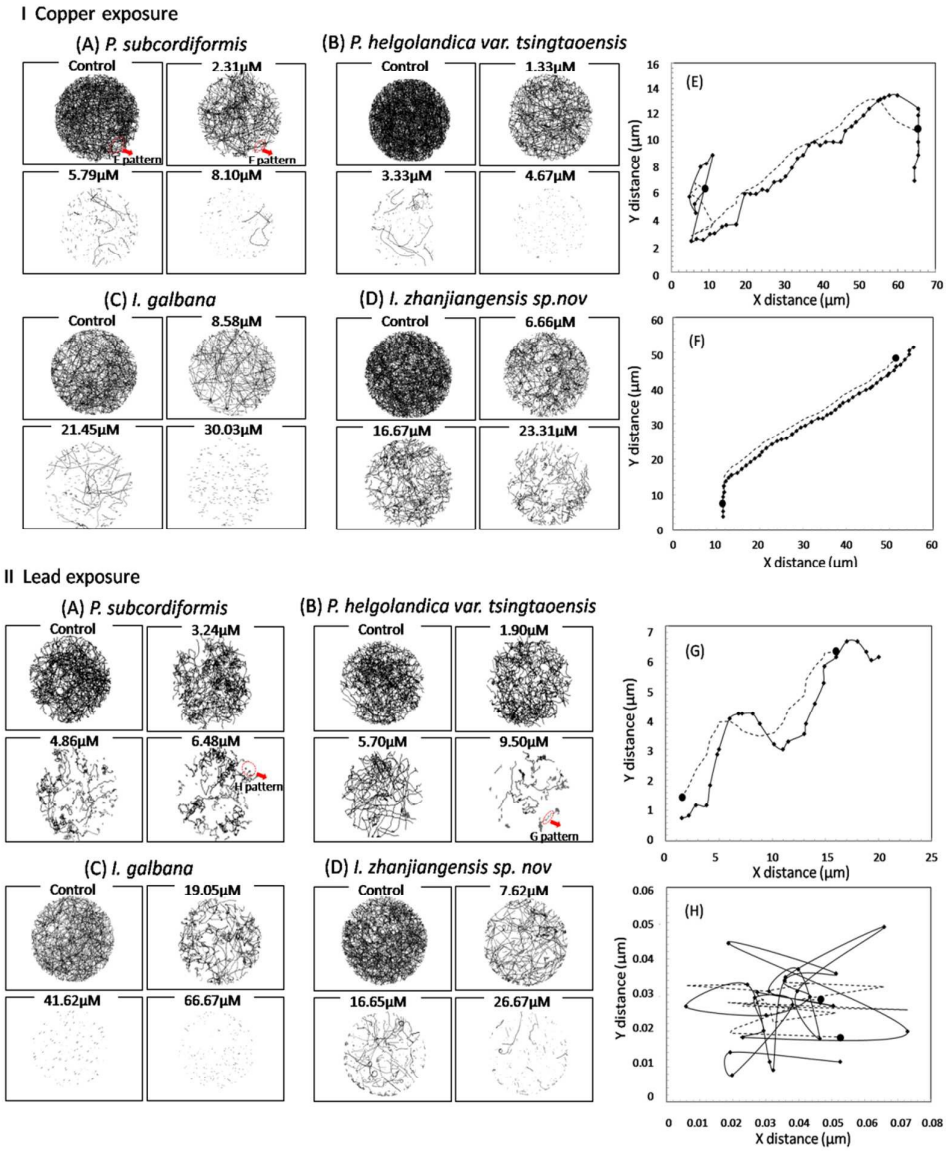
				5.1(mM）		LC_{50} luminescent bacteria test /1min	(40)
NP	14.47,18.87, 29.65, 20.81	*P. subcordiformis*, *P. helgolandica var. tsingtaoensis,* *I. galbana,* *I. zhanjiangensis sp.nov/* 2h, swimming inhibition		[1]6.26, [3]6.67	[2]2.17,	*P. helgolandica var. tsingtaoensis, Chlorella vulgaris, Dunaliellasalina* (microalga)/96h, growth inhibition	[1] (41), [2](42), [3](41)
				4.4, 2.6		luminescent bacteria test/15min *D.magna* (Daphnia)/48h, immobility test	(43)

3.3 Movement tracks and toxic effect on swimming manner

The CASA-generated movement tracks of *P. subcordiformis*, *P. helgolandica var. tsingtaoensis*, *I. galbana* and *I. zhanjiangensis sp.nov* versus pollutant concentrations in the optimal physical conditions appeared at Fig. 4 I-N. (A-D). Each black path corresponds to a single trajectory. The relationship between movement tracks and contaminants can be totally described by a decreasing trend. The trajectory densities were gradually declined with the densest trajectories found in the control for all the tested microalgae. Differences were evident in a limited number of cases where microalgal paths were eliminated, or where microalgae were eliminated from analysis because they swam out of the view. The length of movement track, representing the distance traveled by the microalga over the tested time period, also declined significantly versus increasing pollutant concentration, until into a dot.

In addition, the coordinates generated by the program were used to plot individual tracks for VCL, VAP, and VSL determination and compare the visually evident character of these swimming pattern (Fig. 4. E-L). The black path represents VCL points utilized by the program, dashed path represents the VAP path as calculated by the program, rondures on the VAP path represent the points used in calculating VSL. In control cases, the four strains exhibited swimming patterns characterized by a relatively straight "runs" periodically intersected by changes in direction, leading to a random dispersal of cells (Fig.4. E and I). *Platymonas*（*Tetraselmis*）algae swim forward propelled by four equivalent flagella with a "cilia-type" beat manner. The algae can change in swimming direction, or "tumble" smoothly when the flagella close to one another, resulting in a uni-directional beating. [44] When *Platymonas* cells responded to a range of pollutants, their swimming ability and tuning frequencies were inhibited. A random dispersal trajectory became into sluggish straight line, until wobble and immobile manner (Fig. 4. E-H). Decreasing plot shapes of VCL, VAP, and VSL determined from such *Platymonas* swimming patterns are generally similar (Fig. S1 A and B, in ESI), and thus the EC values calculated from these VCL, VCL, VAP, and VSL are comparable (Table 1). The two *Isochrysis* cells both have two nearly equal smooth flagella, where *I. zhanjiangensis sp.nov* has higher ratio of flagella length to cellular length than *I. galban.* [45] They prefer to erratic run in comfortable environment and would like to rotate under press (Fig. 4 I-L). *I. galban* swim slowly and steadily in rotating or swirling motion, and always in one direction.[45] *I.*

zhanjiangensis sp.nov rotate steadily along the long axis of the body propelled by two equivalent flagella with a straight forward manner.[45] The percentage of *Isochrysis* cells in rotating motion manner of total cell numbers increased with the contaminant concentration increasing. Therefore, the EC values calculated from the total point to point distance over the time period (VCL) were significantly higher than those calculated from average path velocity (VAP) and straight line velocity (VSL), especially in EC_{90} values (Table 1). Among all the motility parameters, MOT provides move sensitive EC results, which could be an important aspect of environmental toxicity measurement. However, when taking fully into account the motion character and non-uniformity of microalgae activity, EC values provided by VCL might to be recommended.



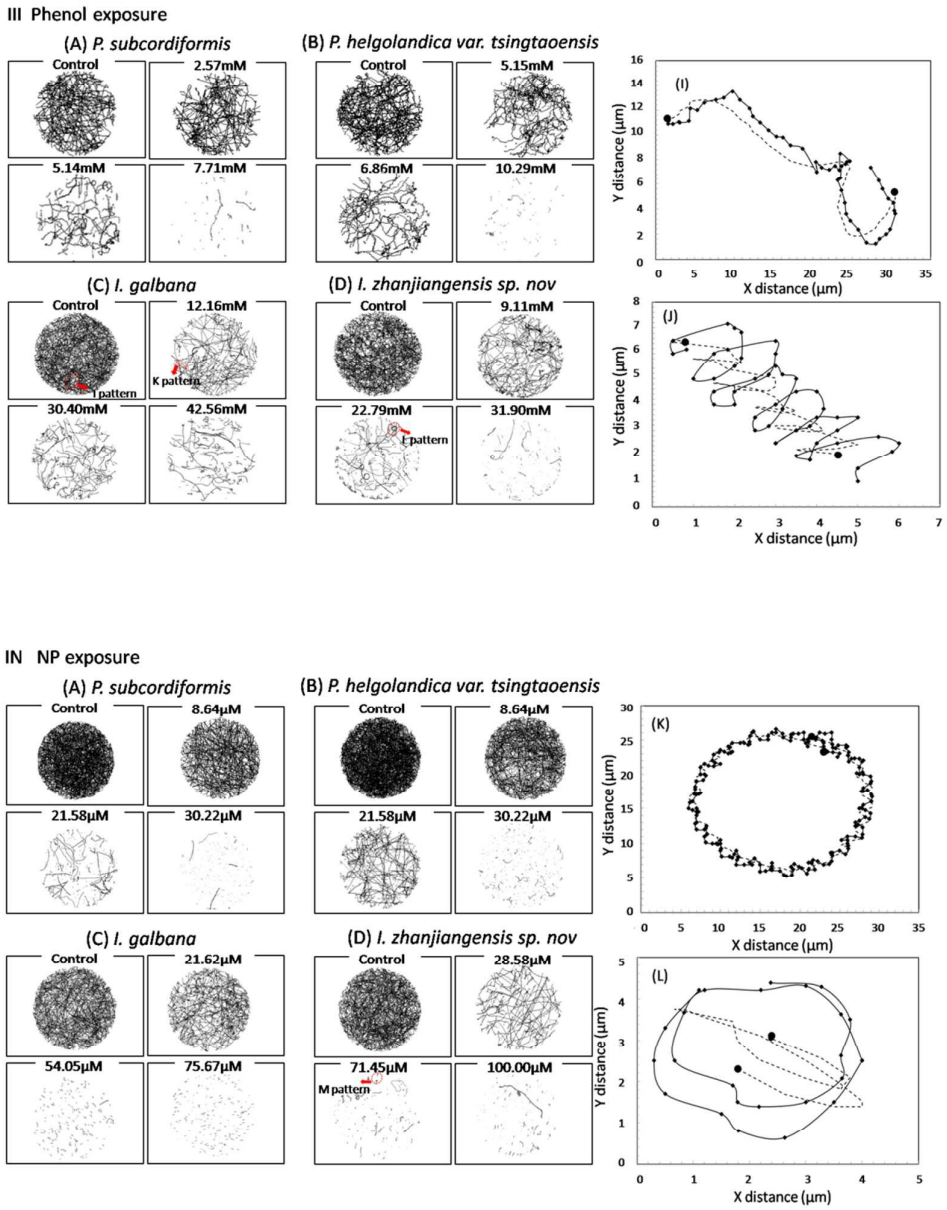


Fig. 4. Movement tracks and swimming manners of the four tested marine microalgae. Each black path corresponds to a single trajectory. I. Trajectories under stress of copper, II. Trajectories under stress of lead, III.Trajectories under of phenol, N. Trajectories under stress of NP, (A) *P. subcordiformis*, (B) *P. helgolandica var. tsingtaoensis*, (C) *I. galbana* and (D) *I. zhanjiangensis sp.nov*, Individual tracks were chosen to quantify motion parameters for microalgal moving in a variety of manners: (E) Erratic motion of a *Platymonas* cell over 2 s, (F) Sluggish straight motion of a *Platymonas* cell over 2 s, (G) Wobbling run of a *Platymonas* cell over 1 s, (H) Wobble of a *Platymonas* cell over 1 s, (I) Erratic motion of a *Isochrysis* cell over 2 s, (J) rotatable run of a *Isochrysis* cell over 2 s, (K) rotatable circulation of a *Isochrysis* cell over 8 s, (L) Rotation of a *Isochrysis* cell over 1 s.

3.4 Microfluidic toxicity algal-test suitability

The microalgae-based toxicity testing is currently done using conventional lab culturing and additional physiological traits detection. While effective, this approach can be labor-intensive. Microfluidics, also known as ‘‘lab-on-a-chip, can create a new opportunity for developing a more streamlined, meaningful screening test by integrating various functional units.

Here we developed two types of PDMS microfluidics chip for the swimming bioassay. PDMS used here for prototyping of microfluidic chips as they are nontoxic, transparent, air-permeable, inexpensive and easy to handle. Since the maintenance of cultures under laboratory conditions (type of culture, abiotic conditions) must be accurately standardized to assure the quality of tests results, we preliminary focus on determining PDMS microfluidic microalgal culture. [50] We have proved that PDMS molding microstructure can be successful in simulating the environment for microalgae's maintenance.[50] High transparency of PDMS also makes it easy to apply visual track recorder system to tracking microalgal behavior. PDMS 12-well cell culture chip used for the tests of illmuniance, temperature, cellular density and exposure time can perform twelve parallel experiments simultaneously. When investigating temperature effect on microalgal motility, the temperature on the chip can be set and stabilized to within 0.5 ℃ by flows of water through flow channels that surrounds the test cell wells within several minutes (Fig. 5 A). CGG chip use for swimming inhibition test is able to generate eight toxicant gradients with well-defined resolutions within 30 s (Fig. 5 A). As streams of the dye travel down the network, they were repeatedly split, combined with neighboring streams in laminar mode at the nodes, and allowed to mix by diffusion in the serpentine channels. A series of solutions with different concentrations of Rh 123 were formed at the outlets of CGG (Fig. 5B). The fluorescent intensities of Rh 123 in the junctions between the CGG and the cell culture module were quantified, corrected by subtracting the background fluorescence, and compared with the theoretical data. As shown in Fig. 5B, there was a good coherence (correlation coefficient = 0.989) between the experimental and theoretical data. Thus, large-scale dose-response tests can be performed in a simple way as the experimental conditions are flexible using CGG and can be optimized by changing either the category of toxicants or the concentration of the toxicants. The downstream functional area is an array of parallel channels with continuous flow through of fresh medium and parallel rows of cell

culture chambers between the channels. The chambers can confine microalgae but let chemicals penetrate. At 15 min time point, the intensity profile across the chamber presented as a nearly straight line. It implied that chemical contents of the medium inside the chambers could be uniform and close to that of the medium in the parallel flow channels (Fig. 5 C). Thus, the mobile microalgae confined in the culturing chamber can be exposed to the same chemical conditions as those in the flow channels connected with CGG and the twenty four (8×3) toxicity tests can be performed simultaneously in a single miniature operation (Fig. 5 D).

A sealed wet layer of accurate height with micron-scale constructed by PDMS microchambers was critical for microalgal motion capture and test results, which can not be easily performed by conventional method. Since slit will inhibit microalgal swimming and thick or unevenly thickened wet layer cause untracked microalgal cell throughout all frames due to movement out of plane of focus. Additionally, evaporation in an open wet layer without sealing will cause liquid flow and thus microalgal swimming might be interfered. Based on our results, with no exceptions, dose-dependent motility inhibitions by several toxicants were evident, which was generally consistent with toxicity data based on traditional tests (eg. 72h microalgl growth inhibition, Daphnia immobility and 96h fish mortality) (Table 2). Compared to such lengthy toxicity tests, investigating toxicity based on motility is a rapid and convenient bioassay to test acute toxicity of pollutant. In general, this method is applicable to the toxicity assessment of tested toxicants and might be expanded to other pollutants with further tests. If the system is valid for other toxicants, this simple and rapid test might be used as an alternative for conventional toxicity test method in lab or for real time bio-monitoring, where immediate toxicity evaluation is required.

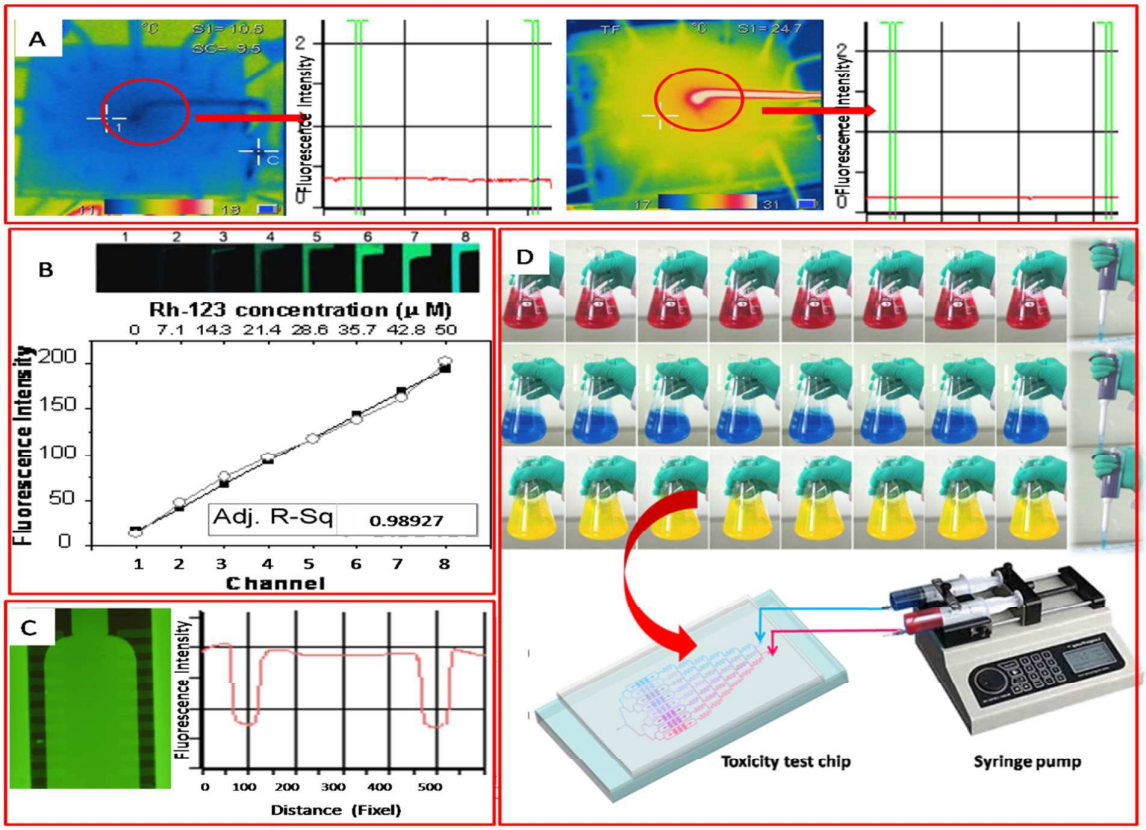


Fig. 5. Schematic and characterization of the CGG chip for twenty four (8×3) toxicity tests.(A) Representative photographs (10℃ and 25℃) of temperature characterization measured with an infrared camera and temperature profile along a circle going through the 12 wells of the chip presented as a nearly straight line.(B)As the fluorescence intensity of Rh123 was proportional to the concentration, the intensities of Rh123 at eight outlets of CGG were imaged by an inverted fluorescence microscope after 30 seconds and quantified by Image- Pro Plus software. Further, the intensities were compared with the theoretical values achieved by the equation. (C) The exiting stream feeds into a downstream array of diffusible chambers. At 15 min time point, the intensity profile across the chamber presented as a nearly straight line. (D) By integrating multiple functional units, lab-intensive toxicity testing procedures such as microalgal culturing, toxicants dispending and adding, sampling and determination can be streamlined and integrated into one single assay.

4 Conclusions

A miniaturized marine microalgae swimming-based toxicity test, by using a microfluidic chip is demonstrated. By integration of several functional elements in a single assay, the chip device allows microalgal motility-based bioassay to be sequentially and automatically

implemented in a high-throughput way. Using the transparent chip device together with a visual track recorder system we investigated the sensitivity of four marine microalgae motility. Light level and temperature are important factors which affected microalgal motility and should be carefully controlled during microalgal swimming-based toxicity test. There appears to be no relationship of microalgal motility inhibition with cellular density under the present test conditions. However, it dramatically affected by exposure time. Under optimized conditions, all values of the relative swimming parameters were shown to be suitable for toxicity tests, where MOT provides move sensitive EC results. The sensitivity difference between velocity parameters (VCL, VAP and VSL) ascribed to the difference in microalgal swimming manner under press. In general, this method is applicable to the toxicity assessment of tested toxicants and might be expanded to other pollutants with further tests. If the system is valid for other toxicants, this simple and rapid test might be used as an alternative for conventional toxicity test method in lab or for on-site testing, where immediate toxicity evaluation is required.

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