Research Paper

Distinct toxicological characteristics and mechanisms of Hg^{2+} and MeHg in *Tetrahymena* under low concentration exposure

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ABSTRACT

Inorganic divalent mercury complexes (Hg^{2+}) and monomethylmercury complexes (MeHg) are the main mercury species in aquatic systems and their toxicity to aquatic organisms is of great concern. *Tetrahymena* is a type of unicellular eukaryotic protozoa located at the bottom of food chain that plays a fundamental role in the biomagnification of mercury. In this work, the dynamic accumulation properties, toxicological characteristics and mechanisms of Hg^{2+} and MeHg in five *Tetrahymena* species were evaluated in detail. The results showed that both Hg^{2+} and MeHg were ingested and exhibited inhibitory effects on the proliferation or survival of *Tetrahymena* species. However, the ingestion rate of MeHg was significantly higher than that of Hg^{2+} . The mechanisms responsible for the toxicity of MeHg and Hg^{2+} were different, although both chemicals altered mitochondrial membrane potential (MMP). MeHg disrupted the integrity of membranes while Hg^{2+} had detrimental effects on *Tetrahymena* as a result of the increased generation of reactive oxygen species (ROS). In addition, the five *Tetrahymena* species showed different capacities in accumulating Hg^{2+} and MeHg, with *T. corlissi* exhibiting the highest accumulations. The study also found significant growth-promoting effect on *T. corlissi* under low concentration exposure (0.003 and 0.01 $\mu\text{g Hg/mL}$ (15 and 50 nM)), suggesting different effect and mechanism that should be more closely examined when assessing the bioaccumulation and toxicity of mercury in aquatic ecosystems.

1. Introduction

Mercury (Hg) has been defined as a global pollutant as a result of its characteristics of long-range transport, persistence, bioaccumulation and toxicity (Fitzgerald et al., 2007; Jiang et al., 2006; Liu et al., 2012). The toxicity of Hg depends largely on its chemical forms, and monomethylmercury complex (MeHg) is the most toxic species of Hg. In aquatic systems, inorganic divalent mercury complexes (Hg^{2+}) and MeHg are the two main species for Hg. Studies have shown that increased generation of reactive oxygen species (ROS) is a toxic mechanism in cell death and fish mortality induced by Hg^{2+} (Yole et al., 2007; Zhang et al., 2016). However, MeHg causes nervous system damage, especially during the early growth phase of the brain (Clarkson, 1997; Tonazzi et al., 2015). With its high rate of bioaccumulation and biomagnification in the food chain, MeHg could do grievous damage to human beings (Hsu-Kim et al., 2013; Meng et al., 2014; Yan et al.,

2010). Determining the biomagnification of Hg species in the food chain is therefore critical to assessing the risks of Hg in the environment.

The mercury uptake of protozoa situated at the bottom of the aquatic food chain can be considered a starting point for mercury accumulation and an essential process affecting the distribution of Hg in food webs (Hammerschmidt et al., 2013; Hsu-Kim et al., 2013; Lin et al., 2013). *Tetrahymena* is a genus of free-living protozoa that is widespread in aquatic systems (Fenchel, 2013). Because of its rapid proliferation, unique nuclear dualism, extensive membrane structure and rapid reaction to external exposure, *Tetrahymena* serves as a useful model organism for the investigation of some exogenous toxic substances. Studies have found that the fatty acid composition of *Tetrahymena thermophila* can be affected by exposure to CuO nanoparticles, and TiO_2 may increase Cd bioaccumulation in *Tetrahymena thermophila* and induce greater joint toxicity (Mortimer et al., 2011; Yang et al.,

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2014).

As a kind of unicellular eukaryotic organisms in aquatic systems, the response of *Tetrahymena* to pollutants can be observed directly and be analyzed in more detail than other animals, such as fish or rats. Considering the unicellular characteristic of *Tetrahymena*, some analysis methods for cell research may be tentatively applied to assess the effect and potential mechanisms of toxicants on the mentioned protozoa. In addition, *Tetrahymena* species are widespread in freshwater and are essential for the transportation and transformation of mercury species in aquatic environments. Thus, *Tetrahymena* is a potentially valuable model organism for evaluating the toxicity of mercury species. In the past few decades, a few studies have been carried out to target the effects of Hg on *Tetrahymena pyriformis*, suggesting that its generation time or bioassay may be affected under exposure to Hg (Carter and Cameron, 1973; Thrasher and Adams, 1972). Apart from the lack of studies about the toxicity and mechanisms of Hg in *Tetrahymena*, only one species of *Tetrahymena* was used in those previous studies and the differences among *Tetrahymena* species were not investigated. Further, the uptake properties and toxicological mechanisms of different Hg^{2+} and MeHg in *Tetrahymena* species remain unclear.

The aim of this work was to study the toxicological characteristics and mechanisms in *Tetrahymena* under exposure to Hg^{2+} or MeHg, the two main Hg species in aquatic systems. To evaluate the effects of these chemicals, five *Tetrahymena* species (*T. corlissi*, *T. pyriformis*, *T. shanghaiensis*, *T. malaccensis*, and *T. thermophila* SB210) were selected. The bioaccumulation properties and toxic effects of Hg^{2+} and MeHg on *Tetrahymena* species were discussed in detail. In order to comprehensively assess the toxic effects of mercury species in the environment, one of the five *Tetrahymena* species was exposed to low concentrations of Hg^{2+} or MeHg (0.003 and 0.01 μg Hg/mL (15 and 50 nM)).

2. Materials and methods

2.1. *Tetrahymena* species and cell culture

The five *Tetrahymena* species (*T. corlissi*, *T. pyriformis*, *T. shanghaiensis*, *T. malaccensis* and *T. thermophila* SB210) were provided by Dr. Wei Miao at the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). The culture medium (SPP medium) used in this study (pH 7.0–7.2) contained 20 g proteose peptone (Becton, Dickinson and Company, USA), 1 g yeast extract (OXOID, Thermo Fisher Scientific, USA), 2 g glucose (Sigma, USA) and 0.03 g Ferric citrate (Sigma, USA) in 1000 mL ultra-pure water (Millipore, Darmstadt, Germany), with 1% (v/v) penicillin-streptomycin solution for preventing infection from bacterium or fungus (10,000 units/mL penicillin and 10,000 mg/L streptomycin, HyClone, GE Healthcare Life Sciences, USA). Considering the living conditions generally suitable for the five *Tetrahymena* species, all *Tetrahymena* cells used in the study were cultured at 28 °C, shaking at 135 rpm (Feng et al., 2007; Gorovsky et al., 1975; Li et al., 2015; Ye et al., 2014).

2.2. Growth curves

Tetrahymena cells were first cultured in medium for about 24 h, until the cells entered the logarithmic growth phase. A portion of these cells was inoculated into 10 mL of fresh medium and cultured in a 20 mL glass bottle (CNW Technologies GmbH, Germany). Experiments were conducted in triplicate. The final cell density for all *Tetrahymena* species was set to be about 3000 cells/mL using a hemocytometer coupled with a microscope following the previous publication (Absher, 1973; Li et al., 2015). Cell densities of all groups were determined and recorded every 3 h using a hemocytometer until the cells entered the decline phase (Absher, 1973; Li et al., 2015; Miao et al., 2006). Before counting, *Tetrahymena* cells were anesthetized by adding a small amount of methanol solution (10%, v/v), in order to immobilize the *Tetrahymena* cells and ensure the accuracy of the cell count.

2.3. Exposure to Hg^{2+} and MeHg at gradient concentrations

The growth responses of five *Tetrahymena* species were observed when exposed to 8 different concentrations of either Hg^{2+} or MeHg for 24 h. Based on the growth curves of the five *Tetrahymena* species, Hg^{2+} or MeHg solutions (GBW08617 and GBW08675, both from National Institute of Metrology, China) were added to the medium early in the logarithmic phase of growth. For the stock solutions, the Hg^{2+} is dissolved in an aqueous nitric acid (3%, v/v) solution and the MeHg is dissolved in methanol. The work solutions were stepwise diluted from the stock solutions (stored at 4 °C) by ultra pure water in brown glass bottles when they would be used. The added concentrations of Hg^{2+} or MeHg were as follows: 0 (the control), 0.03, 0.1, 0.3, 0.6, 1, 3, 6 and 10 μg Hg/mL (0, 0.15, 0.5, 1.5, 3, 5, 15, 30 and 50 μM). To make the work condition more clear and easy for comparison, we expressed each concentration in two representing ways. Three parallel experiments were conducted for each concentration. Following exposure for 24 h, the cell densities of all groups were determined. The concentration producing 50% of the maximum effect (EC_{50}) was calculated according to the growth inhibition of the five *Tetrahymena* cells in terms of the added Hg concentrations, represented by the fold change (%) in growth when compared to the control (Schramm et al., 2011). The medium containing cells was then centrifuged at 5500 rpm, 4 °C for 15 min and the supernatant was quickly discarded. Cell samples at the bottom of the tubes were cleaned with mercury-free SPP medium three times before the cells were recounted. Then the concentrations of total Hg (THg, expressed by the mean Hg mass per 1000 cells) in the samples were analyzed to determine the correlativity between uptake of mercury species and growth inhibition in *Tetrahymena*.

Based on growth curves of the five species tested and EC_{50} values at 24 h, *Tetrahymena* cells were exposed to two relatively low dose (in terms of non-inhibitory effect) of mercury species (0.1 μg Hg/mL (0.5 μM) for Hg^{2+} and 0.03 μg Hg/mL (0.15 μM) for MeHg, respectively) in the early logarithmic phase of growth. Both concentrations had the similar non-inhibitory effects on cell proliferation. Medium containing cells was collected at 12 h, 24 h, and 48 h after exposure. Each treatment was conducted in triplicate. After cleaning and counting, THg concentrations (ng Hg/1000 cells) were analyzed to compare the Hg^{2+} and MeHg bioaccumulation capacity of five *Tetrahymena* species following long-time exposure.

2.4. Hg analysis

The THg concentrations in the cell samples were analyzed using a Hydra II C mercury analyzer (Teledyne Leeman Labs, Hudson, USA) according to the USEPA method 7473 (Shao et al., 2016; USEPA, 1998). Briefly, a 50 μL cell suspension of every treated sample was pipetted into a nickel boat. The boats containing samples were then burned at high temperature to reduce all mercury species to elemental mercury, which was trapped with a gold amalgam. After decomposition, the Hg content of each sample was determined with a Hydra II C mercury analyzer. Finally, the concentrations of THg were calculated as the mean total Hg mass per 1000 cells (ng Hg/1000 cells).

2.5. Measurement of cell death, intracellular reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) using flow cytometry

Cell death (judged through cell membrane permeability), intracellular ROS and MMP were measured using flow cytometry (Novocyte 1040, ACEA, USA). Based on the 24 h EC_{50} , the exposed gradient concentrations of Hg^{2+} and MeHg were relatively low to maintain *Tetrahymena* cell density abundant enough for analysis: 0 (control), 0.03, 0.1, 0.5 μg Hg/mL (0.15, 0.5, 2.5 μM) for Hg^{2+} and 0 (control), 0.03, 0.1 μg Hg/mL (0.15, 0.5 μM) for MeHg. The 0.1 ‰ (v/v) H_2O_2 (diluted from 30% H_2O_2) was used for the positive control. To ensure the viability of *Tetrahymena* cells and for the convenience when

centrifuging, the organisms were cultured in 1.5 mL tubes (Axygen, Corning, USA). *Tetrahymena* were exposed at the early stage of logarithmic phase for 24 h. Briefly, cell membrane status was examined by detecting the increase in red fluorescence after propidium iodide (PI for short, BD Biosciences, San Jose, CA, USA) staining described in previous research (Chan et al., 2006). Dichlorofluorescein diacetate (DCF-DA, Sigma, USA) was added in the cell solutions and the final concentration was 10 μM . The solutions were incubated in the dark for 30 min to study the intracellular ROS level of *Tetrahymena* after exposure (Chen et al., 2014). *Tetrahymena* cells were then rinsed three times with PBS and augmentation of green color was expressed according to the FITC results for flow cytometry. MMP was analyzed by measuring the decrease in red fluorescence intensity after adding the dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide, Invitrogen, Thermo Fisher Scientific, USA) as discussed in previous research (Chen et al., 2014). Each treatment was repeated three times.

2.6. Effects of Hg^{2+} and MeHg on the growth of *T. corlissi* at low concentration ($\leq 0.01 \mu\text{g Hg/mL}$ (50 nM))

T. corlissi was selected for exposure to low concentrations of Hg^{2+} or MeHg ($\leq 0.01 \mu\text{g Hg/mL}$ (50 nM)). The specific gradient concentrations for Hg^{2+} and MeHg were 0 (the control), 0.003 and 0.01 $\mu\text{g Hg/mL}$ (15 and 50 nM). 0.1 % (v/v) H_2O_2 treatment was used as a positive control. The exposure was initiated in the early plateau phase to ensure the abundance of cell number and cell density was determined by flow cytometry following exposure for 24 h (Czechowska and van der Meer, 2011). The effects of mercury species on the growth of *T. corlissi* were expressed by fold change (times) of the cell numbers in Hg-treated groups compared to the control. Experiments were performed in triplicate.

2.7. Quality control and statistical analysis

For the analysis of THg concentrations in this work, we also measured certified reference material (DORM-4, fish protein) and analytical blanks for quality controls. The mean THg concentration of DORM-4 here was determined to be $402 \pm 27 \text{ ng/g}$ ($n = 5$), which was consistent with the certified value ($410 \pm 50 \text{ ng/g}$). The detection limit was about 10 ng Hg in terms of absolute mass and the analytical blanks were lower than the detection limit. Statistical analysis was performed using SPSS 19.0. Independent *t* test and analysis of variance (ANOVA) were used to evaluate the significance of mean difference among groups. The statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. Both Hg^{2+} and MeHg showed inhibitory effect on the growth of *Tetrahymena*

Due to the good living condition during logarithmic phase of *Tetrahymena*, the exposure was determined to be carried out during this phase. Thus, growth curves of the five *Tetrahymena* species were illustrated. Logarithmic phases were determined using the concentration interval of EC_{20} and EC_{80} with Origin 9.1 logistic fitting (Fig. S1). As shown in Table 1, the time required for different *Tetrahymena* species to reach the logarithmic phase of growth was varied after inoculating at the same density (approximately 3000 cells/mL). Of the five *Tetrahymena* species mentioned, *T. corlissi* and *T. pyriformis* entered the logarithmic phase of growth more slowly (at about 27 h) than the others, while *T. malaccensis* achieved logarithmic phase the earliest (at about 14 h). For *T. thermophila* SB210 and *T. shanghaiensis*, the time to reach the logarithmic phase of growth was about 17 h. So the selected exposure time was 27 h for *T. corlissi* and *T. pyriformis*, 14 h for *T. malaccensis* and 17 h for *T. thermophila* SB210 and *T. shanghaiensis*,

Table 1

Logarithmic phases and 24 h EC_{50} of five *Tetrahymena* species exposed to Hg^{2+} and MeHg.

<i>Tetrahymena</i>	logarithmic phase (h)	24 h EC_{50} for Hg^{2+} ($\mu\text{g Hg/mL}$)/(μM)	24 h EC_{50} for MeHg ($\mu\text{g Hg/mL}$)/(μM)
<i>T. corlissi</i>	27–44	0.403/2.015	0.092/0.46
<i>T. pyriformis</i>	27–57	1.546/7.73	0.294/1.47
<i>T. shanghaiensis</i>	17–30	0.584/2.92	0.648/3.24
<i>T. malaccensis</i>	14–28	1.633/8.165	0.562/2.81
<i>T. thermophila</i> SB210	17–29	1.289/6.445	0.246/1.23
average	20.4–37.6	1.091/5.455	0.368/1.84

which were all synchronous their respective early logarithmic phases of growth.

After exposure to mercury species of gradient concentrations, the cell density of both the Hg^{2+} - and MeHg-treated groups decreased significantly compared with the control. Herein, EC_{50} was evaluated based on GraphPad Prism 5.0 fitting (log (inhibitor) versus. response – Variable slope) following Hg incubation for 24 h, in order to investigate the effects of mercury species on the proliferation of *Tetrahymena* (Fig. S2). The growth inhibition of Hg in *Tetrahymena* cell density is presented by percentage (%) when compared with the control. The average EC_{50} of MeHg was 0.368 $\mu\text{g Hg/mL}$ (1.84 μM) and the value ranged from 0.092 to 0.648 $\mu\text{g Hg/mL}$ (0.46–3.24 μM) (Table 1). However, the average EC_{50} of Hg^{2+} was much higher than that of MeHg, which varied from 0.403 to 1.633 $\mu\text{g Hg/mL}$ (8.165 μM) (averaged 1.091 $\mu\text{g Hg/mL}$ (5.455 μM)). It is worth noting that the concentrations used for the calculation of EC_{50} were nominal. Considering the existence of organic matter in the medium, the practical Hg^{2+} dose may be lower than expected. Therefore, this parameter might be lower in terms of the practical concentrations in medium than in terms of the originally added concentrations. Even so, these results demonstrated that MeHg displayed higher toxicity than Hg^{2+} . Of the five *Tetrahymena* species, EC_{50} of *T. corlissi* for both Hg^{2+} and MeHg was much lower than the others, suggesting it had the highest sensitivity to mercury species. The unique sensitivity of *T. corlissi* may be explained by its slower proliferation rate than the other four species (Fig. S1) and, thus, the relatively longer suffering time to toxic Hg species than the others. The inhibitory effect found in our work was similar to the findings of other research in which Hg was also observed to have suppressing effect on the proliferation of immune cells (Kakuschke et al., 2009). In addition, some metal oxide nanoparticles (e.g. ZnO and TiO_2) have shown inhibitory effect on the growth of some microbial species (Wu et al., 2010; Wang et al., 2010.). However, due to the immature fabrication of Hg nanoparticles, the study about toxicity of Hg nanoparticles is still lacking (Ramesh et al., 2011). Therefore, the potential toxicology risk of Hg nanoparticles merits further research.

3.2. Ingestion properties of different *Tetrahymena* species for Hg^{2+} and MeHg were distinct

As mentioned before, the work solutions of Hg^{2+} and MeHg (0.1 $\mu\text{g Hg/mL}$ (0.5 μM) for Hg^{2+} and 0.03 $\mu\text{g Hg/mL}$ (0.15 μM) for MeHg, respectively) had the similar non-inhibitory effects on the proliferation of *Tetrahymena* and they were used to compare the potential differences of the five *Tetrahymena* species in the bioaccumulation characteristics of Hg^{2+} and MeHg. As shown in Fig. 1(A), for both the Hg^{2+} - and MeHg-treated groups, the mean THg in all five *Tetrahymena* species increased with the extended exposure time generally. Because the dosage of MeHg was 3.3 times lower than the Hg^{2+} concentration, the absolute Hg mass per 1000 cells in the Hg^{2+} -treated groups was higher than that in the MeHg-treated groups. Our focus here is the dynamic uptake trends of *Tetrahymena* cells with exposed time increasing. In Fig. 1(A), the accumulation properties of different *Tetrahymena* species

Fig. 1. THg (ng Hg/1000 cells) in *Tetrahymena* cells (A) and accumulation percentages of all *Tetrahymena* cells (B) under exposure to 0.1 µg Hg/mL (0.5 µM) Hg^{2+} or 0.03 µg Hg/mL (0.15 µM) MeHg for 48 h (data shown by mean $\pm$ SD, $n = 3$).

to Hg^{2+} and MeHg were observed to be different. For the MeHg-treated groups for all *Tetrahymena* species, the mean THg concentration per 1000 cells was highest after 24 h exposure and showed nearly no alteration at 48 h, suggesting the saturation of *Tetrahymena*'s uptake within 24 h. However, the accumulation of mean THg in *T. corlissi*, *T. pyriformis* and *T. shanghaiensis* did not reach its peak after 24 h exposure to Hg^{2+} because the mean THg per 1000 cells continued to increase from 24 h to 48 h.

In addition, accumulation percentages of the five *Tetrahymena* species to the exposed Hg^{2+} and MeHg were calculated via dividing the THg mass in all cells by the added THg mass. The results were displayed in Fig. 1(B). Generally, the accumulation percentages of different *Tetrahymena* species for Hg species performed distinctly though the large part of Hg species in the medium entered into the protozoa. In detail, *T. corlissi* and *T. pyriformis* seemed to be similar and their maximum accumulation percentages were lower than those of the other species, which can be explained by their less cell numbers than the other three as the exposure was carried out in their early logarithmic phases and the cells kept proliferation (in Fig. S1). Moreover, the accumulation of *T. corlissi* and *T. pyriformis* to Hg species for the whole medium reached to dynamic equilibrium (about 90% and 60%, respectively) after exposure for 12 h, which is significantly diverse (ANOVA, $p < 0.05$) from that of the other three species at 12 h whose bioaccumulation percent (about 100%) got the peak at 24 h. In particular, the bioaccumulation percentages of *T. corlissi* and *T. pyriformis* for MeHg were significantly higher (independent t test and ANOVA, $p < 0.05$) than those of the other three species at 12 h. When comes to 24 h, their percentages were significantly lower (independent t test and ANOVA, $p < 0.05$) than those of the other three species, indicating their probably stronger uptake and excretion ability to Hg during relatively short time than others. These results further indicated the species difference of *Tetrahymena* in the uptake of Hg species with the exposed time increasing. To assess the species difference more clearly, the mean THg mass per 1000 cells should be used which can normalize the difference in cell numbers. In terms of the mean THg mass per one cell or 1000 cells as shown in Fig. 1(A), the accumulation levels of the five *Tetrahymena* species to Hg in decreasing order were as follows: *T. corlissi* > *T. pyriformis* > *T. thermophila SB210* > *T. shanghaiensis* > *T. malaccensis*. It is worth mentioning that *T. corlissi* cells displayed the highest THg levels for both Hg^{2+} - and MeHg-treated groups, revealing its high capacity to absorb Hg species (refer to Fig. S3 for more

information), which might be a reasonable explanation its lowest EC_{50} values (represent highest toxicity). The high degree of Hg accumulation in *T. corlissi* may be partially explained by the fact that this species has a slower proliferation rate and, thus, the individual cell suffered from the Hg for longer time than the others (Fig. S1).

In order to explore the relations between the inhibition effect and the uptake of Hg species, THg concentrations in *Tetrahymena* cells were analyzed for all gradient-concentration Hg-treated groups after the cell counting. As shown in Fig. 2(A), there was an increase in the intracellular THg concentration (ng Hg/1000 cells) coupled with a decrease in the cell density (cell count by fold change to the control, %) with the enhancement of mercury concentration, indicating that the uptake of Hg caused a dramatic decline in the population of *Tetrahymena* cells. These results suggest that the inhibition effect of Hg species on *Tetrahymena*'s growth was induced by the uptake of *Tetrahymena* cells to Hg species, which was related to the Hg concentrations.

Furthermore, the uptake rates of Hg^{2+} and MeHg for the five *Tetrahymena* species were studied by comparing the THg concentrations per 1000 cells in the same and non-toxic dosages (0.03 µg Hg/mL, equal to 0.15 µM) Hg^{2+} - or MeHg-treated groups for the same exposed time (24 h). The mentioned results were shown in Fig. 2(B). As shown in Fig. 2(B), the mean THg concentrations per 1000 cells in MeHg-treated groups were significantly (ANOVA, $p < 0.05$) higher than those in Hg^{2+} -treated groups for all the five *Tetrahymena* species. Considering the same dosage and time for exposure, our results indicate that the MeHg uptake rate for *Tetrahymena* was significantly higher than that of Hg^{2+} , which may result from the different abilities of the two mercury species penetrating the lipid bilayer of *Tetrahymena* cells. Actually, MeHg can strongly be bound to the proteins and, therefore, be integrated on cell membranes more readily than Hg^{2+} .

3.3. Toxicological mechanisms of Hg^{2+} and MeHg were distinct

As a powerful tool, flow cytometry (FACS) has been widely used to describe the biological toxicity of chemicals in cells and has demonstrated great range as analysis tool in some studies (Czechowska and van der Meer, 2012; Zhao and Ibuki, 2015). To reveal a mechanism responsible for growth inhibition caused by Hg^{2+} and MeHg in *Tetrahymena*, propidium iodide (PI) staining was used to evaluate the membrane damage induced by Hg. PI penetrates the damaged cell membrane and labels the cell nucleus with orange color, and is often

Fig. 2. (A) THg in different *Tetrahymena* cells along with the growth inhibition percentage following exposure to Hg^{2+} or MeHg for 24 h: right axis represents THg concentration shown as mean THg in 1000 cells, and left axis represents growth inhibition expressed by fold change to control (%); (B) Uptake of Hg species by *Tetrahymena* in the above 0.03 $\mu g/mL$ ($0.15 \mu M$) Hg^{2+} - or MeHg-treated groups expressed by mean THg mass per 1000 cells (“BLK” represents the group without Hg addition; data shown by mean $\pm$ SD, $n = 3$, “*” represents $p < 0.05$).

Fig. 3. Biological effect on *Tetrahymena* cells presented by fold change to control: (A) PI for cell membrane status; (B) DCF for intracellular ROS; (C) JC-1 for MMP (the presence of yellow or red represents toxic response. $n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

used to analyze cell cycles and cell apoptosis (Fried et al., 1976; Riccardi and Nicoletti, 2006). Mean fluorescence intensity (MFI) was measured after exposure for 24 h (Fig. S5 (A) in Supplementary Materials). The MFI of *T. corlissi* after exposure to 0.1 $\mu g/mL$ ($0.5 \mu M$) Hg^{2+} was similar to that of the control group, suggesting that Hg^{2+} exhibited nearly no toxic effect on the integrity of *Tetrahymena* cell membranes. However, MeHg displayed a significant disruptive effect on the cell membrane of *Tetrahymena*. As depicted in Fig. 3A, under 0.1 $\mu g/mL$ ($0.5 \mu M$) MeHg exposure, the MFI of PI in *T. corlissi*, *T. malaccensis* and *T. thermophila SB210* increased to more than 1.5 times

(1.60, 1.52 and 2.05, respectively) when compared to the control. Only *T. malaccensis* displayed an increase in MFI of approximately 1.2 times (1.20 and 1.26) under 0.03 and 0.1 $\mu g/mL$ ($0.5 \mu M$) Hg^{2+} exposure. The notable damage of cell membranes was also detected in the positive control group in which the average MFI value was 15.5 times of the control (from 3.2 to 37.9). The results were consistent with the different uptake of Hg species as mentioned above. These findings implied that MeHg was more effective in causing cell death by damaging cell membranes for *Tetrahymena* than Hg^{2+} . Because of its readiness to interact with sulphhydryl groups, MeHg might be more ready to bind to

Fig. 4. Promoting effect on the proliferation of *T. corlissi* at low concentration Hg^{2+} or MeHg exposure (0.003 and 0.01 $\mu\text{g Hg/mL}$ (15 and 50 nM)): (A) Representative scatter plots of cell counts by flow cytometry (The plots in the circle were regarded as the live cells in good living condition according to the normal cell morphology in the control groups.), (B) Growth-promoting effect of mercury species on *T. corlissi* by fold change to control (data was shown by mean $\pm$ SD, $n = 3$).

the membrane protein and integrate in the cell membranes compared to Hg^{2+} (Simpson, 1961; Clarkson, 1972). Therefore, MeHg may induce severer damage to the *Tetrahymena* membranes than Hg^{2+} .

Previous studies suggest that exposure to pollutants may cause abnormal elevation in intracellular reactive oxygen species (ROS), which can induce oxidative stress on biological molecules and result in DNA damage and denaturing of proteins (Li et al., 2013; Parman et al., 1999). Therefore, we measured the intracellular ROS using dichlorofluorescein diacetate (DCF-DA) staining. The MFI in green fluorescence represents the relative amount of intracellular ROS. For example, the MFI in *T. thermophila* SB210 cells reached to about 2 times when compared to the control, indicating the elevation of intracellular ROS in the 0.1 $\mu\text{g Hg/mL}$ (0.5 μM) Hg^{2+} -treated groups (Fig. S5 (B)). As shown in Fig. 3B, four out of five of the *Tetrahymena* species exhibited significant ROS elevation in the Hg^{2+} -treated groups. The average increase in ROS generation was 1.37 times, ranging from 1.05 to 1.97 times. In comparison, MeHg displayed either a low degree of alteration or no alteration in ROS generation, as the average change was 0.95 times when compared to the control. For the positive control groups,

the average MFI value of the five *Tetrahymena* species was about 1.19 times of the control (1.02–1.52), also indicating the generation of ROS. Our results suggested that the generation of intracellular ROS in *Tetrahymena* cells was associated with the species of Hg. In our study, elevation of ROS triggered by Hg^{2+} exposure is shown to be attributable to its toxicity, which is consistent with some other studies (Zhang et al., 2016). However, MeHg showed nearly no stimulatory effect with respect to the ROS generation in *Tetrahymena*, although some researchers have asserted that MeHg may also induce neural cell death by generating ROS (Sarafian et al., 1994). The different toxic performances of MeHg may be related to the different experimental objects used in these studies. Indeed, the cell membrane structure of *Tetrahymena* was much more complex than the cells commonly used in other studies (Miao et al., 2006; Mortimer et al., 2011). The extensive membrane structure might make some influence during MeHg damaging *Tetrahymena* cells and the underlying mechanism might need further study.

Mitochondrial membrane potential (MMP) is an important indicator of the status of mitochondria. Some pollutants have showed disruptive effects on cell mitochondria that lead to changes in MMP, indicating a

potential cell signal pathway of some toxicants (Chen et al., 2014; Sanderson et al., 2013). For instance, ROS production in aerobic cells may lead to oxidative stress on mitochondria which can trigger the release of cytochrome c and result in cell apoptosis (Ott et al., 2007). To evaluate changes in MMP after mercury treatment, we stained cells with JC-1 and applied FACS analysis to examine them (Fig. S5 (C)). We found that both mercury species could lead to the alteration of MMP in most species of *Tetrahymena*. While the MMP of the organisms in the H₂O₂-treated group did not show the same change as Hg species did, indicating the potential different toxic pathways. As shown in Fig. 3C, alterations to MMP were found in *T. pyriformis*, *T. shanghaiensis* and *T. malaccensis* after exposure to Hg²⁺ because the mean fluorescence intensity averaged 73% compared to control (48%–89%). In the MeHg-treated groups, all five species showed changes in MMP compared to the controls, with the average being 36% (11%–48%). These results suggested that the changes in MMP induced by Hg species were tightly linked with the cell toxicity for *Tetrahymena*. To evaluate the effects of Hg species on *Tetrahymena*, our work tentatively applied some analysis methods for cell research and observed effective response of the organism.

3.4. Both Hg²⁺ and MeHg triggered promoting effect on the proliferation of *T. corlissi* under low concentration exposure

In addition to the understanding of acute toxicity, examining the effects of Hg species at low concentrations could provide useful information in accurately assessing the risks of mercury in natural environments (Du et al., 2015). In light of *T. corlissi*'s unique Hg uptake characteristics, we evaluated the effect of mercury species on the proliferation of *T. corlissi* at low concentration (0.003 and 0.01 µg Hg/mL (15 and 50 nM)) after exposure for 24 h. Upon FACS analysis, the morphology and the intracellular structure of *T. corlissi* cells can be evaluated using Forward Scattering (FSC) and Side Scattering (SSC). We used these parameters to examine the number of normal *T. corlissi* cells. We applied a strong oxidant reagent H₂O₂ (0.1 %, v/v) as a positive control. In Fig. 4A, the exposure of 0.1 % H₂O₂ can dramatically decrease FSC and SSC, suggesting the disruption of cell morphology and the intracellular environment. According to the normal cell morphology in the control groups, the plots in the circle region 1 were determined as the cells in good living condition. We then analyzed the number of cells with normal SSC and FSC in circled region 1 (Fig. 4A) to calculate the number of live *T. corlissi* cells. As shown in Fig. 4A, there was a noticeable growth boost in *T. corlissi* in terms of the increased cell number after exposure to low concentration Hg²⁺ or MeHg (0.003 and 0.01 µg Hg/mL (15 and 50 µM)). As depicted in Fig. 4B, a significantly (both independent *t* test and ANOVA, *p* < 0.05) growth-promoting effect was observed in *T. corlissi* (about twice the control) under low dose Hg exposure for both Hg²⁺- and MeHg-treated groups. In the positive control group (0.1 % H₂O₂, v/v), cell proliferation was severely reduced as a result of cell death. After the mathematical conversion, the dosages used here were 15 and 50 nanomolar and were comparable to some previous studies. It has been reported that subnanomolar dose MeHg could induced strongly abnormal gene expression of the microalga *Chlamydomonas reinhardtii* (Beauvais-Fluck et al., 2016). Also, submicromolar concentration mercury could induce Akt signaling-related pancreatic β-cell dysfunction through oxidative stress and PI3K activation (Chen et al., 2006). Our study revealed a significant growth-promoting effect caused by low concentrations of mercury species in *T. corlissi*, which may result from the stress induced by Hg species commonly known as hormesis. The hormesis induced by low dose toxicants has been reported a lot (Calabrese, 2017; Stebbing, 1982). Indeed, the hormesis effect of low dose Hg has also been mentioned in previous studies which can be an explanation of the growth-promoting effect of low dose Hg species on *Tetrahymena* observed in our work (Duchemin et al., 2008; Hammerschmidt et al., 2002; Wang et al., 2013). However, the underlying mechanisms of the growth-promoting

effect and whether or not the promoting effect of Hg species at low concentration influences other *Tetrahymena* species are questions that merit further study. Since *T. corlissi* displayed a high capacity to engulf Hg and promoted proliferation following low dose mercury uptake, it is a likely candidate for use in the treatment of wastewater to eliminate mercury.

4. Conclusions

As the main species of Hg in the aquatic environment, the toxicity of Hg²⁺ complexes and MeHg complexes merits particular attention. In this work, we found that both Hg²⁺ and MeHg can enter into the selected five *Tetrahymena* species, and that these mercury species induced inhibitory effects on cell proliferation. But the dynamic accumulation properties, toxicological characteristics and mechanisms of Hg²⁺ and MeHg in the *Tetrahymena* species were distinct. These results provide essential information for understanding the toxicity of Hg²⁺ and MeHg and are essential for accurately evaluating the environmental risks of Hg. Moreover, the promoting effect on the proliferation of one of the *Tetrahymena* species studied (*T. corlissi*) was found under low concentration exposure to both Hg²⁺ and MeHg (0.003 and 0.01 µg Hg/mL (15 and 50 nM)), a result that provides new insight for comprehensive assessment of the toxic effects of mercury species in the natural environment. However, the promoting effect of Hg²⁺ and MeHg on other *Tetrahymena* species and the related mechanisms require further study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aquatox.2017.10.014>.

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