Quantitative Evaluation of Methylmercury Bioaccumulation in Rotifer *Brachionus plicatilis* by AIEgen

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A B S T R A C T

Mercury ion (Hg^{2+}) can be transformed to methylmercury (MeHg) by a variety of aquatic microorganisms in water. MeHg accumulates in fish through the food chain and eventually poses a particular challenge to public health. In this study, we used a new tool named AIEgen (Aggregation-induced emission fluorogen) to quantify the process of MeHg bioaccumulation in vivo on the species of rotifers *Brachionus plicatilis*. We analysed the relationship between the ratio of photoluminescence (PL) intensities (I_{585}/I_{480}) and MeHg concentration (C_{MeHg}) to create a master curve for calculating MeHg concentration based on the measurement of PL intensities. Quantitative results showed that the bioaccumulation of MeHg in the rotifers increased from 1.65 μg at 30 min to 2.33 μg at 180 min and reached a plateau concentration at the time of 180 min. After being transferred into artificial seawater without MeHg for 30 min, the rotifers released 0.38 μg MeHg into the medium, and the concentration of MeHg in water was kept relatively stable in the next 150 min. This study indicates that the bioaccumulation of MeHg in the body of zooplankton can be quantified by AIEgen effectively, and the main process of MeHg accumulation by rotifers in water may via passive transport.

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INTRODUCTION

Mercury (Hg), a toxic element, is widely spread in the environment (Ullrich *et al*., 2001). Hg exists in the environment in the forms of elemental Hg (Hg^0), inorganic Hg (Hg^{2+}), and organomercury compounds, such as monomethyl mercury (CH_3Hg^+, methylmercury, MeHg) (Ma *et al*., 2017). Besides, Hg^{2+} is convertible to organic mercury and MeHg is the most toxic organic form of mercury. MeHg is a potent toxin, bioaccumulated and concentrated through the aquatic food chain (Mergler *et al*., 2007). Atmospheric deposition of mercury (Hg) to surface water is one of the primary sources of Hg in aquatic environments and eventually drives MeHg toxin accumulation in fish (Luo *et al*., 2017), a greatly nutritious food, with known benefits for human health. Thus, methylmercury pollution poses human health risks via dietary fish consumption (Kucharzyk *et al*., 2015).

In 2001, the concept of aggregation-induced emission (AIE) was coined by Ben Zhong Tang (Luo *et al*., 2001). The fluorogens with AIE attribute have been referred to as AIEgens, which have attracted considerable attention due to their highly emissive behavior of aggregated illumination (Mei *et al*., 2015). AIEgen (m-TPE-RNS), a kind of Hg^{2+} sensor with high selectivity and high sensitivity has been developed (Chen *et al*., 2017; Ruan *et al*., 2015) and used to detect and quantify the Hg^{2+} bioaccumulation in algae, and Hg^{2+} release from algae after bioaccumulation (Jiang *et al*., 2016). In previous research, we found that the MeHg detection mechanism for a specific AIEgen is similar to the reaction between Hg^{2+} and AIEgen. Thus, we quantitatively evaluated the bioaccumulation of MeHg inside the waterflea *Daphnia carinata* by AIEgen (He *et al*., 2022).

Zooplankton play an important role in the biological carbon pump and serve as a trophic link between primary

producers and predators (Petrik *et al.*, 2022). *Brachionus plicatilis* is a widely-distributed small invertebrate in the global oceans, which is raised in the aquaculture as food for fish, shrimp and crab. Rotifers have been widely used as a model organism for toxicological studies due to their high sensitivity to various toxicants and ease of culture in the lab (Zheng *et al.*, 2017).

In the present study, we investigated the toxic effects of MeHg on rotifer *B. plicatilis*. The main aim of this study was to use a novel AIEgen method to quantitatively evaluate the bioaccumulation of MeHg inside the rotifer. Thus, it would supplement the MeHg toxicity data to marine organisms and improve our understanding of the dynamics of MeHg transfer in aquatic systems.

MATERIALS AND METHODS

Materials

Rotifers *Brachionus plicatilis* used in this study were resting eggs provided by College of Fisheries, Southwest University (Chongqing, China). The resting eggs were originally collected from Ningbo, China. *B. plicatilis* used in our research were hatching from the same resting eggs which represented the same strain. The rotifers were cultured with the density of 1000 ind mL^{-1} in artificial seawater (salinity of 20-35‰) under conditions with a L:D 12:12 h photoperiod at 3000 lx in an illumination incubator with the temperature maintained at 26 °C. The artificial seawater comprised NaCl 21.1 g/L, Na$_2$SO$_4$ 3.55 g/L, KCl 5.99 g/L, NaHCO$_3$ 2.94 g/L, KBr 86.0 mg/L, H$_3$BO$_3$ 23.0 mg/L, NaF 3.00 mg/L, MgCl$_2$·6H$_2$O 9.96 g/L, CaCl$_2$ 10.1 g/L, SrCl$_2$·6H$_2$O 22.0 mg/L. All reagents were obtained from Aladdin (Shanghai, China) unless otherwise specified. The AIEgen (m-TPE-RNS) was provided by AIEgen Biotechnology (Hong Kong, China) and dissolved in acetonitrile (ACN). Stock solutions of AIEgen and CH$_3$HgCl (MeHg) at a concentration of 0.1 mmol L^{-1} were prepared. The test solutions for MeHg determination contained 1 μmol L^{-1} m-TPE-RNS and different concentrations of MeHg. PL intensities were read on a fluorescent spectrometer (Edinburgh, England) with the excitation wavelength at 350 nm. The maximum photoluminescence (PL) intensity of m-TPE-RNS occurred in water/ACN mixtures with 60% water volume fractions (f$_w$, vol%). Therefore, the f$_w$ was fixed at 60% in water/ACN mixtures in the subsequent experiments. The intense emission peak of PL was displayed at 480 nm and 585 nm in the reaction of m-TPE-RNS and MeHg and the PL intensity ratio was calculated at these two wavelengths (I$_{585}$/I$_{480}$) (He *et al.*, 2022).

Survival rate of rotifer after incubation with MeHg

Rotifers were harvested from an aquarium tank with a 50-μm mesh net and introduced into a series of water samples containing different MeHg concentrations (0, 1, 3, 5 and 10 μmol L^{-1}) with the rotifer density of 200 ind mL^{-1} for 1 h, followed by a period of recovery in artificial seawater without MeHg for 48 h. Survival was calculated based on the number of live and dead animals in each vial to determine the toxicity of MeHg to *Brachionus plicatilis* at each concentration.

Time optimization for staining MeHg with AIE

After mixing 10 μL of the m-TPE-RNS stock solution with 390 μL acetonitrile (ACN) and 590 μL Milli Q water, 10 μL of MeHg stock solution was added to reach the water: ACN ratio of 3:2. The photoluminescence (PL) intensities were measured at 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 min on the fluorescent spectrometer to determine value of the ratio of PL intensities (I$_{585}$/I$_{480}$) over time.

Development of the master curve for MeHg determination

To develop the master curve for MeHg determination, a series of MeHg concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4 and 1.5 μmol L^{-1}) were detected at a constant AIE concentration (1 μmol L^{-1}) over the abovementioned time periods. After the PL intensities were obtained, the PL master curve was developed to show the relationships between PL intensity ratio (I$_{585}$/I$_{480}$) and MeHg concentrations.

Rotifers density-dependent MeHg absorption

To determine the MeHg absorption by rotifer, a series of rotifer densities (0, 100, 200, 400, 600, 800 and 1000 ind mL^{-1}) were prepared by diluting the stock rotifer culture with artificial seawater. MeHg was added to each rotifer sample to reach a MeHg concentration of 1 μmol L^{-1}. After incubation for 30 min, the rotifers were removed by a mesh net. Then, the PL intensities of each residue solution were determined by fluorescence spectrometer and the amount of MeHg in each residue solution was calculated by the AIE method using the developed master curve.

Quantification of MeHg bioaccumulation in Brachionus plicatilis

Rotifers were harvested by the method above, and were adjusted to a density of 400 ind mL^{-1} with artificial seawater, to which MeHg was added to the final concentration of 1 μmol L^{-1}. Group 1 was designed to determine the directional movement of MeHg dispersal in rotifers from the environment. The supernatant (600 μL) was collected from the *B. plicatilis*-MeHg solution at the

times of 30, 60, 90, 120, 150 and 180 min, respectively. Subsequently, each of these supernatants was mixed with 400 µL AIEgen in the ACN solution at the water to ACN ratio of 3–2. The PL intensities of each stained solution were determined by fluorescence spectrometer. Group 2 was set up to quantify the release of MeHg from rotifers after incubation in artificial seawater containing MeHg (1 µmol L⁻¹) for 60 min. The rotifers were rinsed twice with artificial seawater at the beginning of the release trial. After incubation for the same time regimes as in group one, 600 µL supernatant was collected from each rotifer solution for MeHg quantification. The MeHg concentration was determined by the PL intensities read on the fluorescence spectrometer.

Statistical analysis

All data were analyzed by Excel 2016 and SPSS18.0 using univariate ANOVA. The amount of bioaccumulation (A_t), bioaccumulation efficiency (E_t) and bioaccumulation ratio (R_t) of MeHg in rotifer were calculated as (Jiang *et al.*, 2016).

$$A_t = (C_0 - C_t) \times V_0 \times M_{MeHg}$$

Where A_t is the amount of MeHg accumulated in rotifer at time t; C_0 is the initial concentration of MeHg in the culture medium; C_t is the concentration of MeHg in the culture medium at time t; V_0 is the volume of culture medium; M_{MeHg} is the molar mass of MeHg.

$$E_t = A_t/(t \times B_m)$$

Where E_t is the bioaccumulation efficiency of MeHg in rotifers at time t; t is the indicated time for MeHg absorption; B_m is the biomass of *Brachionus plicatilis* (1000 ind ≈ 0.6 mg) in the medium.

$$R_t = \frac{C_t}{C_m} = C_t/(C_0 - C_t)$$

Where R_t is the bioaccumulation ratio of MeHg in rotifers at time t; C_t is the amount of MeHg absorbed by rotifers at the indicated time t. C_m is the amount of MeHg in the medium at the indicated time t. C_0 is the initial amount of MeHg added with $C_0 = C_t + C_m$.

RESULTS

Survival rate of rotifer after incubation with MeHg

An acute toxic experiment was conducted to further determine the effect of MeHg toxicity on rotifer *B. plicatilis*. Figure 1 shows the survival rate of rotifer after 1-h incubation at different MeHg concentrations, followed by 48-h recovery in clean artificial seawater. After incubation with 1 µmol L⁻¹ MeHg, 90% rotifers survived, which was not significantly different from the control group (C_{MeHg} = 0 µmol L⁻¹), but rotifer survival rates decreased to 65%,

40% and 0 (after 36 h recovery) after MeHg incubation at 3 µmol L⁻¹, 5 µmol L⁻¹ and 10 µmol L⁻¹, respectively. Therefore, the MeHg concentration of 1 µmol L⁻¹ was used in subsequent studies.

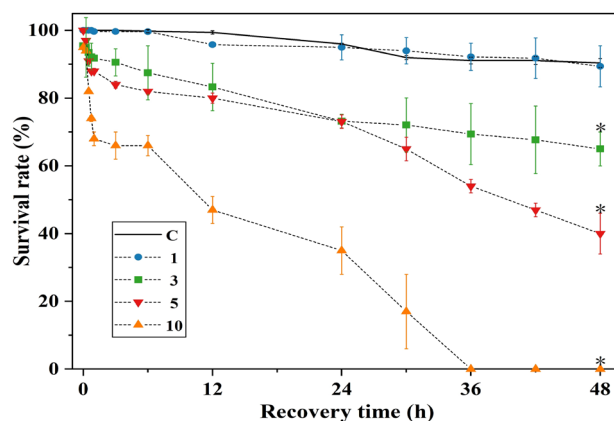Fig. 1. Survival rate of rotifer after 1 h incubation at different MeHg concentrations (0, 1, 3, 5 and 10 µmol L⁻¹) followed by recovery in clean artificial seawater for 48 h. * means the survival rate of the treatment group at 48 h is significantly different from control group.
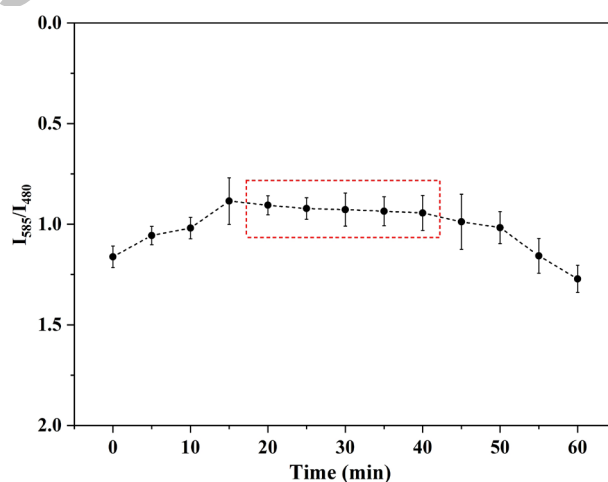

Fig. 2. The ratio of photoluminescence (PL) intensity of AIE concentration of 1 µmol L⁻¹ and MeHg concentration of 1 µmol L⁻¹ at different time elapse of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55.

Development of the AIE method for MeHg determination

The effect of time elapse on PL intensity ratio associated with AIE and MeHg reaction is shown in Figure 2. With fixed AIE and MeHg concentrations at 1 µmol L⁻¹ and time elapse at 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 min, the PL intensity ratio increased with reaction

time during the initial 20 min, and reached a plateau at 20 min to 40 min. A slight decrease was observed from 40 min to 60 min. Therefore, 30 min was used as the reaction time of mixtures with AIE and MeHg in the subsequent study.

After adding 1 μmol L^{-1} AIEgen to MeHg with different concentrations, we separately calculated the PL intensity ratio and developed the regression equation between the PL ratio (I_{585}/I_{480}) and MeHg concentration (C_{MeHg}). As shown in Table I, a variety of function curves were fitted through SPSS, and the results showed that the power function equation had the highest confidence, $R^2 = 0.996$, so the power function equation was used as the standard curve for detecting MeHg. As shown in Figure 3, the power function between C_{MeHg} and I_{585}/I_{480} was yielded as:

$$C_{MeHg} = 0.844(I_{585}/I_{480})^{1.349}$$

Table I. Fitting function equations between the PL ratio (I_{585}/I_{480}) and MeHg concentration (C_{MeHg}).

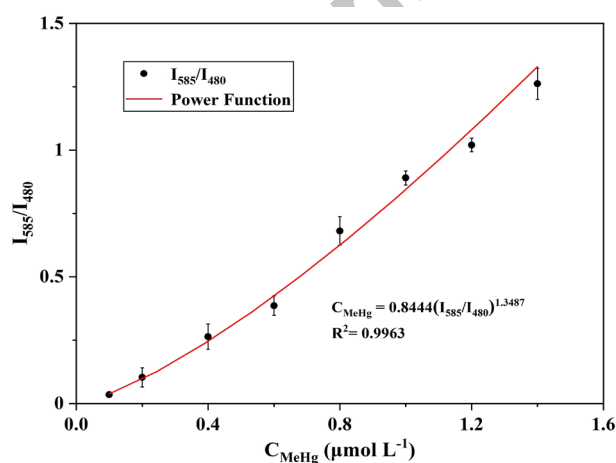| Functional relationship	Equation	R^2	Adjusted R^2	Std. Error
Power function	$y = 0.844x^{1.349}$	0.996	0.996	0.082
Ternary function	$y = -0.313x^3 + 0.823x^2 + 0.355x - 0.005$	0.995	0.991	0.043
Binary function	$y = 0.119x^2 + 0.78x - 0.060$	0.993	0.991	0.044
Linear function	$y = 0.955x - 0.101$	0.991	0.990	0.045
Exponential function	$y = 0.062e^{2.481x}$	0.869	0.847	0.490
Logarithmic function	$y = 0.449\ln(x) + 0.863$	0.850	0.824	0.189

Fig. 3. The ratio of photoluminescence (PL) intensity of MeHg (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4 and 1.5 μmol L^{-1}) at the AIE concertation of 1 μmol L^{-1} at time.

Quatitative evaluation of bioaccumulation of MeHg by the rotifer

As shown in Figure 4, with an increase in rotifer density, the amount MeHg bioaccumulation increased in the rotifer. The linear equation between rotifer density and the amount MeHg bioaccumulation (A_t) was yielded as: y = 0.008x + 0.7494, $R^2 = 0.9968$. In the solution with MeHg (1 μmol L^{-1}), the bioaccumulation efficiency (E_t) of MeHg decreased from 2.30 μg mg^{-1} h^{-1} at a low rotifer density of 100 ind ml^{-1} to 0.40 μg mg^{-1} h^{-1} at a high rotifer density of 1000 ind ml^{-1}.
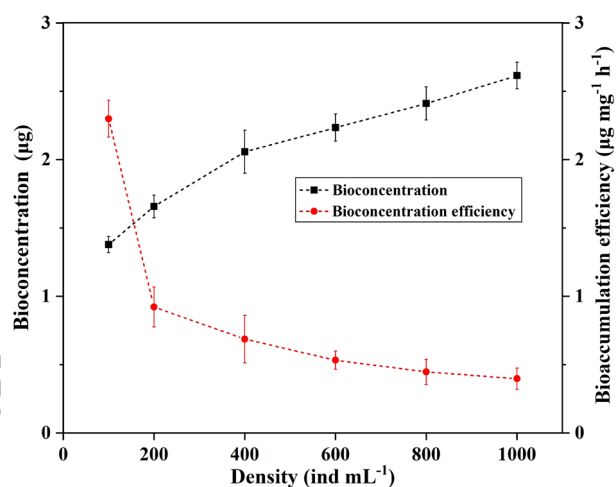

Fig. 4. Bioaccumulation and bioaccumulation efficiency of MeHg (1 μmol L^{-1}) at different rotifer densities (100, 200, 400, 600, 800 and 1000 ind mL^{-1}) within 30 min.
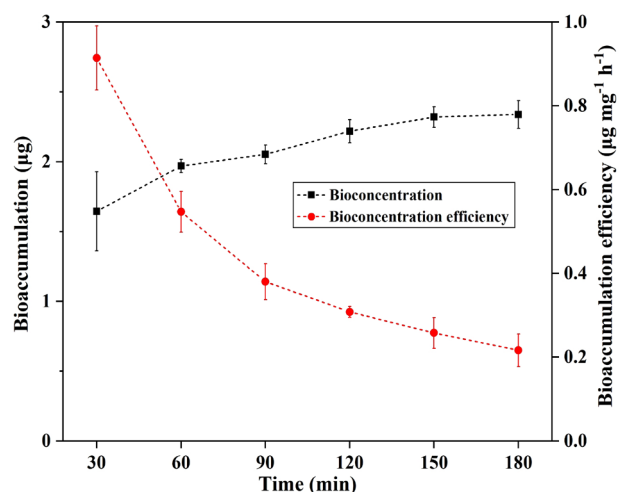

Fig. 5. Bioaccumulation and bioaccumulation efficiency of MeHg (1 μmol L^{-1}) in rotifers (400 ind ml^{-1}) at different times (30, 60, 90, 120, 150 and 180min).

Figure 5 shows the amount of bioaccumulation (A_t) and bioaccumulation efficiency (E_t) of MeHg inside rotifers over time. The bioaccumulation was a time- and MeHg concentration-dependent process. In the medium with 1 μmol L^{-1} MeHg, the amount of MeHg inside rotifers increased from zero to 1.65 μg (5.02 μg in the control group) within the first 30 min, followed to 2.33 μg at 180 min and reached a plateau concentration at the time of 180 min. The bioaccumulation efficiency (E_t) of MeHg decreased from 0.91 μg mg^{-1} h^{-1} at 30 min to 0.22 μg mg^{-1} h^{-1} at 180 min. The bioaccumulation ratio (R_t) of MeHg in rotifer were calculated, the results showed that a total of 86.6% MeHg in the 1 μmol L^{-1} MeHg medium was transferred from the environment to the rotifers within 180 min.

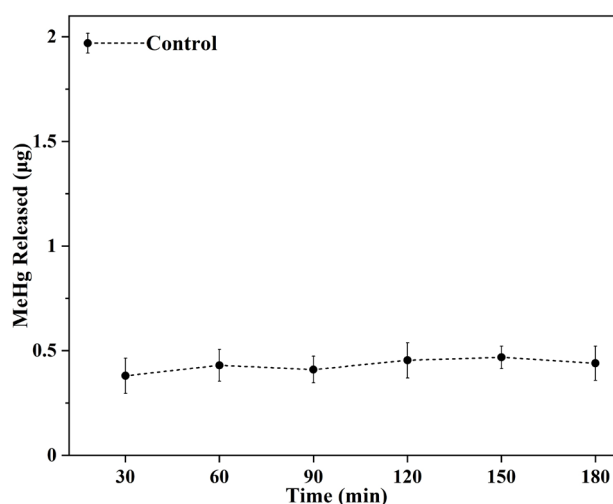Fig. 6. Release of MeHg (1 μmol L^{-1}) in rotifers (400 ind ml^{-1}) at different times (30, 60, 90, 120, 150 and 180 min).

After incubation for 60 min in the medium with 1 μmol L^{-1} MeHg, the amount of MeHg inside rotifers increased from zero to 1.97 μg. Figure 6 (Note: Control: The amount of MeHg bioaccumulation in rotifers within 60 min) shows the MeHg release from rotifers to water at different times. After being transferred into artificial seawater for 30 min, the rotifers released 0.38 μg MeHg into the clean water, and the MeHg concentration in water was kept relatively stable in the next 150 min (the amount of MeHg between 0.38 to 0.46 μg). Calculations showed that a total of 23.7% MeHg in rotifers was released to the environment within 180 min, and most MeHg retained in rotifers.

DISCUSSION

Rotifers have been an effective indicator of environmental risk assessment because of their widely distributed and sensitive to environmental change (Duggan et al., 2002). The effects of mercury on the life parameters of rotifers have been reported. The estimated LC$_{50}$ values for rotifer Brachionus koreanus at 24 h exposure was 2.964 μg L^{-1} MeHg (Lee et al., 2017). It was also reported that 0.005 mg L^{-1} Hg^{2+} would affect the survival rate and reproductive capacity of Brachionus rubens (Sarma et al., 2005). However, the non-lethal effect and inchoate biological response of low-concentration pollution are always not enough in the environmental risk assessment (Maier et al., 2004; Van Der Oost et al., 2003). In the present study, we found that high concentration MeHg (10 μmol L^{-1}) in short term (60 min) can lead to irreversible damage to rotifers. For example, after incubation for 60 min with 10 μmol L^{-1} MeHg, the essential biological functions of rotifers were impaired, including the stop of movement and ingestion, and all rotifers were dead within 36 h after transfer to clean water. Nevertheless, after incubation for 60 min with 1 μmol L^{-1} MeHg, 90% rotifers survived, which was not significantly different from the control group (C$_{MeHg}$ = 0 μmol L^{-1}).

Rotifer density is an important parameter influencing the process of bioaccumulation. In this study, the bioaccumulation of MeHg in rotifers increased with the climb of rotifer density, while the bioaccumulation efficiency showed a decreasing trend. This is similar to the results of the study on Hg^{2+} accumulation by rotifers Brachionus plicatilis (Jiang et al., 2017). In the solution with 1.355 μg mL^{-1} HgCl$_2$ (5 μmol L^{-1}), the bioaccumulation efficiency of Hg^{2+} decreased from 5.28 μg mg^{-1} h^{-1} at the rotifer density of 0.093 mg mL^{-1} to 2.61 μg mg^{-1} h^{-1} when the rotifer density increased to 0.375 mg mL^{-1}.

In this study, the amount of bioaccumulation and bioaccumulation efficiency of MeHg in rotifers changed continuously over time. The bioaccumulation of MeHg in rotifers increased with the passage of time, while the bioaccumulation efficiency showed a decreasing trend. This is similar to the results of Hg^{2+} accumulation in Brachionus plicatilis (Jiang et al., 2017) and Euglena gracilis (Jiang et al., 2016). There is evidence that passive transport seemed to be the major pathway for most phytoplankton to acquire MeHg (Lee and Fisher, 2016). The speed of passive transport decreases with the increase of concentration in vivo, which is similar to the result of MeHg accumulation by rotifers in this study, indicating that the main process of MeHg accumulation by rotifers in water may via passive transport. Rotifers could rapidly absorb MeHg from the environment, and nearly half of the supplied MeHg was absorbed within 180 min. This is similar to MeHg uptake by phytoplankton (Liu et al., 2011). The MeHg released into the water by rotifers became stable for a short time (30 min) and showed similar amount of release in the next 150

min. Only a small fraction (23.7%) of MeHg was excreted by rotifers. It is inferred that the MeHg absorbed by rotifers is likely to combine with proteins, forming MeHg-binding proteins and thus being accumulated by the rotifers.

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IRB approval

All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Southwest University.

Ethical statement

All state and institutional guidelines for the care and use of animals were followed in this research.

Statement of conflict of interest

The authors have declared no conflict of interest.

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