Dynamics and mechanisms of bioaccumulation and elimination of Nonylphenol in Zebrafish

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ABSTRACT

Nonylphenol (NP) has been widely concerned for its endocrine disrupting effects and bioconcentration in aquatic organisms. However, the knowledge on the dynamic uptake and depuration in model organisms - zebrafish (Danio rerio) were still limited. In this study, we investigated the accumulation and elimination of NP for the whole body and trunk of zebrafish by a modified pretreatment and analysis method with fewer samples. The results of acute toxicity test showed that the LC50 values of NP in zebrafish ranged from 474 μg·L^{-1} for a 24-h exposure to lower than 238 μg·L^{-1} for a 96-h exposure. Meanwhile, the NP concentrations in zebrafish during the depuration stage fitted the first-order kinetic model well, and the depuration rate constant (K2) was reduced with higher NP concentrations. Both the accumulation and elimination of NP in the trunk were faster than those in the whole fish, indicating the preferential transfer from viscera to muscle and rapidly diffusion in reverse. The bioconcentration factors at a steady state (BCFSS) of NP were 104 - 112 L·kg^{-1} in the whole body and 76 - 104 L·kg^{-1} in the trunk, respectively, suggesting that the muscle was a major position for NP storage. The BCFSS were increased within a certain concentration due to the decreasing depuration capacity inhibited by NP toxicity. To our knowledge, this study was the first to emphasize the correlation between NP exposure concentration and bioconcentration capacity.

**Keywords:** Nonylphenol; Zebrafish; Acute toxicity; Bioconcentration.

1. Introduction

Nonylphenol (NP), a ubiquitous exogenous endocrine disruptor, is widely used in production of nonylphenol polyethoxylated surfactants (NPEOs) and as additives of industrial raw materials.
Abundant NP was discharged in the form of NPEOs and their degradation products with sewage and sludge from wastewater treatment plants (WWTPs) to aquatic environment which may be further degraded under various biochemical conditions (Giger et al. 1984). The concentration of NP in river water of Chongqing area reached 7.3 μg·L⁻¹ while NP were 0.9 - 20700 ng·g⁻¹ (dry weight) in marine sediments and 6.6 - 47798 ng·g⁻¹ (dry weight) in major river sediments of Taiwan (David et al. 2009, Lee et al. 2013, Shao et al. 2005). NP has been reported that can cause various acute and chronic toxicity effects to fish, mammals, crustaceans, mollusks and algae, etc. (Shirdel et al. 2020). Servos (1999) found that the median lethal concentration (LC₅₀) of NP to fish ranged from 17 to 3000 μg·L⁻¹, while those of NOEC for fish and invertebrates were 6 μg·L⁻¹ and 3.7 μg·L⁻¹, respectively. NP generates endocrine disrupting effects by competing for binding site of estrogen receptor (White et al. 1994), thus disrupting the reproductive, immune and nervous system functions of vertebrate and the sexual differentiation of juvenile (Günther et al. 2017). Although NP in surface water would not cause acute toxicity to aquatic organism, the concentration of NP that cause endocrine disrupting effect was far below the LC₅₀ value.

NP has strong bioaccumulation potential in aquatic organism due to its strong hydrophobicity (5.4 mg·L⁻¹ in water, log Kow = 4.48) and long half-life which can last for 1-2 months in water, and even more than 60 years in sediment (Ahel & Giger 1993, Shang et al. 1999, Ying et al. 2002). The accumulation of NP in fish is mainly through ingestion (water, suspended particles and dietary) and respiration (Arnot & Gobas 2006). NP has been detected in wild fish of Dianchi Lake (Yu et al. 2012), Chongqing area (Shao et al. 2005), Pearl River Delta (Lv et al. 2019), and Taiwan (Lee et al. 2015), ranging from ND to 18.9 ng·g⁻¹, 100 ng·g⁻¹, 243 ng·g⁻¹, and 277 ng·g⁻¹, respectively, even reaching 3000 ng·g⁻¹ in fish (wet weight) at downstream of WWTPs (Zheng et al. 2015). estimated that
bioconcentration factor (BCF) of NP in fish tissues ranged from 24 to 1250 L·kg⁻¹. The BCF values reported by Lee et al. (2015) even reached 2.60×10⁴ L·kg⁻¹, which might be attributed to the differences in species. Different fish species have diverse bioaccumulative potentials, which are due to their habitats, feeding habits, trophic level and behavior, etc. (Lv et al. 2019). Furthermore, Fan et al. (2019) found that the biomagnification effect through detritivore-fish trophic level, which cannot only affect the stability of aquatic ecosystem but also endanger the health of aquatic products consumers (Cheung et al. 2008). However, most previous studies merely considered the individual exposure concentration of NP and were lacked of investigation on the dynamic process of both accumulation and purification.

In this study, we investigated the dynamic bioaccumulation and elimination behavior of NP in the whole body and trunk of zebrafish (Danio rerio), which was a model organism for toxicology. The acute toxicity test was conducted for determining the LC₅₀ of NP in zebrafish (OECD 2019). Then toxicokinetic parameters were quantified from bioaccumulation and elimination experiments through the route of uptake from water directly. Moreover, multiple exposure concentrations in bioaccumulation experiments were conducted, expecting to provide a reference for aquaculture on bioaccumulation and elimination at environmental concentration.

2. Materials and methods

2.1. Chemicals and standards

Nonylphenol (mixture of ring and chain isomers, purity ≥ 98%, CAS: 25154-52-3) purchased from Adamas-beta (Shanghai, China), was prepared in ethanol (30 g·L⁻¹). Internal standard nonylphenol-D₈ (4-n-NP-D₈, purity ≥ 98%) was purchased from Dr. Ehrenstorfer GmbH
The structures of NP and NP-D8 were shown in Fig. S1. Ethanol (GR) was supplied by Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). N-Hexane, ethyl acetate, methanol and acetonitrile from Merck (Darmstadt, Germany) were all HPLC grade. The stock solutions of standards (1000 mg·L⁻¹) were prepared in methanol, stored at -20 °C in the dark. Ultrapure water was prepared by Milli-Q water purification system. Oasis® HLB extraction cartridges (6mL, 500mg) used for SPE were purchased from Waters (Milford, MA, USA). Unhatched artemia cysts was obtained from Tianjin Fengnian Aquaculture Co., Ltd (Tianjin, China).

2.2. Zebrafish cultivation

Zebrafish (Danio rerio) were purchased from a local fishery farm (Shanghai, China). Sexually mature zebrafish were used for bioconcentration test and cultivated in PMMA tanks (30 cm × 30 cm × 30 cm) in laboratory. The stock population of zebrafish were domesticated for more than two weeks in tap water, which had aerated for 2 day, and fed throughout with artemia cysts. Mortalities of zebrafish were below 10% of the population in seven days. To maintain the size and fat content of zebrafish, the amount of feed was about 2% of body weight. Juvenile zebrafish were chosen for acute toxicity test, which were originated from the same source with mature zebrafish.

Tap water after aeration for 48 h was used as test water, the temperature and photoperiod were maintained at 23 ± 1 °C and 12-14 hours a day in domestication as well as trial period, respectively. The test water conditions were shown as follows: pH = 7.8-8.0; SS ≤ 2.7 mg L⁻¹; TDS = 150-200 mg·L⁻¹; hardness = 127 mg·L⁻¹ (CaCO₃); TOC ≈ 4.6 mg·L⁻¹; DO ≥ 5.0 mg·L⁻¹.

2.3. Acute toxicity test
The concentrations of exposure solutions in acute toxicity test were 0, 75, 125, 250, 500, 750, 1000 and 1500 μg·L⁻¹, respectively. The control group was conducted with ethanol as cosolvent (0.05 mL·L⁻¹). Each glass beaker of 2 L was added 10 fishes at the start of exposure, solutions were renewed every 24 h to maintain the valid concentration. All zebrafish were held in the laboratory for 9 days and fed to satiation before exposure. Feeding was terminated from the day before exposure and continued to the end of test. The test lasted for 96 h, and the mortalities of zebrafish were recorded every 24 h. The observation was conducted in variational interval time, and dead zebrafish were removed as soon as possible.

2.4. Uptake and depuration experiment

Semi-static method was carried out for uptake and depuration test according to OECD (2012) for testing of chemicals. Zebrafish were randomly assigned to 2 - 3 test treatments and a solvent control (0.067 mL·L⁻¹) in triplicate within PMMA tanks. The uptake test lasted for 28 d and depuration test for 14 d. Test waters were renewed for every 48 h in exposure phase. The initial pH of all prepared test solution with NP was 7.3 - 7.9 and TOC ranged from 20 to 34 mg·L⁻¹. Zebrafish were fed with hatched artemia cysts twice a day. After feeding for 5 min, uneaten food and faeces were siphoned from the tanks as soon as possible to keep NP from being adsorbed throughout the test. Zebrafish were severally transferred into dechlorinated tap water at the end of exposure phase. The uptake and depuration test of zebrafish trunk (whole body except viscera) were added with higher concentration of NP according to the consequence of accumulation in the whole zebrafish. The period of the uptake and depuration test were adjusted to 3 weeks and 1 week, respectively.
2.5. Analysis of NP concentration in zebrafish

All samples were anesthetized in DI water (4 °C), and then processed immediately or stored in aluminum foil at -20 °C until analysis. All glass containers were washed with DI water and rinsed with acetone twice, then baked at 150 °C. The extraction procedure for fish samples was improved from the method of Xu et al. (2010). Briefly, samples were homogenized with ultrapure water, then transferred into a glass tube, 2 × 10 mL ethyl acetate and 3 mL Na₂CO₃ (100 g·L⁻¹) were added. Vortex shaking was performed for 1 min after spiking with 100 ng internal standard NP-D₈, followed by ultrasonic extraction for 10 min. The tube was centrifuged for 10 min at 3000 rpm in order to separate water and organic phase. An aliquot of organic phase was transferred to an evaporating pipe, the residue was added into 10 mL ethyl acetate for second extraction, another aliquot of organic phase was combined with the first extract. The solvent was concentrated to dryness under nitrogen, then reconstituted into 1 mL of methanol, followed by dilution with 19 mL water (pH = 3.0 ± 0.1). The mixture was passed through HLB cartridges for purification, which had been preconditioned by sequentially passing 10 mL methanol and 6 mL water (pH = 3.0 ± 0.1) through it. After the extract loading on the HLB cartridges, the cartridges were rinsed with 10 mL of MeOH: H₂O (1: 9, v: v) and 10 mL n-hexane in sequence, then eluted with 10 mL methanol after drying out. The elute was concentrated to dryness under a gentle stream of nitrogen, then the residue was reconstituted into 1 mL of ACN: H₂O (8: 2, v: v), and filtered through a 0.22 μm nylon filter prior analysis.

2.6. Analysis of NP concentration in water

The actual concentrations of NP in test solution were lower than nominal concentration(Snyder
et al. 2001), since NP was dispersed inhomogeneously on account of its low water solubility. NP in water was collected from the test solutions at the beginning and the end of uptake phase. In addition, the concentrations of test solutions were determined both at the start and end of solution-exchange period. The average concentrations of upper water were detected in triplicate, owing to living habit of zebrafish. Solid phase extraction (SPE) method was applied to enrich NP. Briefly, 1 L water sample spiked with 100 ng NP-D8 was adjusted to pH value of 3.0 ± 0.1, then passed through Oasis HLB cartridges which were previously conditioned with 5 mL METB, 5 mL methanol and 5 mL DI water in sequence. After water samples loading on the cartridges, the columns were rinsed with 6 mL MeOH: H2O (1: 9, v: v), 6 mL acidified water (pH = 3.0) and 6 mL NH4OH: MeOH: H2O (2: 10: 88, v: v: v), followed by eluting with 10 mL methanol. Evaporation of extracts with nitrogen was performed at 40 °C using Reeko AutoEVA-20L concentrator, then reconstituted with 1 mL of ACN: H2O (8: 2, v: v).

2.7. Determination of lipid content

Fish growth during the experiment can lower the measured chemical concentrations in the fish with the effect that the overall depuration rate constant K2 is greater than what arise from removal processes such as respiration, metabolism, egestion. Lipid contents of test fish, which are strongly associated with the bioaccumulation of hydrophobic chemicals, is necessary to present both kinetic and steady-state bioconcentration factors. To correct a kinetic BCF for growth dilution, the depuration rate constant should be corrected for growth. The lipid content of zebrafish was determined on average for three times during the whole examination by the previous method (Smedes 1999). Briefly, several zebrafish individuals were weighed and homogenized in a glass jar,
then 16 mL 2-propanol and 20 mL cyclohexane were added, followed by vortex. Taking into account the water in homogenized tissue, water was added to achieve a total weight of 22 g. The mixture was shaken and centrifuged to separate water phase and organic phase. Organic phase was transferred into a flask, followed by second extraction with 2.6 mL 2-propanol and 17.4 mL cyclohexane. The combined extract was evaporated to dryness on the top of water bath, then dried in an oven thoroughly at 105 °C. The lipid content was calculated by the mass of residue and the sample mass.

2.8. Quantitation of compounds

The final samples were analyzed by LC-MS/MS (Shimadzu LC-30A coupled with AB SCIEX 5500 Q-trap) equipped with Shim-Pack XR-ODSII C18 Column (75 mm × 2 mm, 1.0 μm particle size) in negative ESI mode. The capillary voltage and ionization source temperature were -4500 V and 550 °C, respectively. The column temperature was set as 40 °C and 4 μL sample was injected with the flow rate at 0.30 mL·min⁻¹. 0.1% ammonia in water and acetonitrile were used as mobile phase, A and B. In order to acquire a good peak, the mobile phase was programmed as follows: 0 - 1.0 min, 30% B; 1.0 - 1.2 min, 30% - 95% B; 1.2 - 5.0 min, maintained at 95% B; 5.0 - 5.1 min, 95% - 30% B; finally backed to 30% for 2.9 min before next sample injection. Quantitation of NP was performed in the MRM mode and done with internal standard method. The quantitative and qualitative ion pairs were at m/z 219.1/133.2 and 219.1/147.2 for NP, while the ion pairs of NP-D8 were at m/z 227.3/112.3 and 227.3/126.4, respectively.

2.9. Quality assurance and control
Quantification of NP adopted the internal standard method. The stock solution of NP was progressively diluted by methanol to prepare a series of concentration of standard solutions, every standard solution of 1 mL was spiked with 100 ng (10 μL × 10 mg·L⁻¹) internal standard, established the standard curve with the concentration ratio and peak area ratio of NP and NP-D8 as the horizontal and vertical coordinates using MultiQuant software, as shown in Fig. S2. The regression equation is \( Y=2.19698X+0.02289 \) (\( R² > 0.9999 \)). Each of fish homogenate was spiked with 10 μL standard solutions and 100 ng NP-D8, performed according to the pretreatment and analysis method. Validation parameters of the method were provided in the Table. S1. The pretreatment and analysis methods could be applied well for the determination of NP, OP and BPA (BPA-D16 for IS) simultaneously, yet we investigated only NP to avoid the interference of similar substances in this study.

2.10. Data analysis

Most bioconcentration data can be described by a simple two-compartment model (OECD 2012), consisting of uptake and depuration phase:

\[
C_f = C_w \cdot \frac{K_1}{K_2} \cdot (1 - e^{-K_2 t}), \quad 0 < t < t_c
\]

\[
C_f = C_w \cdot \frac{K_1}{K_2} \cdot (e^{-K_2(t-t_c)} - e^{-K_2 t}), \quad t > t_c
\]

Where \( C_f \) is the NP concentration in fish (\( C_{\text{fish}} \), μg·kg⁻¹, wet weight), \( C_w \) is the NP concentration in water (\( C_{\text{water}} \), μg·L⁻¹), \( K_1 \) is the first order rate constant for NP uptake into fish (L·kg⁻¹·d⁻¹), \( K_2 \) is the first order rate constant for NP depuration from fish (d⁻¹), and \( t_c \) is the time at the end of uptake stage (d).

The BCF for NP, defined as the concentration of NP in fish uptake only through respiratory
and dermal only divided by its concentration in water, was calculated according to the following equation:

\[
BCF = \frac{C_f}{C_w} \tag{3}
\]

\[
BCF_{SS} = \frac{C_{f,steady\ state}}{C_w} \tag{4}
\]

\[
BCF_k = \frac{K_1}{K_2} \tag{5}
\]

Where \( BCF_{SS} \) represents the bioconcentration factor at a steady state, and \( C_{f,steady\ state} \) represents the mean concentration of NP in zebrafish at a steady state (\( \mu g \cdot kg^{-1} \), wet weight); \( BCF_k \) is the kinetic bioconcentration factor. An estimate of \( K_2 \) can be obtained from the empirical relationship:

\[
lg K_2 = -0.414 lg P_{ow} + 1.47 (r^2 = 0.95) \tag{6}
\]

In most case, the concentration variation of depuration confirmed to the first order kinetic model:

\[
C_f = C_{f,0} \cdot e^{-K_2 t} \tag{7}
\]

Where \( C_{f,0} \) is the initial concentration in fish at a depuration stage. The half-life of depuration is calculated by the following equation:

\[
t_{1/2} = \frac{ln2}{K_2} \tag{8}
\]

The calculation of the first order rate constant for uptake into fish:

\[
K_1 = \frac{C_f K_2}{C_w \times (1-e^{-K_2 t})} \tag{9}
\]

Linear-regression analysis was performed on concentration-death effect with the IBM SPSS Statistics 24.0 (IBM corp., USA) to calculate acute toxicity \( LC_{50} \) of different exposure time, and use correlation coefficient to indicate the fitting degree. All the other statistical analyses were carried out with originPro 2018C.
3. Results and discussion

3.1. Acute toxicity

The mortalities of zebrafish after 24 h, 48 h, 72 h, and 96 h of each treatment were shown in Fig. 1. The results showed that the range of toxicity for NP with mortality of maximum limit of 100% was below 1000 μg·L⁻¹. There were no mortalities or clinical sighs of toxicity observed in control treatment throughout the exposure period. All tested zebrafish died after exposed to 1000 μg·L⁻¹ and 1500 μg·L⁻¹ of NP for 24 h indicating that NP has strong lethal effect on zebrafish. The fish under 750 μg·L⁻¹ NP treatment were all dead in 48 h. The mortality for tested zebrafish exposed to 75 μg·L⁻¹ and 125 μg·L⁻¹ NP were stable at 10% after 48 h to 96 h, while the mortalities exposed to medium level concentration (250 μg·L⁻¹ and 500 μg·L⁻¹) were positively correlated with exposure time. The lethal toxicity of NP to aquatic organisms are frequently related to its evident estrogenicity not just its oxidative stress, suggesting adverse effects on immune response, cell signaling, apoptosis, and reactive oxygen species (ROS) generation, etc. (Duan et al. 2016, Matozzo et al. 2004, Rastgar et al. 2019, Yao et al. 2005).

The acute toxicity results of zebrafish exposing to NP were shown in Table. 1. The LC₅₀ of NP after 48 h exposure was 299 μg·L⁻¹, which was similar to that of 332 μg·L⁻¹ in Chen et al. (2018) ’ s study. By contrast, 96 h LC₅₀ of sizable rosy barb (Puntius conchonious) was up to 379 μg·L⁻¹, while the values of remarkably bigger grass carp and chub were 155.84 μg·L⁻¹ and 187.01 μg·L⁻¹ (Bhattacharya et al. 2008, Lv et al. 2012), respectively. Based on the acute toxicity values, although the native species (grass carp and chub) were more sensitive to NP, the small-sized zebrafish was more suitable for the evaluation of NP toxicity. Detoxification of exogenous chemicals generally consists of oxidation, reduction and hydrolysis reactions in phase I, and conjugation with the
products of previous phase in phase II. The oxyradical scavenging capacity of antioxidant enzymes was limited and the enzyme activities could be affected by NP (Shirdel et al. 2020). High content of NP in vivo was demonstrated to inhibit biotransformation activities in phase I and II and antioxidant enzymes activities (Pickford et al. 2003), thus continuously assimilating NP and producing ROS accumulated to a certain extent and cause lethal toxicity to zebrafish. Nevertheless, different species with specific metabolic pathways have different susceptibility to modifications in activity or expression of enzymes related to the metabolism or detoxification of NP (Smith & Hill 2004). Hence, the tolerance of NP seemed to be species-dependent rather than size-dependent, which was related to the tolerance threshold of metabolic and detoxifying enzyme systems to NP, and the balance of uptake and depuration in fish relied on the above enzymes. Anyhow, the toxicity of NP to zebrafish (Danio rerio) was at the level of highly toxic pesticides which is consistent with other results according to the SECP of China (GB/T 31270.12-2014), drawing conclusion that nonylphenol has great toxicity to aquatic organisms. The acute toxicity data were also employed for reference to set exposure concentrations.

3.2. Accumulation and elimination in the whole zebrafish

The variation range of mean NP concentrations in all treatments after 48 h of the first and the last period were 83% - 106%, and the actual average concentrations of upper water, which were effective, were illustrated in Table 2. Average lipid contents and the weight of zebrafish throughout the test of whole body were maintained at 3.91% - 4.14% and 0.30 ± 0.05 g, respectively.

Variations of NP concentration in the whole zebrafish during the uptake and depuration stages were shown in Fig. 2a. During the uptake stage, NP concentrations in the whole fish were increased
over time, especially fast in the first two days. The $C_{\text{fish}}$ of low-level and high-level treatments stopped increasing basically from 21 d in uptake stage, suggesting that the concentration of NP in zebrafish had reached steady state. The half accumulation of NP was performed before 4 d in uptake stage for both treatments, revealing an initially fast uptake of NP from water. During the depuration stage, the concentrations of NP in fish under low-level and high-level treatments were reduced to that of control at one-week and two-week, respectively, indicating that the complete purification process were basically finished. The routes of uptake into body of NP are mainly through gill and gut. Besides, dermal absorption is another potential way of assimilating waterborne toxicant due to a large specific surface area of zebrafish. NP is transported with oxygenated blood from gill to other target organs directly while the NP absorbing into blood through gut has undergone degradation (Pickford et al. 2003).

As shown in Fig. 3b, the depuration data were fitted well by the first-order kinetic model based on the linear regression of $\ln C_{\text{fish}}$ and time. The depuration rate constant ($K_2$) obtained from the empirical Eq. (6) confirmed with the fitted value $K_2$ ($K_2 = 0.4303 \text{ d}^{-1}$) estimated from the model of low-level, while the fitted $K_2$ of high-level was $0.2827 \text{ d}^{-1}$. NP concentration in the whole fish under high NP treatment kept decreasing within the whole depuration stage, yet, it was still much higher than that under low NP treatment. Correspondingly, the $K_2$ value of high NP treatment was much lower than that of low NP treatment, indicating the inhibited metabolic ability of NP in zebrafish. Meanwhile, the $t_{1/2}$ of NP in the whole fish exposed to high NP was longer than Low NP.

The uptake rate constant ($K_1$) of low and high NP treatments, calculating by graphical method with $K_2$ and midpoint in uptake stage from smooth uptake curve in Fig. 3a, were $32.66 \text{ L} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ and $27.75 \text{ L} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, respectively. Moreover, after fitting well into the two-compartment model as
Eq. (1) with Origin software, the $K_1$ values in low and high NP treatments were 36.83 L·kg$^{-1}$·d$^{-1}$ ($R^2 > 0.854$) and 30.28 L·kg$^{-1}$·d$^{-1}$ ($R^2 > 0.765$), respectively. The growth of NP content in high-level treatment was obviously faster than that in low-level treatment, and the BCF of NP in high-level treatment stayed greater than other treatments. The BCF got to 112 L·kg$^{-1}$ and 104 L·kg$^{-1}$ severally when $C_{fish}$ reached equilibrium (Fig. 3b). NP bioconcentration factors for the killifish ($Oryzias latipes$), salmon, and fathead minnow ($Pimephales promelas$) performed in laboratory were determined to be 167 ± 23 L·kg$^{-1}$, 282 L·kg$^{-1}$, and 270 - 350 L·kg$^{-1}$ (McLeese et al. 1981, Naylor 1995, Tsuda et al. 2001), respectively. Lower bioaccumulation abilities for NP in zebrafish might be due to the differences in habitats, feeding habits, their respective behavior (Lv et al. 2019), as well as their metabolic abilities. In addition, (Lv et al. 2012) reported that order of BCF values of NP in three cyprinid species were low-level < medium-level (and high-level), suggesting a higher exposure concentration increase the BCF of NP in fish.

3.3. Accumulation and elimination in the trunk

All test solutions were performed in same way during the uptake stage in the trunk (whole body except viscera), and the actual mean concentrations were depicted in Table 2. The highest test concentration was approximately 3% of 96 h LC$_{50}$, and the mortalities were lower than 5%. Average lipid contents of zebrafish throughout the test period were 4.15% - 4.30%, and the average weight was maintained at 0.24 ± 0.04 g.

As shown in Fig. 4a, NP concentrations in zebrafish trunk hadn’t achieved steady state within 21-d uptake phase for the high-level treatment yet, but appeared to be approaching the maximum. The NP concentrations in the trunk accumulated to the maximum level within about 3 days for low-
level and medium-level treatments, while the NP concentration in the whole fish became stable after 3-week exposure. The reason for trunk reaching equilibrium earlier than whole fish is that NP in viscera was transferred to muscle when the content of NP exceeded the capacity of metabolic organs (Orias et al. 2015). It agreed with the rapid uptake of whole zebrafish at the early stage of accumulation, followed by gradually decline of growth rate until steady state since NP in muscles was closed to be saturated. Liver is the main metabolic organ in viscera to eliminate NP (Pedersen & Hill 2000), including phase I and phase II metabolism via oxidation reactions and conjugation reactions with purpose of transform NP to polar metabolites, which mainly were glucuronidated and hydroxylated NP metabolites (Burkina et al. 2016), finally excrete through urine, bile, etc. (Jonsson et al. 2008, Thibaut et al. 1999). The BCFss of NP in the trunk of low-level and medium-level treatments were 76 L·kg⁻¹ and 96 L·kg⁻¹, respectively (Fig. 4b). Under the similar exposure concentration, BCFss of whole fish were slightly greater than trunk on account of higher bioconcentration potential of viscera. Muscles with major mass served as dominating storage part whereas reached a lower equilibrium concentration with low fat.

The lnC_fish of trunk in depuration stage had a good linear relationship with time, apparently for medium-level (R² = 0.987) and high-level treatment (R² = 0.988) (Fig. 5), confirming that elimination of NP in the trunk of zebrafish also fitted the first-order kinetic model. The C_fish from low and medium NP treatments declined to that in the control after depuration for 3 days, indicating most NP was excreted from trunk at the early depuration phase. On account of the low concentrations of depuration, the fitting data were deducted with the average value of control group. The K₂ values in medium and low NP treatments had no significant difference in fitting with all data. Actually, the C_fish value of 7 d was supposed to be lower according to the first-order kinetic
model, but those were subject to accuracy in a low concentration by the inevitable blank contamination from procedure and laboratory environment (Salgueiro-González et al. 2012). Thus, the $K_2$ of the low-level treatment tended to be underestimated considering that the $K_2$ value of medium NP treatment was significantly greater than that of high NP treatment in the whole fish ($P < 0.05$). In conclusion, the $K_2$ values of NP in zebrafish were positively correlated with the level of NP. Moreover, the $K_2$ values of NP in the trunk about three treatments were all greater than those in the whole fish, indicating that the elimination rate of NP in muscle was faster than viscera, although muscle is not the main metabolic organ. The rapid transfer of NP from muscle to viscera with extensive diffusion could explain the disparity (Guo et al. 2019). On the other hand, NP concentration in muscle reached apparent maximum earlier through the diffusion from muscle to viscera. Besides comparing the equilibrium time before steady-state, it could also be estimated from $K_1$ that the rate of concentration growth of trunk was greater than that of whole fish. Similar order of $K_1$ about NP was obtained from whole fish and trunk (Table. 2), the assimilation rate of NP seemed to be inhibited by toxicity of NP. These results were consistent with previous study on hydroxylated polybrominated diphenol ethers (OH-PBDEs) and broflanilide (Jia et al. 2020, Zhao et al. 2014), whereas the $K_1$ decline of NP with increasing exposure concentrations were not as great as those of OH-PBDEs and broflanilide. The hydrophobic alkyl residue might alter the cell membrane of gill that caused the fusion of gill lamellae, thus reduced the uptake rate of NP through the dominating route of gill (Bhattacharya et al. 2008).

The $t_{1/2}$ of NP in the whole fish were 1.75 d and 2.45 d for low NP and high NP treatments, respectively, while the $t_{1/2}$ in the trunk of low-level, medium-level and high-level treatments were 0.56 d, 0.64 d, and 0.86 d, respectively, indicating the short half-life of NP in the fish trunk. The
shorter half-lives of NP in the trunk than while fish is beneficial for consumers, since edible fish were gut off before eating. In addition, the risk for intake of fresh fish from muscle would be substantially lowered after fresh fish transferring to fresh water for a day or two.

3.4. Relationship of BCF and exposure concentration

As shown in Fig. 4b, the BCFSS of trunk was positively correlated with concentration, even though Cfish of NP in high-level treatment had not reached equilibrium. The order of BCFSS about different NP concentration in the trunk was low-level < medium-level < high-level, which was consistent with the consequence of whole fish (Fig. 2b). Yet, both Lv et al. (2012) and Snyder et al. (2001) reported that the inconsistent order of BCFSS about NP in red carp, grass carp, sliver carp and fathead minnows. The most probable interpretation is that the exposure of NP in this study was much lower than that in high-level set by Lv et al. (2012), and the NP solutions in this study had not reached the concentration of maximum BCFSS about zebrafish. While for fathead minnows exposed to 0.33 - 2.36 μg·L⁻¹ NP, which was below high-level in this study, the lower inflection concentration may lie in the greater sensitivity of fathead minnows to NP with lower LC₅₀ values (Snyder et al. 2001). More research should be conducted to investigate the correlation with LC₅₀ and the inflection concentration. For most fish, environmental concentration is hard to reach their inflection points of BCF maximum. In other words, BCF of NP in most of wild and cultured fish generally increase with exposure concentration. The toxicity kinetic model of organic compounds in fish could be described with critical body residue, which was the product of LC₅₀ × BCF (Ai et al. 2019). In other words, the extent of hazard was closely connected with the content of organics in the body. The exposure concentrations in water and the regularly predicted BCF values were both important in the
risk assessment of aquatic animals.

The BCF\textsubscript{SS} of NP in zebrafish increased initially with increscent exposure concentration, then followed by decreasing, while the consequence of chlorpyrifos was consistent with our study (El-Amrani et al. 2012). However, there is a different relationship between BCF\textsubscript{SS} and the exposure concentration with lower BCF\textsubscript{SS} for higher concentration of exposed chemicals, such as chlorophenols (PCP and 2,4-DCP), hydroxylated polybrominated diphenyl ethers (2'-OH-BDE68 and 4-OH-BDE90), broflanilide, and Atrazine (El-Amrani et al. 2012, Jia et al. 2020, Kondo et al. 2005, Zhao et al. 2014). This could be attributed to the increasing pollutant content in body that lead to the activation or induction of metabolic enzyme associate with the chemical, which further result in the augment of elimination rate (Zhao et al. 2014). However, when zebrafish embryos and juvenile sea bass exposing to high concentration of NP (Teles et al. 2004, Wu et al. 2011), antioxidant and detoxifying enzyme GST (Glutathione S-transferase) showed inhibiting effect. In addition, the expression of CYP1A1 gene, which participate in biotransformation phase I, and the activity of its catalytic probe EROD (7-ethoxyresorufin-O-deethylase) of \textit{Gobius niger}, Atlantic cod and male sea bass were suppressed significantly (Maradonna et al. 2005, Sturm et al. 2001, Vaccaro et al. 2005). Interestingly, the BCF\textsubscript{SS} of \textit{Tapes philippinarum} to NP was negatively correlated with exposure concentration, the sublethal toxicity to assimilation and filtration function, etc. after exposing to high NP concentration inhibited the uptake rate of NP (Lietti et al. 2007). It might explain the results of Lv et al. and Snyder that the BCF\textsubscript{SS} of high-level was lower. There is no accordant conclusion on the relationship between exposure concentration and BCF\textsubscript{SS}, which is related to the modification mechanism of test species by individual compounds with enzymes about assimilation and metabolism.
4. Conclusion

The LC$_{50}$ of NP to zebrafish in acute toxicity ranged from 238 to 474 μg·L$^{-1}$ during 24 to 96 h. The NP concentrations in the whole body and trunk of zebrafish fitted the first-order kinetic model well during the depuration stage. The depuration rate constant of NP in zebrafish were positively related with exposure concentration, which might be due to the inhibited metabolic enzyme activity refer to NP by toxicity. The larger $K_2$ values of NP in the trunk than those in the whole body suggested the faster depuration of muscle than viscera. The BCF$_{SS}$ of NP were 104 - 112 L·kg$^{-1}$ in the whole zebrafish and 76 - 104 L·kg$^{-1}$ in the trunk, respectively, indicating that muscles served as the major storage of NP. In addition, the muscles reached a lower-level equilibrium earlier than viscera. Within a certain concentration range, BCF$_{SS}$ value of NP in zebrafish increased with exposure concentration. The increasing of relative accumulation potential mainly lies in the decrease of depuration rate and slightly descending uptake rate. The relationship between BCF and exposure concentration of NP may be related to the modification and sensibility to NP that associated with the biotransformation enzymes. Therefore, further studies to investigate the dynamic reaction between metabolic activities and NP contents in vivo through metabolomics technique or exclusive metabolites are necessary, as well as the functional relation between BCF and exposure concentration.

Ethical approval

All authors declared that they had no known competing financial interests or personal relationships that seemed to affect the work reported in this article. All authors followed the ethical responsibilities of
this journal.

Consent to participate and publish

All authors participated and approved the final manuscript to be published.

Authors contributions

Yinjie Zhang: Writing-Original draft preparation, Conceptualization, Methodology
Cheng Peng: Data curation, Investigation
Wei Zhang: Supervision, Validation
Siyuan Ling: Software, Validation
Shuangqing Hu: Visualization, Supervision

Funding

This study was supported by the Shanghai Agriculture Applied Technology Development Program, China (20190307). Sample analysis, sample collection and interpretation of data were also funded by China (20190307).

Conflict of interest

The authors declare that they have no conflict of interest.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding
author on reasonable request. // All data generated or analyzed during this study are included in this published article.

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**Table Captions:**

Table 1. Acute toxicity LC$_{50}$ of NP to zebrafish.

Table 2. Toxicokinetic parameters and BCF obtained from accumulation and elimination exposed to different concentration of NP.
Table 1. Acute toxicity LC$_{50}$ of NP to zebrafish.

| Parameters                  | Exposure time (h) |
|----------------------------|-------------------|
|                            | 24                | 48                | 72                | 96                |
| LC$_{50}$ (µg·L$^{-1}$)    | 474               | 299               | 250               | 238               |
| R$^2$                      | 0.83              | 0.85              | 0.79              | 0.81              |
| 95% Confidence intervals (µg·L$^{-1}$) | 157 - 791 | 109 - 480 | 77 - 423 | 75 - 401 |
Table 2. Toxicokinetic parameters and BCF obtained from accumulation and elimination exposed to different concentration of NP.

| Fish part                  | Exposure concentration (μg·L⁻¹) | K₁ (L·kg⁻¹·d⁻¹) | K₂ (d⁻¹) | LogBCFss | LogBCFk |
|----------------------------|---------------------------------|-----------------|----------|----------|---------|
| Whole fish                 | 0.534                           | 32.66           | 0.412    | 2.02     | 1.90    |
|                            | 1.160                           | 27.75           | 0.283    | 2.05     | 1.99    |
| Trunk (fish except viscera)| 0.741                           | 67.44           | 1.237    | 1.88     | 1.75    |
|                            | 3.057                           | 59.74           | 1.076    | 1.98     | 1.72    |
| Viscera                    | 7.017                           | 60.40           | 0.809    | 2.02     | 1.89    |

BCFss is the bioconcentration factor at a steady-state and BCFk is the kinetic bioconcentration factor (K₁/K₂).
**Figure Captions:**

Figure 1. Mortalities of zebrafish exposed to different concentrations of NP during 96 h.

Figure 2. Concentration (a) and BCF (b) of NP in the whole zebrafish exposed to low and high NP during the uptake and depuration stages.

Figure 3. Curves of logarithm of NP concentration with time in the uptake stage (a) and first-order kinetic fitting curves in the depuration stage (b).

Figure 4. Concentration (a) and BCF (b) of NP in fish trunk under low, medium and high NP exposure during the uptake and depuration stages.

Figure 5. First-order kinetic fitting curves of NP in the trunk during the depuration stage.
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