Ecotoxicology and Environmental Safety 222 (2021) 112528

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Ammonia nitrogen exposure caused structural damages to gill mitochondria of clam *Ruditapes philippinarum*

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ARTICLE INFO

Edited by: Dr. Caterina Faggio

Keywords:
Ammonia nitrogen
Gill mitochondria
Swelling
MtDNA copy
Succinic dehydrogenase
Mitochondrial ultrastructure

ABSTRACT

Ammonia nitrogen has been one of the key pollution indicators along the Chinese coastline for quite a few years. Our previous studies have proved that ammonia nitrogen is harmful for *Ruditapes philippinarum* clam in several aspects. Environmental concentrations of ammonia nitrogen were found to significantly decrease ATP contents and disturb ATP metabolism, in addition to reducing the potential across the mitochondrial membrane in clam gill tissues. Accordingly, mitochondrion is considered as one of the target organelles of ammonia nitrogen toxicity in clams. However, there is a lack of direct evidence to prove it. In order to reveal detail information of ammonia nitrogen toxicity on clam mitochondria and screen the related biomarker to indicate ammonia nitrogen pollution, mitochondrial parameters in gill tissues including swelling, mtDNA copy number and marker enzyme (succinic dehydrogenase, SDH) activity were measured after the clams were exposed to 0.1 mg/L and 0.5 mg/L ammonia nitrogen for 3 days and 21 days, respectively. Moreover, adverse effects of ammonia nitrogen exposure on clam mitochondrial ultra-structures, mitochondrial swelling and division were also discriminated under transmission electron microscope (TEM). Final results showed that ammonia nitrogen exposure to both concentrations significantly induced mitochondrial swelling, reduced the number of mitochondria and messed their normal structure, decreased the number of mtDNA copies and down-regulated SDH activity, all in a concentration and duration dependent manner. So, the present study helps us to better understand the structural damage of ammonia nitrogen on mitochondria in clam gill cells and provides fundamental data for ammonia nitrogen control in aquaculture.

1. Introduction

With the high development of modern agriculture and industry, a lot of inorganic pollutants are produced and discharged into the rivers. According to the annual bulletins for Chinese marine environment, inorganic nitrogen has been a kind of long-lasting pollutants around the Chinese coastline for quite a few years. Among the different forms of inorganic nitrogen compounds, ammonia nitrogen is a prominent one because of its high toxicity for the marine animals, especially for the highly intensive fishes, crustaceans and mollusc aquaculture (Armstrong et al., 2012; Keppler, 2007; Maas et al., 2012; Randall and Tsui, 2002). Piles of marine toxicological studies of ammonia nitrogen come from fishes, in which ammonia nitrogen is found to diffuse across the membrane system and cause influxes of excessive Ca^{2+} and K^{+}, resulting in cell apoptosis, dysfunction of nervous system and even death in fishes (Randall and Tsui, 2002; Arafa and Atteia, 2013).

In comparison, toxicological mechanism of ammonia nitrogen is not clear in marine invertebrate yet. In marine bivalve, the related toxicological studies focused on the adverse effects of ammonia nitrogen in cellular and immune responses, cumulative survival rates (EC50 and LC50), as well as energy allocation (Keppler, 2007; Widman et al., 2008; Ferretti, Calesso, 2011; Maas et al., 2012; Wang et al., 2012). Till now, the underlying toxicity mechanism of ammonia nitrogen in marine bivalve hasn't been well clarified and needs more effort to push forward.

Our previous studies have revealed that ammonia nitrogen is detrimental to the marine mollusk *Ruditapes philippinarum*. Environmental concentrations of ammonia nitrogen can cause many deleterious effects to the clams, including decreasing the stability of lysosome, reducing the

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https://doi.org/10.1016/j.ecoenv.2021.112528
Received 9 February 2021; Received in revised form 12 July 2021; Accepted 14 July 2021
Available online 23 July 2021

potential across the mitochondrial membrane in clam gill cells, decreasing ATP contents, disturbing the ATPase activities and ATP-consuming activities such as membrane synthesis, feeding and respiration (Cong et al., 2017, 2019). It is known that mitochondria are the major place for ATP generation by oxidative phosphorylation during TCA cycles and play vital roles in many cellular activities, such as energy production, information processing, signal transduction, muscle metabolism, apoptosis, and calcium metabolism (Díaz-Vegas et al., 2018; Wen, 2018). So, the gill mitochondrion is considered to be one of the toxic target organelle for the ammonia nitrogen exposure on clam *R. philippinarum* (Cong et al., 2019). However, there are some important problems are not clear. Firstly, it is not known which structure inside mitochondria will be affected by ammonia nitrogen? Secondly, whether the structural changes of mitochondria is accompanied by instability of mitochondrial DNA? Thirdly, whether there is typical biomarker to present the structural damage of ammonia nitrogen on clam mitochondria?

It is known that mitochondria are made of two-layered membranes, so that reduced mitochondrial trans-membrane potential suggested that structure integrity of clam mitochondria was probably affected by ammonia nitrogen. Once the mitochondrial membrane system was damaged, ion barriers function of their inner membrane would be damaged and water would influx into mitochondrial matrix, leading to mitochondrial swelling (Javadov et al., 2018). In addition, reactive oxygen from oxidative phosphorylation during TCA cycles would cause damage to other important mitochondrial element, especially mitochondrial DNA (mtDNA), which lacks histone protection and DNA repair mechanism (Quiros et al., 2017). Clam mtDNA is reported to encode 13 proteins (subunits of the oxidative phosphorylation complexes I, III, IV, and V), in addition to 2r RNAs and 22 tRNAs (Ma et al., 2018; Hu et al., 2019; Liu et al., 2020). These genes are essential for mitochondrial function in oxidative phosphorylation (OXPHOS) during ATP synthesis. Recent studies also reveal that variation in mtDNA copy number contributes to the formation of certain cancer (Kopinski et al., 2021). If mtDNA genome is instable, it will not only affect ATP synthesis in mitochondria but also induce cancer to the organisms (Quiros et al., 2017; Girolimetti et al., 2020; Kopinski et al., 2021). Besides, significant changes in ATP contents suggested that ammonia nitrogen might heavily interfere with the activities of related enzymes involved in TCA cycles (Cong et al., 2019). As an important enzymes involved in the TCA cycles, succinic dehydrogenase (SDH) is the only dehydrogenase locating in the inner membrane of mitochondria (IMM) and plays important roles in connection of oxidative phosphorylation and electron transfer. Then SDH is regarded as a marker enzyme to represent mitochondrial function in ATP generation. It has been widely accepted that reduction in SDH activity is the result of mitochondrial destruction (Beal et al., 1993; Fattoretti et al., 2001; Hafez et al., 2021).

Accordingly, in the present study, *R. philippinarum* clams were used as the target animals to receive an ammonia-nitrogen exposure of environmental concentrations for 21 days. Mitochondrial swelling was determined by optical absorbance to find out if the ammonia nitrogen induced changes in mitochondrial volume. Clam gill tissues were made into ultrathin slices and observed under transmission electron microscopy (TEM) to discriminate minute changes in gill mitochondria. Besides, mtDNA copy number was determined to indicate harmful effects of ammonia nitrogen on mtDNA stability. SDH activity was measured to indicate the adverse effect of ammonia nitrogen on the IMM. In general, the present study was aimed to investigate the toxicological effects of ammonia nitrogen on mitochondrial structure in clam gill cells and screen the related biomarker to indicate ammonia nitrogen pollution in marine environment.

2. Materials and methods

2.1. Clams and ammonia nitrogen exposure

R. philippinarum clams (averaging 3.53 ± 0.42 cm in shell length) were collected from Yanda supermarket in Yantai city. Before the exposure experiment, about 600 clams were cultured in tanks with aerated and filtered seawater (18 ~ 19 ℃, pH 8.0, 32 psu), and fed with microalgae mixture of *Isochrysis galbana* and *Chlorella vulgaris Beij*. During the experiment period, the seawater was completely changed twice (before and 2-hours after the feeding) each day. Based on the data from annual bulletins for Chinese marine environment, 0.1 mg/L and 0.5 mg/L of ammonia nitrogen were regarded as environment related and used as the exposure concentrations in the present study. After a two-week acclimation period, the clams were randomly divided into three groups, e.g. the control group (named as Control), 0.1 mg/L ammonia nitrogen exposed group (named as 0.1 mg/L) and 0.5 mg/L ammonia nitrogen exposed group (named as 0.5 mg/L), each with three replicate tanks respectively (N = 65 in each tank). A newly prepared solution (1 mol/L) of high purity NH_4Cl was used as the source of ammonia nitrogen. According to Emerson et al. (1975), the real concentrations of ammonia nitrogen in the exposure environment are affected by the temperature and pH of the seawater. So the addition volume of NH_4Cl solution to the seawater was adjusted based on the values of temperature and pH every day.

During a 21-day exposure period, the gill tissues were collected after the clams were exposed to ammonia nitrogen for 1 day, 3 days, 7 days and 21 days in the control, 0.1 mg/L and 0.5 mg/L groups for further investigation. In detail, gill tissues at the 3rd and 21th day were collected (N = 5) and divided into two parts, with one part to isolate mitochondria to detect mitochondrial swelling and the other part to observe the ultrastructure of mitochondria under transmission electron microscopy (TEM). At the same time, gill tissues (N = 5) at the 1st, 3rd, 7th and 21th day were divided into two parts, with one part to detect the variation of mtDNA copy number and the other part to test activities of SDH in TCA cycles.

2.2. Mitochondrial isolation and swelling determination

Mitochondria were isolated from gill tissues according to the protocol of Mitochondrial Extraction Kit (Solarbio, China). Briefly, 200 mg of gill tissues were washed in sterilized seawater and then cut into small pieces. After addition of iced lysis buffer, the tissue mixture was homogenized 20 times by glass grinder, and low-speed differentially centrifuged at 800 g for 5 min, 4 °C to remove nucleus, membrane fragments and intact cells. The supernatant was transferred to another tube and concentrated at 12,000 g for 10 min, 4 °C to collect the gill mitochondria. Mitochondria pellets were suspended in store buffer and the corresponding protein concentration was determined by using Bradford method, with BSA as the standard.

Absorbance changes of gill mitochondria at 540 nm were monitored according to Catanzaro et al. (2009) and Huang et al. (2018) with a little modification. Briefly, 200 μL of mitochondria were added softly in respiration buffer (210 mM mannitol, 70 mM sucrose, 5 mM succinate, 10 μM EGTA, 1 μM rotenone, and 10 mM Hepps/KOH buffer, pH 7.4). The mixture was incubated at 25 °C for 10 min, then the absorbance was monitored at 540 nm every 30 s for 10 min, by using a multiskan spectrum microplate spectrophotometer (Infinite M200, TECAN). Final results of mitochondrial swelling were expressed as the maximum value of ΔA_{540} per min and per mg protein.

2.3. TEM observation of mitochondrial ultrastructure

In order to detect the adverse effects of different duration of ammonia nitrogen exposure on the ultrastructure of mitochondria, gill tissues were collected in the control, 0.1 mg/L and 0.5 mg/L groups at the 3rd day and 21th day. To avoid potential disturbance of long-term breeding under laboratory condition on the normal structure of mitochondria, the control group at the 3rd day was used as the control in TEM observation. Fresh gill tissues were cut into about 1 mm^3 by sterilized scissors and prepared into ultrathin slices. In detail, fresh gill tissues were firstly double fixed with 2.5% glutaraldehyde for over 4 h and 1% OsO_4 for 1.5 h respectively. After each fixation, the tissues were rinsed three times in 0.1 M phosphate buffer saline (PBS, pH 7.0), 15 min each time. Secondly, the tissues were dehydrated by a graded series of acetone (50%, 70%, 90%, 100% acetone) for 15 min each step, and then dehydrated by 100% acetone twice. Thirdly, the gill tissues were embedded in 2:1 mixture of 100% acetone and Epon812 resin at room temperature for 0.5 h, in 1:2 mixture of 100% acetone and resin at 37 °C for 1.5 h, then in 100% resin at 37 °C for 3 h. Fourthly, the embedded gill tissues were hardened gradually at 37 °C, 45 °C, 60 °C, each for 24 h respectively. Then the gill tissues were ultrathin sectioned by ultratome (Reichert-Jung ULTRACUT E, Austria) into slices of 70 nm thick. After the slices were stained by uranyl acetate and alkaline lead citrate for 15 min respectively, they were observed under a high-resolution TEM (JEM1200, Japan).

In order to well elucidate the adverse effects of ammonia nitrogen on mitochondria numbers, a histogram about the relative frequencies of mitochondria number per cell was constructed in Excel based on the result of TEM observation, using the method described by Grech (2018) with slight modifications. Considering the deteriorated mitochondria in the stressed groups (especially in the 0.5 mg/L group) at the 21th day, 15 gill cells were used to construct the histogram for each group to keep the data uniform at each time point. The relative frequency (frequency/numbers of total sample) was used instead of frequency on the Y-axis to better indicate the variation profile of mitochondria number.

2.4. Detection of mtDNA copy number

DNA was isolated from gill tissue according to the manual protocol of TIANamp Marine Animals DNA Kit (TianGen Biotech, Beijing). Specific mitochondrial gene (COX I) and nuclear gene (β-Actin) were used to determine mtDNA copy in gill tissues by using SYBR Green real-time PCR (Niemann et al., 2017). The PCR amplifications were carried out in an ABI 7500 Real-Time Detection System (Applied Biosystems) as described previously (Cong et al., 2018). The PCR program was 50 °C for 2 min and 95 °C for 5 min, followed by 40 cycles of 15 s at 94 °C, 30 s at 60 °C. Final data were expressed as mean ± S.D. (N = 5) and analyzed by one-way analysis of variance followed by least significant difference analysis using SPSS19.0.

2.5. Measurement of succinic dehydrogenase activity

Succinic dehydrogenase (SDH, EC1.3.5.1) is an important marker enzyme in TCA cycle. It catalyzes succinic acid into fumaric acid and hydrogen, which is transferred by phenazine dimethyl sulfate (PMS) to 2, 6-dichlorophenol indophenol (DCPIP). DCPIP has a characteristic absorption peak at 600 nm. So the activity of SDH can be presented as the reducing rate of DCPIP through the absorbance changes at 600 nm. In the present study, 0.1 g of gill tissue was homogenized and centrifuged at 11,000 g for 10 min, 4 °C. The supernatant was collected to detect the activities of SDH according to the protocol of SDH Test Kit (Solarbio, China). Similarly, the protein concentration for each sample was also determined by BSA method. SDH activity was expressed as mean ± S.D. (U/mg protein) and subjected to one-way analysis of variance (one-way ANOVA) followed by least significant difference (LSD) analysis using SPSS 19.0 software.

3. Results

3.1. Mitochondrial swelling

After a 3-day exposure, mitochondria exhibited slight swelling, but the differences were not significant in the two ammonia nitrogen-exposed groups compared to the control. As the exposure duration extended, extremely significant increments were detected in the 0.1 mg/L and 0.5 mg/L groups compared with that of the control group ($P < 0.001$, $P < 0.001$ respectively) at the 21th day (Fig. 1).

3.2. TEM observation of mitochondrial ultrastructure

TEM observation on mitochondrial ultrastructure were shown in Fig. 2–Fig. 4. In the control group, many mitochondria with round or oval shapes distributed around the gill cell (Fig. 2A, 12,000×). Most of the mitochondria had clear and intact mitochondrial membrane (Fig. 2B, 20,000 ×). Insides their membrane, many long and homogenous cristae were arranged orderly and densely. One secondary lysosome was found among the mitochondria.

In the 0.1 mg/L group, the amount of mitochondria was obviously decreased when the clams were exposed for 3 days (Fig. 3A, 40,000×). So did the number of cristae in most of the mitochondria. Some of the cristae had indistinct or broken shapes. In some mitochondria, the remaining cristae with normal shapes gathered and arranged uniformly at one part of the mitochondrion, leaving the other part of mitochondrion empty (Fig. 3A). In addition, divided mitochondria and phagosome appeared. In the 0.5 mg/L group (Fig. 3B), more auto-phagosomes and lysosomes appeared in the cell after ammonia nitrogen exposure for 3 days. Some of the mitochondria had elongated shapes. The mitochondria could be discerned by their shapes, but with a few less cristae than those of the control and 0.1 mg/L groups. Most of the cristae were broken or dissolved (Fig. 3B, 40,000×). In each mitochondria of the 0.5 mg/L group, there was only 2–4 cristae in normal shapes.

After a 21-day exposure, there were quite a few of mitochondria in the gill cells of the 0.1 mg/L group. But many of the mitochondria exhibited anamorphic shapes, such as swollen, elongated, and other shapes. A swollen mitochondrion might be 5–10-times larger than the normal ones (Fig. 4A, 40,000×). The cristae became shortened, swollen, dissolved, and fragmented. They were in a disordered manner in each mitochondrion. A mitochondrion could divide into four small ones (Fig. 4A). In the 0.5 mg/L group, the number of mitochondria was much smaller. Most of the remaining mitochondria were in round shapes, with more phagosomes and autophagosomes around (Fig. 4B, 40,000 ×). The mitochondria in abnormal shapes deteriorated further with fewer cristae relic insides (Fig. 4D, 40,000 ×). The double membranes dissolved in

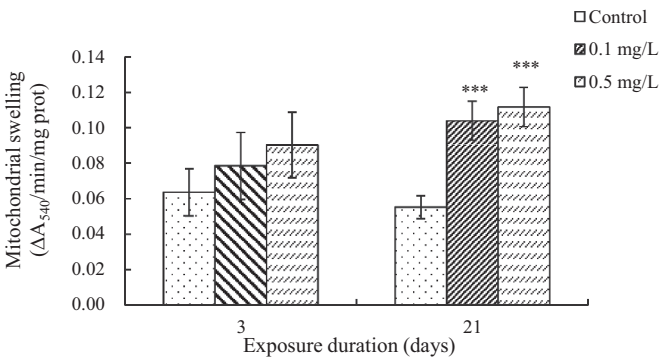

Fig. 1. Mitochondrial swelling in the gill cells after ammonia nitrogen exposure. Notes: (1) Control, clams in the control group; 0.1 mg/L, clams exposed to 0.1 mg/L NH_3-N; 0.5 mg/L, clams exposed to 0.5 mg/L NH_3-N. (2) * ** Denotes statistically extremely significant differences ($P < 0.001$) between the marked group and the control group.

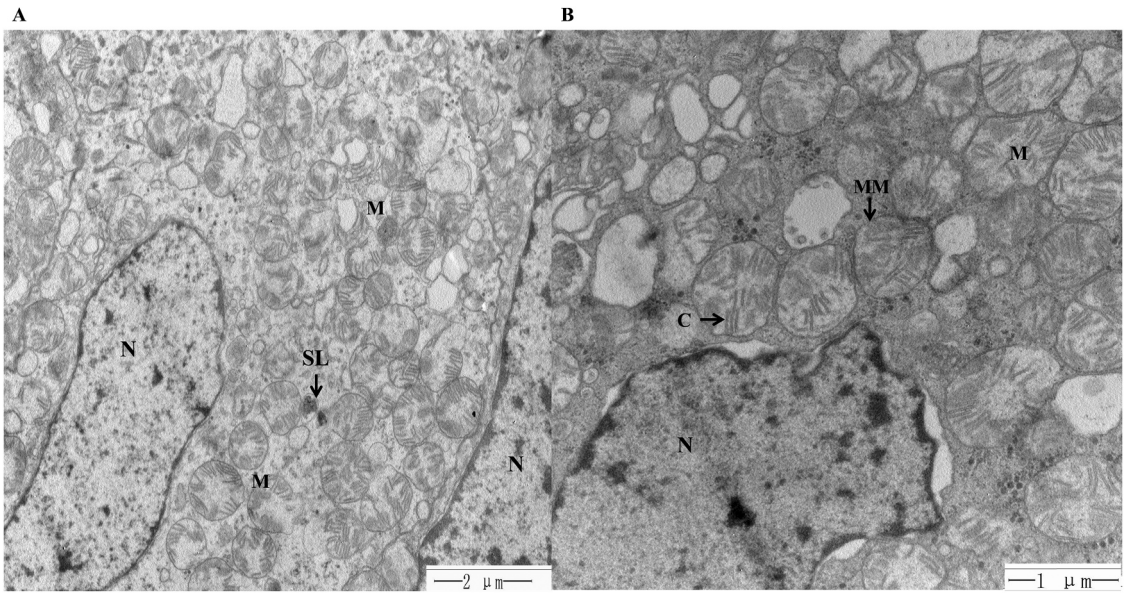

Fig. 2. Ultrastructure of mitochondria in gill cells of clams in the control group at the 3rd day. Notes: A. Control group (12,000 ×); B. Control group (20,000 ×). Abbreviations: C, Cristae; M, Mitochondria; MM, Mitochondrial membrane; N, nucleus; SL, secondary lysosome.

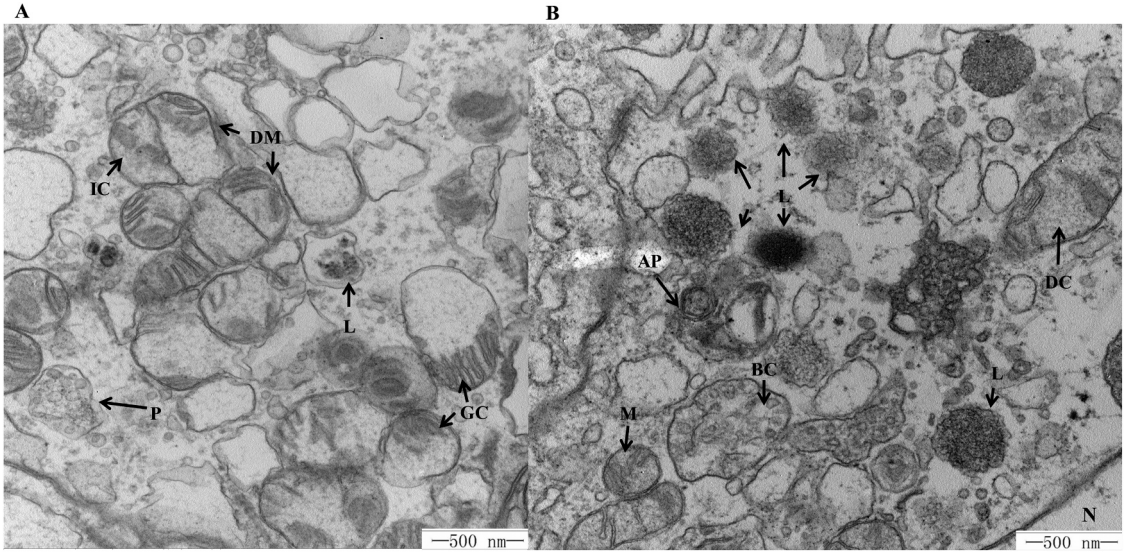

Fig. 3. Ultrastructure of mitochondria in gill cells of clams in the 0.1 mg/L group and 0.5 mg/L group at the 3rd day. Notes: A. 0.1 mg/L group (40,000 ×); B. 0.5 mg/L group (40,000 ×). Abbreviations: AP, autophagosome; BC, broken cristae; DC, dissolved cristae; DM, divided mitochondria; GC, gathered cristae; IC, indistinct cristae; L, lysosome; M, mitochondrion; N, nucleus; P, phagosome.

some of the mitochondria. On the other hand, some mitochondria with discerned membrane systems had no cristae insides. The whole gill tissue seemed in chaos.

Histogram of mitochondria numbers per cell (Fig. 5) indicated that mitochondria number in the control group distributed in a wide scope of 11 ~ 55 per cell. About 50% of gill cells had 11–19 mitochondria and over 20 mitochondria could be found in the rest of gill cells. However, ammonia nitrogen exposure extremely decreased mitochondria numbers in both of the stressed groups ($P < 0.001$) compared with the control at either the 3rd day or the 21th day. In the stressed group, gill cells with 1 ~ 10 mitochondria accounted for over 60% except that in the 0.1 mg/L groups at the 21th day, which might be a consequent of mitochondria division. No cell had more than 20 mitochondria in the stressed groups. In addition, there were gill cells with only one mitochondrion in the 0.5 mg/L group at the 21th day.

3.3. Effects of ammonia nitrogen exposure on mtDNA copy

The relative copy numbers of COX 1 gene (Fig. 6) were slightly decreased after the clams were exposed to either 0.1 mg/L or 0.5 mg/L of ammonia nitrogen for 1 day ($P > 0.05$). At the 3rd day, slightly increment was observed in the 0.1 mg/L group to a normal level ($P > 0.05$), but significant decrement appeared in the 0.5 mg/L group ($P < 0.05$). When the clams were exposed to the ammonia nitrogen for 7 days, significantly decrements were observed in both of the 0.1 mg/L and 0.5 mg/L groups ($P < 0.01$, $P < 0.05$ respectively). However, no significant difference was detected in either of the stressed group at the 21th day.

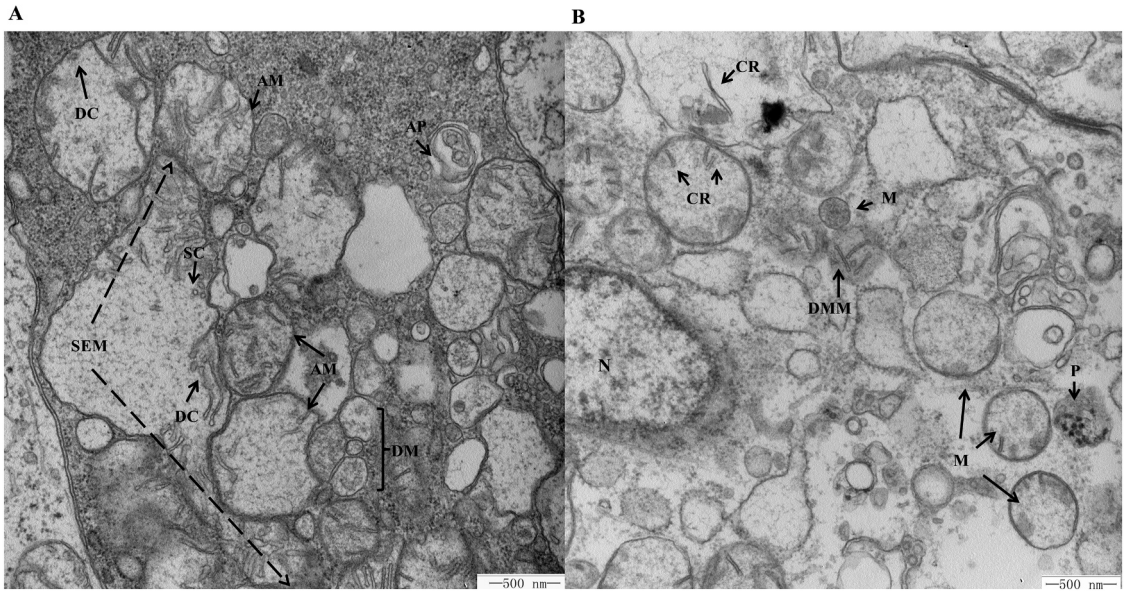

Fig. 4. Ultrastructure of mitochondria in gill cells of clams in the 0.1 mg/L group and 0.5 mg/L group at the 21th day. Notes: A. 0.1 mg/L group (40,000 ×); B. 0.5 mg/L group (40,000 ×). Abbreviations: AM, anamorphic mitochondrion; AP, autophagosome; CR, cristae relic; DC, dissolved cristae; DM, divided mitochondrion; DMM, dissolved mitochondrial membrane; M, mitochondrion; N, nucleus; P, phagosome; SC, shortened cristae; SEM, swollen and elongated mitochondrion.

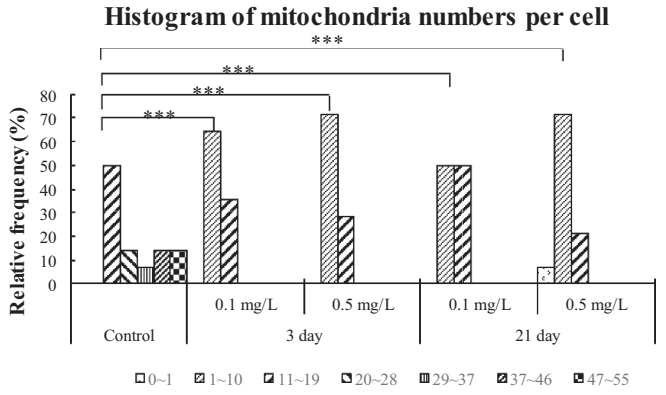

Fig. 5. Relative frequencies of mitochondria per cell in different groups. Notes: Mitochondria numbers per cell have been divided into 7 regions, e.g. 0–1, 1–10, 11–19, 20–28, 29–28, 29–37, 38–46, 47–55. Significant differences between different groups were marked as * ** above the columns ($P < 0.001$).

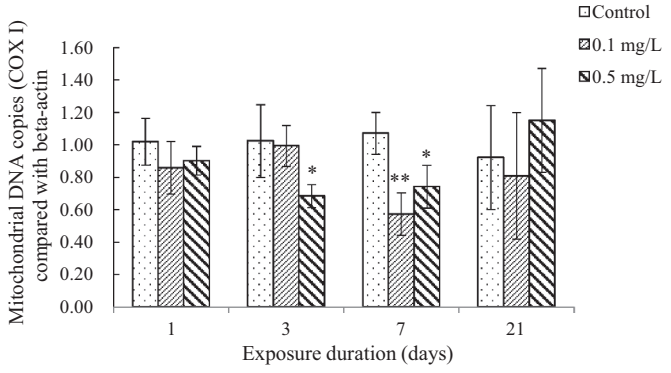

Fig. 6. Relative mtDNA copies compared with nuclear gene. Notes: (1) Control, clams in the control group; 0.1 mg/L, clams exposed to 0.1 mg/L NH_3-N; 0.5 mg/L, clams exposed to 0.5 mg/L NH_3-N. (2) * Denotes statistically significant differences ($P < 0.05$) between the marked group and the control group. * * Denotes statistically extremely significant differences ($P < 0.01$) between the marked group and the control group.

3.4. Activities of succinic dehydrogenase

According to Fig. 7, succinic dehydrogenase (SDH) activities in the 0.5 mg/L group were significantly decreased at the 1st day ($P < 0.05$), 3rd day ($P < 0.05$), 7th day ($P < 0.01$), and 21th day ($P < 0.001$). In addition, SDH activities in the 0.1 mg/L group exhibited significantly decrements at the 7th day ($P < 0.05$), and 21th day ($P < 0.01$).

4. Discussion

Massive and intensive human activities bring continuous and heavy pollution to the marine environment. Ammonia nitrogen is one of the prominent pollutants to the marine organisms. Previous toxicological studies have found that ammonia nitrogen can significantly decrease ATP contents in gill tissue of *Ruditapes philippinarum* clam (Cong et al., 2017). It is known that mitochondria are the powerhouse for the cell to produce ATP through oxidative phosphorylation (Rolfe et al., 1994). Significant decrement of ATP suggested that clam mitochondria were probably to be affected by ammonia nitrogen exposure. It suggested that mitochondrion was one of the toxicological targets of ammonia nitrogen on clam. As our research further advanced, mitochondrial trans-membrane potentials of clam gill cells were found significantly decreased after ammonia nitrogen exposure (Cong et al., 2019). Based on these results, it can be speculated that the two-layered membrane system of mitochondria would be destroyed and its function in ATP generation would be affected by ammonia nitrogen in gill cells.

The integrity of two-layered membrane system of mitochondria is their basic structure for their normal functions, such as ATP generation, ion homeostasis, lipid oxidation, synthesis and redox signaling (Javadov et al., 2018). According to Javadov (2018), mitochondrial swelling begins with osmotic imbalance from ion homeostasis of the matrix, such as K^+, H^+ and Ca^{2+} influx/efflux across the inner mitochondrial membrane (IMM) through permeability transition pores (PTPs). Mild mitochondrial swelling may be a normal physiological regulation for the mitochondria to maintain the normal function (Kaasik et al., 2007). But excessive swelling can induce apoptosis or necrosis depending on the availability of ATP (Javadov et al., 2018). In the present study, a 3-day exposure of

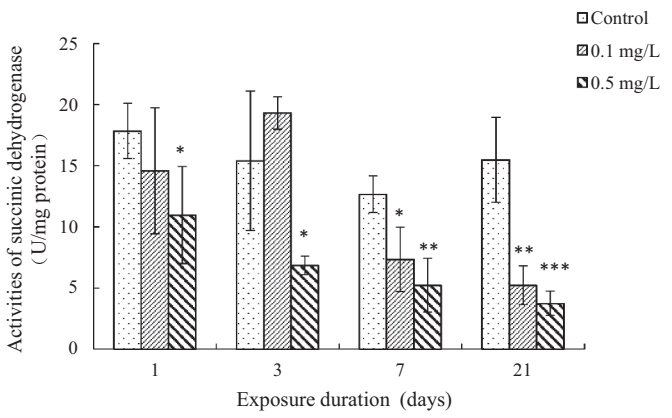

Fig. 7. Succinic dehydrogenase activities in gill cells after ammonia nitrogen exposure. Notes:* Denotes statistically significant differences ($P < 0.05$) between the marked group and the control group. * * Denotes statistically extremely significant differences ($P < 0.01$) between the marked group and the control group. * ** Denotes statistically extremely significant differences ($P < 0.001$) between the marked group and the control group.

ammonia nitrogen slightly increased mitochondrial swelling with no significant difference among the three groups. It probably meant that mitochondria were under a normal physiological range after ammonia nitrogen exposure of 0.1 ~ 0.5 mg/L for 3 days. But mitochondrial swelling was significantly increased in both 0.1 mg/L and 0.5 mg/L groups after 21 days, which suggested that mitochondria in the gill cells were in extremely swelling state and their structural integrities especially their inner membrane were probably destroyed.

Changes of mitochondrial morphology observed by transmission electron microscopy can provide direct clues for the above deduction. In the control group, there were a lot of mitochondria in the gill cells with round or oval shapes, clear and intact mitochondrial membrane, and many long and distinct cristae. However, in the 0.1 mg/L group at the 3rd day, the number of mitochondria was obviously decreased. But there was an interesting phenomenon that although there were some cristae with indistinct or broken shape, the remained cristae with normal shape gathered in mitochondrion. In addition, some of the mitochondria were divided into two ones. These phenomena suggested that the mitochondria were in remolding state to regulate their structure, shape and number. According to Kageyama et al. (2011), such kind of dynamic remodeling is a key mechanism for the mitochondria to maintain their structural integrity, distribution and functional competence in response to physiological or environmental changes. But in the 0.5 mg/L group, some of the mitochondria became elongated, with most of the cristae broken or dissolved at the 3rd day. In addition, quite a few of large lysosomes and auto-phagosomes appeared. Recent studies have revealed that lysosome plays prerequisite roles in necrosis execution (Reviewed by Alu et al., 2020). Accordingly, it can be concluded that there is massive necrosis in gill tissue of the 0.5 mg/L group after a 3-day exposure.

Longer duration of ammonia nitrogen exposure induced more serious results to the clam gill tissues. At the 21th day, mitochondria with anamorphic shapes in the 0.1 mg/L group suggested that mitochondria were still in a dynamic regulation state to combat against the external stress from ammonia nitrogen exposure. But extensive increment of mitochondrial volume revealed that mitochondria were in extremely swelling, which was in accordance with the result of the significant increment of mitochondrial swelling at the 21th day. It meant that ion homeostasis across the inner mitochondrial membrane was out of balance, and more water influx into the matrix. Although some mitochondria still remained in the gill cells, but majority of the cristae were shortened, swollen, dissolved, and fragmented. It would compromise most of the mitochondrial functions, especially the synthesis of ATP. In the 0.5 mg/L group, the gill state was in the worst condition. Mitochondria without membrane or without cristae in the gill cells indicated that the membrane system of the cell was heavily destroyed by ammonia nitrogen exposure, not to mention their normal functions in ATP generation, electron transportation. More phagosomes and auto-phagosomes implied that the gill cells were in necrosis to a greater extent.

Detection on expression levels of mitochondrial specific genes revealed that exposure of ammonia nitrogen could significantly induce mtDNA damage by decreasing the amount of mtDNA copies. For the clams in 0.1 mg/L group, slight decrement of mtDNA copies was observed at the 1st day and slight increment was observed at the 3rd day. Its variation profile was in accordance with the observation of mitochondrial division under transmission electron microscope. The result suggested that 1–3 days' exposure of 0.1 mg/L did harm to the mitochondrion but clams were active to remold mitochondrion by division to combat against ammonia nitrogen exposure. However, extended exposure duration of 0.1 mg/L ammonia nitrogen to 7 days induced more genetic damage by decreasing mtDNA copies significantly. In addition, higher concentration of ammonia nitrogen caused more harmful damage to mitochondrial DNA. For the clams in the 0.5 mg/L group, significant decrements in mtDNA copies were detected at the 3rd and 7th day. At the 21th day, mtDNA copies recovered to normal levels in the 0.1 mg/L group and 0.5 mg/L group compared with the control. It suggested that mtDNA copies increased gradually from the 7th day to the 21th day in the stressed groups. Since mtDNAs were isolated from the gill tissues, not only mtDNAs in intact mitochondria but also cell-free mtDNA were supposed to be isolated. In the present study, cell-free mtDNA might contribute to the increased mtDNA copies because the membrane system of gill cell was almost destroyed according to the result of TEM observation and more mtDNAs would be released from gill mitochondria after a 21-days ammonia nitrogen exposure. A newly report suggests that increased level of cell-free mtDNA is a marker of cellular stress to present mitochondria damage (Gonçalves et al., 2021). Accordingly, the recovery of mitochondrial copies might suggest that more mitochondria were damaged by ammonia nitrogen exposure. Further, large standard deviations among the samples suggested sub-chronic exposure of ammonia nitrogen induced different changes to mtDNA copies in different individual clam within the same group, so that more than five samples should be collected to mitigate such deviations in the future study.

As a constitutive element of the mitochondrial respiratory chain, succinic dehydrogenase is one of the important enzymes involved in Krebs Cycles. Given that SDH is located at the IMM, its activity has been regarded to reflect the physico-chemical condition of mitochondrion (Gutman, 1978; Fattoretti et al., 2001). In the present study, slight variations of SDH activities in the 0.1 mg/L group with no significant difference to the control group suggested that gill mitochondria were adjusted to adapt to ammonia nitrogen exposure from the 1st day to the 3rd day. Significant decrement of SDH activities in the 0.1 mg/L group the 7th day to the 21th day suggested that longer exposure of 0.1 mg/L ammonia nitrogen brought more toxic effects to the inner membrane of the gill mitochondria. On the contrary, significant decrements of SDH activities were detected in the 0.5 mg/L group from the beginning to the end during the exposure. It indicated that 0.5 mg/L of ammonia nitrogen was toxic enough for the clam gill mitochondria to lose their normal physical and chemical condition of their inner membrane. The variation profiles of SDH activities were in accordance with the changing profiles of mtDNA copy and TEM observation. Thus, SDH can be used as a potential marker enzyme to indicate toxicity of ammonia nitrogen on clam gill mitochondria.

5. Conclusion

Environmental concentrations of ammonia nitrogen induced toxic effects to the clam gill mitochondria by inducing mitochondrial swelling, reducing the number of mitochondria and messing their normal structure, decreasing the number of mtDNA copies and down-

regulating SDH activity, all in a concentration and duration dependent manner. Gill mitochondria remolded their structure, shapes and numbers in the 0.1 mg/L group after acute (3 days) ammonia nitrogen-exposure. However, acute exposure (3 days) of 0.5 mg/L ammonia nitrogen induced necrosis phenomena in clam gill tissue. Sub-chronic exposure (21 days) of 0.1 mg/L ammonia nitrogen caused extremely swelling in clam gill mitochondria, while exposure of 0.5 mg/L ammonia nitrogen drastically destroyed the mitochondrial membrane and cristae. These significant changes suggested that ammonia nitrogen could significantly disrupt the mitochondrial structure of the clam *R. philippinarum*, which would compromise most of the clam mitochondrial functions such as ATP generation, ion homeostasis, lipid oxidation, synthesis and redox signaling. Furthermore, SDH can be used as a potential biomarker to indicate the structural damage of ammonia nitrogen on clam gill mitochondria.

Ethics approval and consent to participate

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Funding

This study was funded by Natural Science Foundation of China (NSFC 41876135).

CRediT authorship contribution statement

MC supervised the whole experiment, edited and finalized the manuscript; YL, HX, YZ did the experiment; JL and HW took part in supervising the experiment.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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