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Effects of acute ammonia exposure and post-exposure recovery on nonspecific immunity in Clam *Cyclina sinensis*

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Abstract

This study aimed to assess the toxicity of ammonia on clam *Cyclina sinensis* and the post-exposure recovery. With increased exposure to TAN, the alkaline phosphatase (AKP) activities after exposure showed a trend of growing initially and subsequently decreasing, whereas the AKP activities after post-exposure recovery showed an increasing trend. The AKP activities after post-exposure recovery were significantly higher than those in control. The acid phosphatase (ACP) activities in T1 and T2 after post-exposure recovery were higher than those in the control, whereas the ACP activities in T3, T4, and T5 after post-exposure recovery were significantly higher than those in the control. The lysozyme (LZM) activities in T1 and T2 after exposure were significantly higher than those in control, whereas the LZM activities in T3, T4, and T5 after exposure were significantly lower than those in the control. Overall, ACP and LZM in the clams exposed to a low level of TAN (≤ 40 mg/L) can recover to the normal levels completely. However, a 48h recovery period scarcely seems adequate to compensate for AKP, ACP, and LZM activities in the clams exposed to a high level of TAN (> 40 mg/L).

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Introduction

It is well-known that ammonia is one of the most severe environmental pollutants in aquaculture systems (Ge et al., 2021a). Ammonia usually exists in two chemical forms: ionized ammonium (NH_4^+) and unionized ammonia (NH_3). As NH3 is of strong fat solubility, it can damage gills and other tissues of marine animals to varying extents (Foss et al., 2009). Studies have demonstrated that ammonia accumulation usually has a negative effect on marine animals (Florence et al., 2015; Gao et al., 2016). In severe cases, the mortality of cultured marine animals can increase sharply (Wang et al., 2012). Therefore, ammonia has been identified as one of the most critical limiting factors for marine animals (Ge et al., 2021b).

There is growing evidence that environmental pollutants can cause immune system disorder in marine animals (Ge et al., 2021b; Sreekakula et al., 2019). Diverse studies have indicated that alkaline phosphatase (AKP), acid phosphatase (ACP), and lysozyme (LZM) play key functions in the nonspecific immune system (Chen et al., 2019; Liu et al., 2004; Yuan et al., 2020). As important phosphatase enzymes, ACP and AKP can resist pathogens by releasing attached phosphoryl groups from pathogenic bacteria and storing them in lysosomes (Long et al., 2021; Xu et al., 2020). LZM is one of the most important regulators of innate immune responses, which can attack the peptidoglycan layer of the bacterial cells (Bayarri et al., 2014). AKP, ACP, and LZM, which are involved in various metabolic processes, have been determined to be indicators to reveal the stress responses of marine animals to the growth environment (Chen et al., 2019; Xu et al., 2020). Effects of ammonia on immune responses of marine animals have drawn a lot of attention recently because immunity variations under environmental stresses are bound up with the occurrence and development of fish diseases (Ge et al., 2021a; Zhang et al., 2019). However, studies on the acute toxicity of ammonia on nonspecific immunity in mollusk are still essential to reveal the sensitivity to this relevant contaminant.

The clam *Cyclina sinensis* is an economically important marine clam (Ni et al., 2020). And the clam distributes widely in coastal areas of East Asia. Because of its rapid growth rate, delicious taste, and resistance to diseases, clam farming contributes a lot to sustainable clam industry development in China (Ni et al., 2021). However, environmental stresses can inhibit mollusks' physiological and immune systems, which can severely influence marine animals (Ching et al., 2009). Previous studies have revealed that toxic ammonia affects the physiological reactions in marine animals (Gao et al., 2016; Yang et al., 2010). Nevertheless, the actual toxic threshold varies greatly in different marine animals (Foss et al., 2009). Furthermore, whether the damage caused by ammonia exposure can recover to the original level remains unclear (Zhang et al., 2019).

Generally, the first toxicological evaluation of a specific combination "organism + toxicant" is to determine the average lethal concentration levels (LC50) (Zhang et al., 2019). It is well accepted that LC50 value can be calculated by acute toxicity experiments of short duration, typically 48 to 96 h (Acar et al., 2018). However, traditional toxicological researches mainly concentrate on the dose-effect relationship between the pollutants and organisms at the given exposure time (Lia et al., 2014). How the poisonous effect of pollutants varies after exposure is often ignored (Chen and Guo, 2015). Therefore, the toxic effect during the post-exposure period should also be taken into consideration to evaluate the toxic effect of pollutants on organisms comprehensively. Within this context, firstly, we determined the median lethal concentrations (LC50) and safe concentration (SC) of TAN and NH3 for *C. sinensis*. And then, we assess the effect of acute ammonia exposure and post-exposure recovery on nonspecific immunity in *C. sinensis*. The results may help to reveal the underlying relationship between immune response and ammonia toxicity.

Materials and Methods

Clam source, nursery and feeds

Healthy clam *C. sinensis* (average body weight 3.38± 0.21g) were obtained from Lianyungang Zhongchuang Aquaculture Company. Test clam *C. sinensis* were acclimated

in polyvinyl chloride tanks (45cm× 30cm× 40cm) containing 30L well-aerated sand-filtered seawater (temperature: 24°C, salinity: 21ppt, pH: 8.0, dissolved oxygen: 5.3 mg/L, and TAN<0.01 mg/L) for ten days before the experiment. Only healthy *C. sinensis* of uniform size without pathological signs were selected as test subjects. During the adaptation period, test clams were fed twice with microalgae. Feeding was ceased one day before the experiment.

The stock solution of high purity ammonium chloride (NH_4Cl) (10g/L) was diluted to the desired concentration of total ammonia (TAN). During the exposure test, the TAN level was measured every 12h with a spectrophotometer (DR 3800, Hach) followed by Ge et al. (2021). To maintain the level of TAN, 100% of seawater was renewed every 12h, and seawater contained the designed concentration of TAN while guaranteeing the other water quality stabilization. During the exposure test, the pH was monitored every 12h using a portable pH meter (PHB-5, Leica, China), and the level was maintained within the range of the control group (8.0 ± 0.3) with diluted HCl and, or KOH (Egnew et al., 2019).

Experimental design

Preliminary experiment

Preliminary experiments were conducted to determine the LC50 concentration of ammonia for *C. sinensis*. Firstly, using the probit analysis method (Acar et al., 2018; Fossog et al., 2013; Ge et al., 2021b), we evaluated the minimum safe dose (0 lethal concentration, MSD) and maximum lethal dose (100% lethal concentration, MLD) were 75.02 and 375.28 mg/ L, respectively. Then, the desired levels of TAN were set as 75.02, 125.06, 175.1, 225.14, 275.18, 325.22, and 375.28mg/L. Each treatment was conducted in triplicate with a density of 20 individuals per tank. The clam, which cannot close the double shell and cannot respond to stimuli, was defined as a dead clam. The dead clam was removed from the tanks every six h, and record the numbers of dead clams. Finally, the LC_{50} for clam *C. sinensis* and the confidence limit of 95% was calculated with Karber's method (Ge et al., 2021a). The level of NH_3 was calculated with TAN, pH, and temperature of the equation: NH_3=TAN/ (10 (p Ka – pH) +1) (Ge et al., 2021a).

Ammonia-N exposure test

According to the 48-h LC_{50} of TAN for clam C. sinensis we determined above, 360 clams were selected and divided into six groups with 18 tanks (six groups of three replicate tanks, 20 individuals per tank) randomly, and exposed to 0 (control), 20 (T1), 40 (T2), 60 (T3), 80 (T4) and 100 mg (T5) TAN /L, respectively. As marine animals exposed to ammonia for 48h can lead to severe toxic effects (Ge et al. 2021), the clam was exposed to ammonia for 48h. The preliminary experimental results show that some immune parameters in low ammonia nitrogen treatment could return to their normal levels in 48h. Therefore, at the cessation of ammonia exposure, the clams were moved to the control conditions for 48 h (the recovery group). During the test, no feed was supplied.

Hepatopancreas collection and enzyme activity assay

Five individuals per tank were randomly collected after exposure and post-exposure recovery in each group. As the main target organs impaired by ammonia is the hepatopancreas (Ge et al., 2021b), the clam *C. sinensis* were decontaminated with 70% ethanol and then dissected to obtain hepatopancreas tissue immediately with sterile scissors. The enzyme activities were determined using diagnosis kits for AKP, ACP, and LZM according to the protocol recommended by the manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing) according to the protocol recommended by the manufacturer (Ge et al., 2021a).

Statistical analysis

Values were calculated using SPSS 18.0 software. Results were expressed as means ± standard deviation. One-way analysis of variance (ANOVA) followed by Duncan's test was conducted to assess the significant differences among treatments (Ge et al., 2019). $P < 0.05$ was considered to be statistically different.

Results

LC_{50} and SC

Exposed to ammonia, the mortality of *C. sinensis* increased along with the increasing concentration of ammonia（**Table 1)**. The LC_{50} in 24, 48, 72, and 96h of TAN were 181.30, 118.17, 105.03, and 80.72 mg/L, respectively. The LC_{50} in 24, 48, 72, and 96h of NH3 were 9.00, 5.87, 5.22, and 4.01 mg/L, respectively. The 96h safe concentration of TAN and NH_3 for the clams was 8.07 and 0.40, respectively.

Table 1 The LC50 and safe concentration (SC) for clam *C. sinensis*

Time	LC_{50} of TAN (mg/L)	LC_{50} of NH_3 (mg/L)	SC of TAN (mg/L)	SC of NH_3 (mg/L)
24	181.30	9.00	18.13	0.90
48	118.17	5.87	11.82	0.59
72	105.03	5.22	10.50	0.52
96	80.72	4.01	8.07	0.40

Effect of acute ammonia exposure on nonspecific immunity enzyme activities in C. sinensis

The AKP activities in the hepatopancreas tissue of clam *C. sinensis* after exposure showed a trend of increasing firstly and then decreasing along with the increasing concentration of ammonia (**Figure 1**). The AKP activities in the groups of exposure were significantly higher than those in the control (P<0.05). The AKP activities in the recovery groups showed an increasing trend along with the increasing concentration of ammonia, and the AKP activities in the recovery groups were significantly higher than those in the control (P<0.05). The AKP activities in the exposure groups were significantly higher than those in the group of post-exposure recovery (P<0.05).

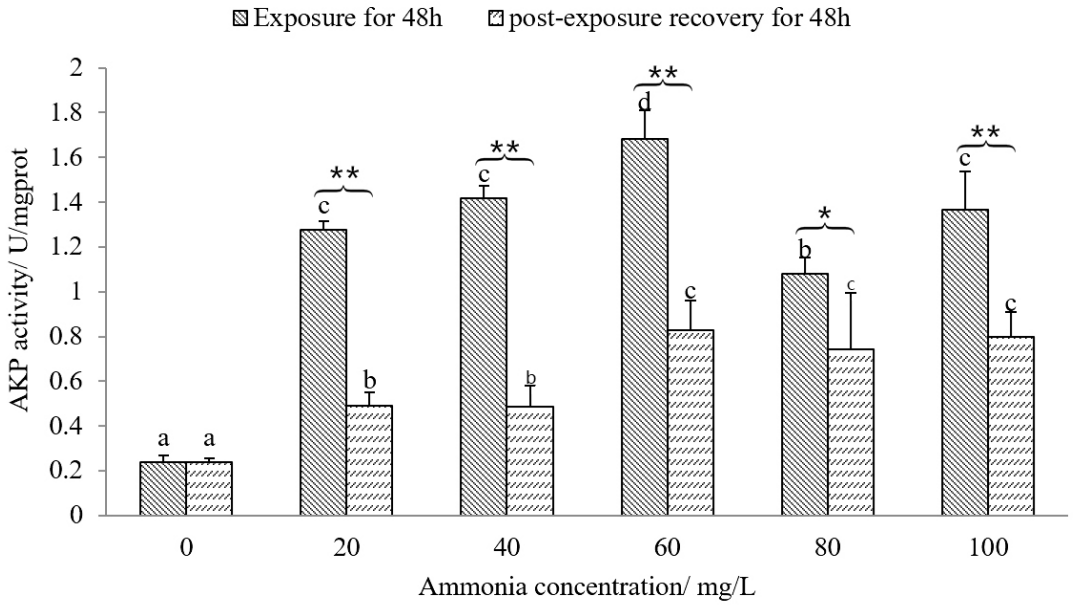

Figure 1 The AKP activity in the hepatopancreas tissue of clam *C. sinensis* after exposed to ammonia. The same lowercase means no significant difference in different exposure concentration of ammonia at the same time (exposure for 48h or post-exposure recovery for 48h), otherwise significant differences (P <0.05). Significant differences in the same concentration of ammonia between exposure for 48h and post-exposure recovery for 48h are indicated by asterisks (* P<0.05, ** P<0.01).

Effect of salinity on IBR of the clams reared in different salinities

The IBR values were calculated using the gill enzyme activities (LZM, NKA, SOD and GPT) (**Table 2**). The IBR values of the clams had a tendency to increase with salinity decreased and they were 11.28, 3.40 and 2.85 in 10‰, 20‰ and 30‰, respectively.

The biomarker star chart for IBR of the clams reared in different salinities (LZM, NKA, SOD and GPT in gills) as is shown in **Figure 3** and the IBR value of the clams under the salinity stress was the area formed by each radius coordinate. In group 10‰, GPT made the biggest contributor to IBR, followed by LZM, NKA and SOD. In group 20‰, LZM made the minimum contributor, followed by SOD and the contributions of GPT and NKA were similar. In group 20‰, GPT made the minimum contributor, and the other three indicators were similar.

As shown in **Figure 2**, the ACP activities after exposure increased significantly along with the increasing concentration of ammonia. The AKP activities in the groups of exposure were significantly higher than those in the control (P<0.05). The ACP activities in the recovery groups increased along with the exposure concentration of ammonia, whereas they were significantly lower than in the groups of exposure (P<0.05). The ACP activities in T1 and T2 after recovery were higher than those in the control (P>0.05), whereas the ACP activities in T3, T4, and T5 after recovery were significantly higher than those in the control (P<0.05).

The LZM activities after exposure showed a trend of increasing firstly and then decreasing along with the increasing concentration of ammonia (**Figure 3**). The LZM activities in T1 and T2 after exposure were significantly higher than those in the control (P<0.05), whereas the LZM activities in T3, T4, and T5 after exposure were significantly lower than those in the control (P<0.05). The LZM activity in T1 after recovery was significantly higher (P<0.05) than that in the control, whereas the LZM activity in T3, T4, and T5 after recovery was significantly lower (P<0.05).

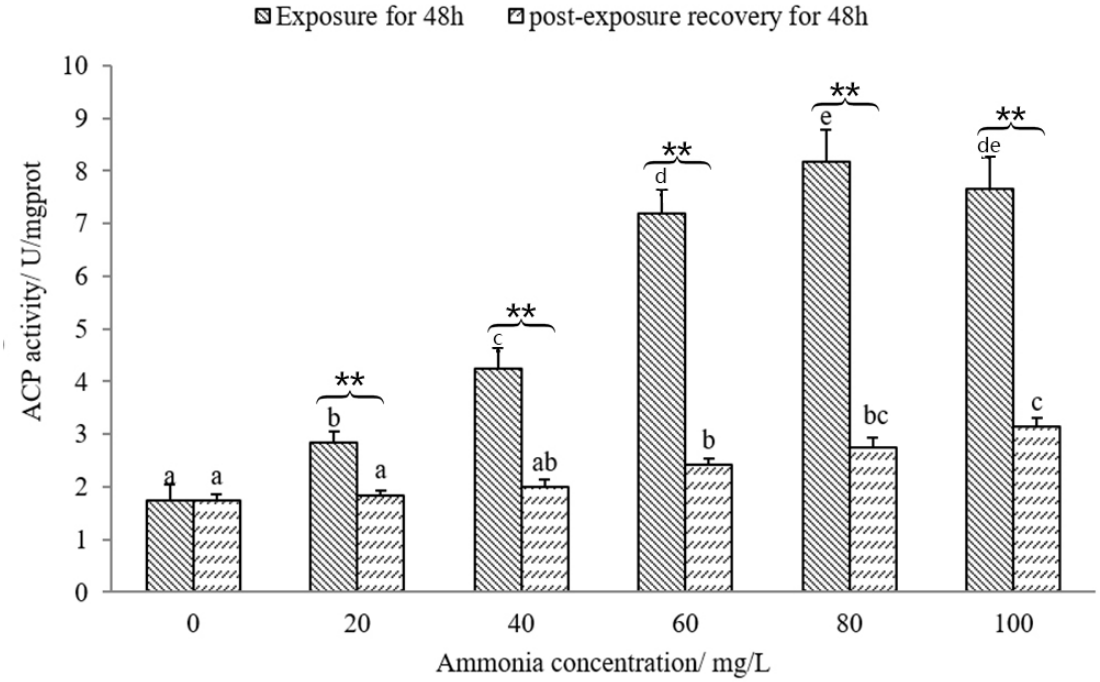

Figure 2 The ACP activity in the hepatopancreas tissue of clam *C. sinensis* after exposed to ammonia.The same lowercase means no significant difference in different exposure concentration of ammonia at the same time (exposure for 48h or post-exposure recovery for 48h), otherwise significant differences (P <0.05). Significant differences in the same concentration of ammonia between exposure for 48h and post-exposure recovery for 48h are indicated by asterisks (* P<0.05, ** P<0.01).

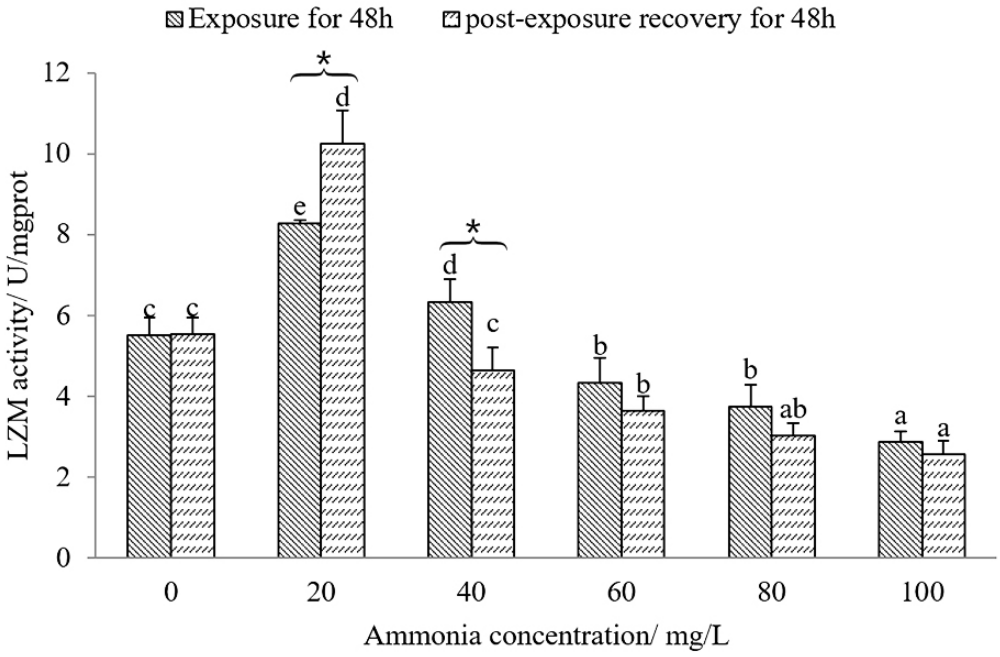

Figure 3 The LZM activity in the hepatopancreas tissue of clam *C. sinensis* after exposed to ammonia. The same lowercase means no significant difference in different exposure concentration of ammonia at the same time (exposure for 48h or post-exposure recovery for 48h), otherwise significant differences (P <0.05). Significant differences in the same concentration of ammonia between exposure for 48h and post-exposure recovery for 48h are indicated by asterisks (* P<0.05, ** P<0.01).

Discussion

Ammonia is harmful to marine animals (Florence et al., 2015; Lu et al., 2016). The toxic effect of ammonia on marine animals has attracted wide attention. Lots of studies have revealed the toxicity of ammonia to mollusks, such as Asian clam *Corbicula fluminea* (Zhang et al., 2019) and clam *Ruditapes philippinarum* (Cong et al., 2019). Ammonia accumulation in water may be a severe threat to marine animals (Ge et al., 2021b). In our present research, the 96h safe concentration of TAN and NH_3 for the clam was 8.07 and 0.40, respectively. They are relatively higher than the SC of other marine animals, such as *Scylla serrate* (Romano and Zeng, 2007) and *Litopenaeus vannamei* (Lu et al., 2016). This is probably because the bivalve mollusks have shells and uniquely efficient mechanisms of detoxification metabolism (Zhang et al., 2019). Generally, the damage aggravated along with the increasing environmental stressor concentration (Ge et al., 2021b). Cong et al. (2017) reported that the clam *R. philippinarum* exposed to the level of 0.5 mg/L ammonia might suffer severe effects, including gill damage and neurotoxicity. James & Diane reported that the LC50-96h for the survival of clam *Spisula solidissima* was 10.6 mg/L TAN and 0.53 mg/L NH_3 (James & Diane, 2011). In the present study, the average lethal concentration levels increased along with the death time reduced. Elevated ammonia in water can accelerate the accumulation of ammonia uptake across the gill epithelium. However, the accumulation of ammonia in organisms usually causes very high ammonia levels in the body fluids, and even leads to death (Sreekakula et al., 2019).

AKP is a lysosomal enzyme and plays an essential role in the nonspecific immune system by catalyzing the hydrolysis of various phosphate-containing compounds in the alkaline environment (Gobi et al., 2016). In the present research, the AKP activities after exposed to ammonia showed a trend of increasing firstly and then decreasing. The result indicates that ammonia exposure can influence AKP activities in *C. sinensis*. Some previous studies have revealed that low levels of potentially environmental toxic pollutants, bacteria, or viruses could cause stimulatory effects on the immune system (Stebbing, 1982; Wai-San

et al., 2011). This phenomenon is so-called "hormesis" (Stebbing, 1982). That's possible because environmental chemicals could inhibit or induce mRNA expression of innate immune-related genes and cytokines and further result in the change of immune enzymes, such as LZM and AKP (Jia et al., 2014; Rogers et al., 2013). In the present research, The AKP activities in the recovery groups showed an increasing trend along with the increasing concentration of ammonia, and the AKP activities in the recovery groups were significantly higher than those in the control. This indicated that AKP activity couldn't recover completely after being transferred to pristine seawater for 48h for those exposed to a high level of ammonia. Compared to the group of exposure, the AKP activity in the group of post-exposure recovery has recovered somewhat. However, it is more difficult for the clam exposed to a high level of ammonia (c > 40mg/L) to resume its original activity than those in a low level of ammonia (c ≤ 40mg/L). This is probably because that the high level of potentially toxic agents caused immune system damage irreparably, or maybe it is because of a 48h recovery insufficient recovery time (Yang et al., 2010).

ACP is one of the marker enzymes of macrophage lysosome in organisms, and ACP plays an essential role by destroying and eliminating foreign bodies in the clam nonspecific immunity (Xia and Wu, 2018). In the present study, the ACP activities in clam *C. sinensis* after exposure increased along with the increasing concentration of ammonia. The result showed that ammonia exposure could cause stimulatory effects on ACP activity. During the same recovery time, the ACP activities in the low level of ammonia (c ≤ 40mg/L) could resume their original level. Whereas the ACP activities in a high level of ammonia (c > 40mg/L) after post-exposure recovery were significantly higher than those in the control. This indicated that ACP activity in the clam which exposed to the lower concentration of ammonia (c ≤ 40mg/L) could recover in 48h. It is possibly because of overcompensation. The occurrence of overcompensation response of exceeding compensation after the organism suffered damaging stress (Xie et al., 2012).

As an important hydrolytic enzyme, LZM could kill bacteria by destroying their cell walls (Bayarri, 2014). Divers studies indicated that ammonia exposure could decrease humoral immune responses of aquatic animals, such as bacteriolytic (Yue et al., 2010). In the present research, the LZM activities after exposure showed a trend of increasing initially and subsequently decreasing along with the increasing concentration of ammonia. It indicated that a low level of ammonia exposure could cause stimulatory effects on LZM activity, whereas a high level of ammonia exposure could cause inhibitory effects on LZM activity. LZM is an essential regulator of innate immune responses, and high-level activity will help to destroy bacterial cells (Jash & Kumar, 2014). Compared to the control, the LZM activity in T1 after post-exposure recovery, indicating that a low level of ammonia stress induces overcompensation (Xie et al., 2012). The LZM activity in T2 was lower, indicating that LZM activity can resume its original level. The LZM activities in groups exposed to the high level of ammonia (c > 40mg/L) after post-exposure recovery were significantly lower than those in the control, indicating that LZM activity in the clam can't recover from ammonia post-exposure recovery in 48h. This is possibly because that the mechanism of LZM synthesis in the clam exposed to a high level (c > 40mg/L) was inhibition, which might even lead to irreversible damage (Xu et al., 2020; Oliveira et al., 2018).

Conclusion

In conclusion, the LC50 of TAN for the clam *C. sinensis* after 24, 48, 72, and 96h were 181.30, 118.17, 105.03, and 80.72 mg/L, respectively. Chronic ammonia exposure for 48h can cause a rise in AKP and ACP activities. However, it causes a reduction in LZM activity. After post-exposure recovery for 48h, activities of ACP and LZM in the clams exposed to a low level of ammonia (≤ 40mg/L) can recover to the normal levels completely, whereas a 48h recovery period scarcely seems adequate to compensate for AKP, ACP, and LZM activities in the clams exposed to a high level of ammonia of TAN (> 40mg/L).

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