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Effect of nitrite on immune response of Taiwan abalone *Haliotis diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus*

Winton Cheng[1], I-Shan Hsiao[2], Jiann-Chu Chen[3,]*

[1]Department of Aquaculture and [2]Institute of Tropical Agriculture, National Pingtung University of Science and Technology, Pingtung, Taiwan, 912, ROC

[3]Department of Aquaculture, College of Life and Resource Sciences, National Taiwan Ocean University, Keelung, Taiwan, 202, ROC

ABSTRACT: Taiwan abalones *Haliotis diversicolor supertexta* held in 30‰ seawater and 26°C were injected with tryptic soy broth (TSB)-grown *Vibrio parahaemolyticus* (1.6×10^5 CFU [colony-forming units] abalone^{-1}), and then placed in water containing different concentrations of nitrite-N (nitrite as nitrogen): 0.01 mg l^{-1} (control), 1.05, 3.04, 5.10 and 10.06 mg l^{-1}. Mortality of the abalones increased in direct parallel to ambient nitrite-N concentration. Over 12 to 48 h, the mortality of *V. parahaemolyticus*-injected abalones held in 3.04 mg l^{-1} nitrite-N was significantly higher than that of abalones in the control solution. Abalones that had been exposed to control, 0.96, 2.95, 5.03 and 10.16 mg l^{-1} nitrite-N for 24, 72 and 120 h were examined for THC (total hemocyte count), phenoloxidase activity, respiratory bursts (release of superoxide anion), phagocytic activity, and clearance efficiency of *V. parahaemolyticus*. The THC increased in abalone after 72 h exposure to 0.96 and 2.95 mg l^{-1} nitrite-N, but decreased in abalones after 24 h exposure to 5.03 and 10.16 mg l^{-1} nitrite-N. Phenoloxidase activity and respiratory bursts increased, while phagocytic activity and clearance efficiency decreased in abalones exposed to ≥0.96 mg l^{-1} nitrite-N for 24 h. It is concluded that nitrite-N in water at concentrations as low as 0.96 mg l^{-1} weakens the immune response and increases mortality of *H. diversicolor supertexta* infected with *V. parahaemolyticus*.

KEY WORDS: *Haliotis diversicolor supertexta* · *Vibrio parahaemolyticus* · Nitrite · Challenge · Phenoloxidase activity · Respiratory burst · Phagocytic activity · Clearance efficiency

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INTRODUCTION

Taiwan abalone *Haliotis diversicolor supertexta* is commercially important in Taiwan as well as on the SE coast of China mainland as a primary culture species. Chen (1984) reported that salinity in the range of 30 to 35‰ and temperature in the range of 24 to 30°C were the optimal levels for growth in this species. Culture of *H. diversiocolor supertexta* has expanded greatly since 1986 due to successful artificial propagation and development of multiple-tier basket systems in grow-out farms (Yang & Ting 1986, Chen & Lee 1999).

Since late 2000, there has been mass mortality of abalones reared in the multiple-tier basket system in grow-out farms, and settlement failure of spat larvae in nursery ponds. The bacteria *Vibrio parahaemolyticus* and *V. alginolytius* isolated from the hemolymph of moribund abalones, have been demonstrated to cause outbreaks of vibriosis associated with warm temperature (Liu et al. 2000, Lee et al. 2001).

The pond water in the multiple-tier basket system often becomes hypoxic, and high concentrations of ammonia may accumulate due to decomposition of organic matters such as unconsumed food and feces, and ammonia in excreta. Nitrite is an intermediate product during bacterial nitrification of ammonia. Elevated concentrations of ammonia have been reported to increase oxygen consumption of the greenlip abalone *Haliotis*

*Corresponding author. Email: jcchen@mail.ntou.edu.tw

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laevigata (Harris et al. 1998), and to decrease the food consumption and growth rate of the ezo abalones *H. discus hannai* and *H. laevigata* (Sano & Maniwa 1962, Harris et al. 1998). Ammonia and nitrite have also been reported to affect the immune response of the blue shrimp *Litopenaeus stylirosyris* (Le Moullac & Haffner 2000), the white shrimp *L. vannamei* (Liu & Chen 2004), and the freshwater prawn *Macrobrachium rosenbergii* (Cheng & Chen 2002, Cheng et al. 2002).

In molluscs, agranular hemocytes (hyalinocytes) and granular hemocytes (granulocytes) are considered to be 2 distinct cell types (Bachère et al. 1995). They are involved in phagocytosis, an important process in eliminating microorganisms or foreign particles (Bayne 1990). During phagocytosis, contact with a pathogen activates the host's NADPH oxidase which, in turn, increases oxygen consumption and produces several species of reactive oxygen intermediates (ROIs) including the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radical (OH•) (Klebanoff 1982, Roch 1999). Superoxide anion is the first product released by respiratory activity bursts, and plays an important role in microbicidal activity. Hemocytes are also involved in the production of melanin via the prophenoloxidase system, which is an important component of the cellular defense reaction (Söderhäll et al. 1996).

Environmental physico-chemical parameters such as salinity and hypoxia, and pollutants such as Cu^{2+} and pentachlorophenol have been reported to affect the immune responses, including circulating hemocytes, phenoloxidase activity, respiratory bursts, phagocytosis and clearance efficiency in the red abalone *Haliotis rufescens*, the black abalone *H. cracherodii* and the Taiwan abalone *H. diversicolor supertexta* (Chen & Yang 1996, Martello et al. 2000, Cheng et al. 2004a,b). It is assumed that increased concentrations of ammonia and nitrite weaken the immune system of *H. diversicolor supertexta*, and lead to its susceptibility to *Vibrio* infection. Therefore, this study examine, the effect of nitrite on the susceptibility of *H. diversicolor supertexta* to *V. parahaemolyticus* and the effect of nitrite on the immune response of *H. diversicolor supertexta*. For the latter purpose, we examined total hemocyte count (THC), phenoloxidase activity, respiratory bursts (release of superoxide anion), and the phagocytic activity and clearance efficiency of *H. diversicolor supertexta* infected with *V. parahaemolyticus*.

MATERIALS AND METHODS

Haliotis diversicolor supertexta. *H. diversicolor supertexta* were obtained from a commercial farm in Kaohsiung, and were acclimated in the laboratory at a salinity of 30‰ for 2 wk at room temperature prior to experimentation. Shell length of the abalones ranged from 3.0 to 3.4 cm (average 3.36 ± 0.96 cm), and body weight from 3.0 to 4.5 g (average of 3.36 ± 0.43 g), with no significant difference among the treatments. During the acclimation period, the abalones were fed the marine alga *Gracilaria tenuistipitata* daily.

Vibrio parahaemolyticus. The bacterium *V. parahaemolyticus*, isolated from moribund abalones and demonstrated to cause 'withering syndrome', was used in the study (Liu et al. 2000). It was cultured in 50 ml tryptic soy broth (TSB) supplemented with 3% NaCl (TSB, Difco) for 24 h at 30°C, and then centrifuged at 7155 × *g* for 15 min at 4°C. The supernatant fluid was removed and the bacterial pellet was re-suspended in saline solution (0.85% NaCl) at concentrations of 8×10^6 and 5×10^8 colony-forming units (CFU) ml^{-1} as the stock bacterial suspension for injection challenge and tests of phagocytic activity and clearance efficiency.

Effect of nitrite on susceptibility of *Haliotis diversicolor supertexta* to *Vibrio parahaemolyticus.* We injected 20 µl of bacterial suspension (8×10^6 CFU ml^{-1}) into the pallial sinus of each abalone with a sterile syringe (Chen 1996, Martello et al. 2000). Challenge tests at a dose of 1.6×10^5 CFU $abalone^{-1}$ were conducted in triplicate. The test and control groups comprised 10 abalones each. After injection, each group of 10 abalones was kept in 15 l PVC tanks (10 abalones each) containing 10 l of water with different concentrations of nitrite-N (zero control and 1, 3, 5 and 10 mg l^{-1}). The test solutions were renewed daily, and the experiment lasted 96 h. The actual measured mean concentrations of nitrite-N in each test solution were 0.01 (control), 1.05, 3.04, 5.10 and 10.06 mg l^{-1} nitrite-N, respectively (Bendschneider & Robinson 1952). The abalone were fed *Gracilaria tenuistipitata* and observed for 96 h. During the experiment, water salinity and temperature were maintained at 30‰ and 26 ± 1°C. Control abalones were injected with an equal volume of sterile saline solution and exposed to the various test solutions. Abalones injected with an equal volume of sterile saline solution (0.85% NaCl) and kept in 10.06 mg l^{-1} nitrite-N served as unchallenged controls (see Table 1). The LC_{50} (median lethal concentration) of nitrite-N was determined using the computer program of Trevors & Lusty (1985).

Effect of nitrite on immune response of *Haliotis diversicolor supertexta.* For hemocyte counts and enzyme-activity assays, *H. diversicolor supertexta* were placed in 8 replicates of 20 l PVC tanks (1 abalone each). Each tank contained 10 l of water with different concentrations of nitrite-N (zero control and 1, 3, 5 and 10 mg l^{-1}) that was renewed daily for 5 d (120 h). The actual measured mean concentrations for each concentration of nitrite-N in each test solution were 0.01 (con-

trol), 0.96, 2.95, 5.03 and 10.16 mg l^{-1} nitrite-N, respectively (Bendschneider & Robinson 1952).

Hemolymph was sampled individually at the beginning of the test, and after 24, 72 and 120 h. Hemolymph (100 µl) was withdrawn from the pallial sinus of each abalone with a 1ml sterile syringe (25 gauge) containing 100 µl PBS (phosphate buffer saline) (0.01 M, osmolality 980 mOsm kg^{-1}, pH 7.4). A drop of diluted hemolymph mixture was placed on a hemocytometer, and a THC was made under an inverted phase-contrast microscope (Leica DMIL, Leica Microsystems Wetzlar); the remainder of the mixture was used for subsequent tests.

Phenoloxidase activity of hemocytes was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) (Coles & Pipe 1994). Briefly, 100 µl of diluted hemolymph in PSB was deposited in triplicate in 96-well microplates, and then centrifuged at 300 × *g* for 15 min. The supernatant fluid was discarded, and then 100 µl of sodium alginate (0.5 mg ml^{-1} in PSB, serving as an activator) was added, and allowed to react for 30 min at 26 to 27°C (Asokan et al. 1997). We then added 50 µl of L-DOPA (3 mg ml^{-1} in PBS) (as receptor), and allowed the hemolymph to react for 10 min. The optical density at 490 nm was measured using a microplate reader (Model VERSAmax, Molecular Devices). The control solution, which consisted of 100 µl of hemolymph, 100 µl PBS (to replace the sodium alginate) and 50 µl L-DOPA, was used to measure the background phenoloxidase activity in all test conditions. The optical density values corresponding to the background phenoloxidase activity were in the range 0.02 to 0.04. The optical density of the phenoloxidase activity of the abalones under all test conditions was expressed as the amount of dopachrome formed in 50 µl hemolymph.

The respiratory burst activity of the hemocytes was quantified by using the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion, as described previously by Song & Hsieh (1994). Briefly, 100 µl of diluted hemolymph in PBS solution were deposited in triplicate onto microplates previously coated with 100 µl poly-L-lysine solution (0.2%) to improve cell adhesion. Microplates were centrifuged at 300 × *g* for 15 min. Plasma was removed and 100 µl sodium alginate (0.2 mg ml^{-1} in PBS) were added, and allowed to react for 30 min at 26 to 27°C. The sodium alginate was discarded and the hemocytes were stained with 100 µl NBT solution (0.3%) for 30 min at room temperature. The NBT solution was removed and the hemocytes were fixed with 100% methanol, and washed 3 times with 100 µl 70% methanol and air-dried. The formazan was dissolved by the addition of 120 µl 2M KOH and 140 µl dimethyl sulfoxide (DMSO). The optical density at 630 nm was measured using a microplate reader. Respiratory burst was expressed as the amount of NBT reduction in 50 µl of hemolymph.

To measure bacterial clearance and phagocytic activity, after 0, 24, 72 and 120 h exposure to different concentrations of nitrite-N, we injected 20 µl bacterial suspension (5×10^8 CFU ml^{-1} in 0.85% NaCl) into the pallial sinus of the abalones, resulting in 1×10^7 CFU $abalone^{-1}$. After injection, each abalone was held in a separate tank containing one of the test solutions for 3 h at 30‰ and 26 ± 1°C. We then collected 200 µl of hemolymph from the pallial sinus and added 200 µl of PBS (pH 7.4, 980 mOsm kg^{-1}). This mixture was divided into 2 equal subsamples, one to measure phagocytic activity, the other to measure clearance efficiency.

Phagocytic activity was measured by the method of Weeks-Perkins et al. (1995). Briefly, 200 µl of the diluted hemolymph sample were fixed with 200 µl 0.1% paraformaldehyde for 30 min at 4°C to fix the hemocytes; the sample was then centrifuged at 800 × *g* with a centrifuge (Model Z323, Hermle) at 4°C. The precipitated hemocytes were washed with 0.4 ml of sterile PBS. We then sampled 50 µl of suspension, which was spread on a glass slide and placed in a cytospin centrifuge (Model Cytospin 3, Shandon) and centrifuged at 113 × *g* for 3 min. The slide was air-dried, stained with Diff-Quick stain, and observed under a light microscope; 200 hemocytes were counted. Phagocytic activity was expressed as percentage phagocytosis:

$$\% \text{ phagocytosis} = [(\text{phagocytic hemocytes})/(\text{total hemocytes})] \times 100$$

Clearance efficiency was measured by the method of Adams (1991). Hemolymph was collected and diluted 200-fold with sterile PBS. We spread 50 µl of diluted hemolymph on separate TSA (tryptic soy agar) plates, and incubated these at 30°C for 24 h. Colonies were then counted with a colony counter. The number of colonies from abalones reared in the control solution at the beginning of the test served as the control group; the number of colonies from abalones exposed to different concentrations of nitrite-N after 24, 72 and 120 h were the test groups. Clearance efficiency of *Vibrio parahaemolyticus* was calculated and expressed as percentage inhibition (PI):

$$\text{PI} = 100 - [(\text{CFU in test group})/(\text{CFU in control group})] \times 100$$

Statistical analysis. Tukey's multiple comparison test was used to compare the significance of differences among treatments using SAS computer software (SAS Institute). The percentage data (from the susceptibility study) were normalized using arcsine transformation before analysis (statistically significant difference = $p < 0.05$).

Table 1. *Haliotis diversicolor supertexta* infected with *Vibroio parahaemolyticus.* Susceptibility (cumulative mortality, %) of abalones exposed to different concentrations of ambient nitrite-N at 30‰ and 26°C. Data in same column with different superscripts on right are significantly different ($p < 0.05$) among time periods (h), and data in same row with different superscripts on left are significantly different ($p < 0.05$) among treatments. Means ± SE (n = 30 abalones in each case)

Bacterial dose (CFU abalone^{-1})	Nitrite-N (mg l^{-1})	Time after challenge (h): 6	12	24	48	72	96
Control[a]	10.06	0	0	0	0	0	0
1.6×10^5	Control[b]	^{d}3.3 ± 0.0^{y}	^{d}6.7 ± 6.7^{w}	^{c}13.3 ± 3.3^{w}	^{b}26.7 ± 6.7^{z}	^{a}36.7 ± 5.8^{z}	^{a}36.7 ± 5.8^{z}
1.6×10^5	1.05	^{c}6.7 ± 6.7^{y}	^{bc}13.3 ± 8.8^{z}	^{b}23.3 ± 3.3^{z}	^{a}36.7 ± 3.3^{z}	^{a}40.0 ± 5.8^{z}	^{a}40.0 ± 5.8^{z}
1.6×10^5	3.04	^{c}10.0 ± 5.8^{xy}	^{b}23.3 ± 8.8^{y}	^{b}30.0 ± 5.8^{yz}	^{ab}40.0 ± 5.8^{y}	^{a}46.7 ± 8.8^{yz}	^{a}46.7 ± 8.8^{yz}
1.6×10^5	5.10	^{d}13.3 ± 3.3^{x}	^{c}26.7 ± 3.3^{xy}	^{b}36.7 ± 6.7^{y}	^{ab}46.7 ± 6.7^{xy}	^{a}53.3 ± 6.7^{y}	^{a}53.3 ± 6.7^{y}
1.6×10^5	10.06	^{d}16.7 ± 3.3^{x}	^{c}30.0 ± 5.8^{x}	^{b}46.7 ± 6.7^{x}	^{b}56.7 ± 6.7^{x}	^{a}70.0 ± 0.0^{x}	^{a}70.0 ± 0.0^{x}

[a]Unchallenged controls injected with sterile saline solution and kept in 10.06 mg l^{-1} nitrite-N
[b]Controls injected with equal volumes of sterile saline solution and exposed to test solutions

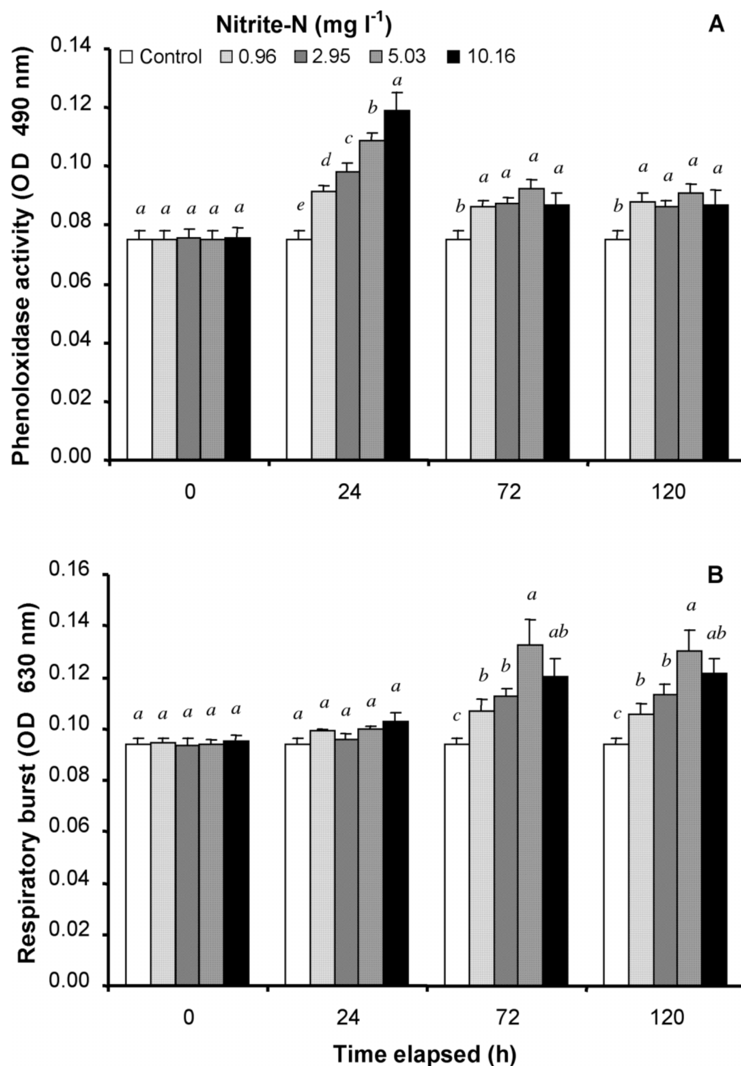

Fig. 1. *Haliotis diversicolor supertexta.* Mean (± SE) (A) phenoloxidase activity and (B) respiratory burst in hemocytes after 0, 24, 72 and 120 h exposure to nitrite-N. Each bar represents mean (±SE) of 8 determinations. Values for same exposure time with different letters = significant difference ($p < 0.05$) among treatments. OD: optical density

RESULTS

Effect of nitrite on susceptibility of *Haliotis diversicolor supertexta* to *Vibrio parahemolyticus*

All unchallenged control abalones survived. In contrast, mortality began to occur at 6 h in the challenged abalones. Cumulative mortality increased in direct parallel to ambient nitrite-N concentration. After 12 h, the cumulative mortality of abalones in 1.05 mg l^{-1} nitrite-N was significantly higher than in the controls. Cumulative mortality over 72 to 96 h was 36.7, 40.0, 46.7, 53.3 and 70.0% for abalones in control, 1.05, 3.04, 5.10 and 10.06 mg l^{-1} nitrite-N treatments, respectively (Table 1). The 48 h and 96 h LC_{50}s of nitrite-N for *Haliotis diversicolor supertexta* were 8.7 and 4.3 mg l^{-1}, respectively.

Effect of nitrite on immune response of *Haliotis diversicolor supertexta*

There were no significant differences in THC for control abalones at and 26°C at the different sampling times; the mean (±SE) varied from $206.0 \pm 19.4 \times 10^4$ to $210.2 \pm 14.6 \times 10^4$ cells ml^{-1}. The THC of abalones exposed to 0.96 and 2.95 mg l^{-1} nitrite-N for 72 h increased significantly by 32 and 23%, respectively, while that of abalones exposed to 5.03 and 10.16 mg l^{-1} nitrite-N for 72 h decreased significantly by 26 and 47%, respectively, as compared to the controls (Table 2).

No significant difference in phenoloxidase activity was observed among the control abalones at the beginning of the experiment and after 24, 72 and 120 h. After 24 h, relative phe-

Table 2. *Haliotis diversicolor supertexta*. Effect of different concentrations of ambient nitrite-N (mg l^{-1}) on THC (total hemocyte counts, $\times 10^4$ ml^{-1}). Data in same column with different superscripts on left are significantly different ($p < 0.05$) among time periods (h), data in same row with different superscripts on right are significantly different ($p < 0.05$) among treatments. Means ± SE (n = 8 abalones in each case)

Sampling time (h)	Control	Nitrite-N (mg l^{-1})			
		0.96	2.95	5.03	10.16
0	$^{a}210.2 \pm 14.6^{x}$	$^{b}210.2 \pm 14.6^{x}$	$^{b}210.2 \pm 14.6^{x}$	$^{a}210.2 \pm 14.6^{x}$	$^{a}210.2 \pm 14.6^{x}$
24	$^{a}208.9 \pm 11.3^{x}$	$^{b}205.1 \pm 28.5^{x}$	$^{b}206.2 \pm 18.1^{x}$	$^{b}143.0 \pm 11.3^{y}$	$^{b}159.4 \pm 25.9^{y}$
72	$^{a}210.8 \pm 14.6^{y}$	$^{a}277.4 \pm 13.0^{x}$	$^{a}258.9 \pm 6.0^{x}$	$^{b}155.8 \pm 16.5^{y}$	$^{c}110.1 \pm 29.6^{z}$
120	$^{a}206.0 \pm 19.4^{y}$	$^{a}294.0 \pm 19.6^{x}$	$^{a}246.6 \pm 11.9^{x}$	$^{b}143.0 \pm 24.0^{y}$	$^{c}105.8 \pm 24.2^{z}$

noloxidase activity (compared to activity of the controls) increased significantly (by 120, 127, 140 and 147%) in abalones exposed to 0.96, 2.95, 5.03 and 10.16 mg l^{-1} nitrite-N. After 120 h, the relative phenoloxidase activity for abalones exposed to 0.96, 2.95, 5.03 and 10.16 mg l^{-1} nitrite-N was 120, 113, 117 and 107%, respectively (Fig. 1A).

No significant difference in respiratory bursts was observed among the control abalones at the beginning of the experiment and after 24, 72 and 120 h. After 72 h, the relative respiratory bursts (compared to activity of the controls) of abalones exposed to 0.96, 2.95, 5.03 and 10.16 mg l^{-1} nitrite-N were 122, 133, 150 and 128%, respectively. After 120 h, the respiratory bursts for abalones exposed to 0.96, 2.95, 5.03 and 10.16 mg l^{-1} nitrite-N were 117, 136, 144 and 133%, respectively (Fig. 1B).

At time 0 h, phagocytic activity was 13.5% in the controls. Phagocytic activity was inversely related to nitrite-N concentration. After 24 h, phagocytic activity decreased significantly to 4.0, 3.0, 3.0 and 2.8% in abalones exposed to 0.96, 2.95, 5.03 and 10.16 mg l^{-1} nitrite-N, respectively (Fig. 2A).

Clearance efficiency was inversely related to nitrite-N concentration. After 24 to 120 h, clearance efficiency of the controls was 15%, while that of abalones exposed to 0.96, 2.95, 5.03 and 10.16 mg l^{-1} nitrite-N had decreased significantly to −60, −100, −190 and −250%, respectively, after 24 h (Fig. 2B).

DISCUSSION

Water temperature, salinity, dissolved oxygen, pH and nitrite have been reported to increase the susceptibility of the freshwater prawn *Macrobrachium rosenbergii* to the pathogen *Lactococcus garvieae* (Cheng & Chen 1998, Cheng et al. 2002). In the present study, we found that the susceptibility of *Haliotis diversicolor supertexta* to *Vibrio parahaemolyticus* increased in direct parallel to increasing ambient nitrite-N concentration. *H. diversi-*

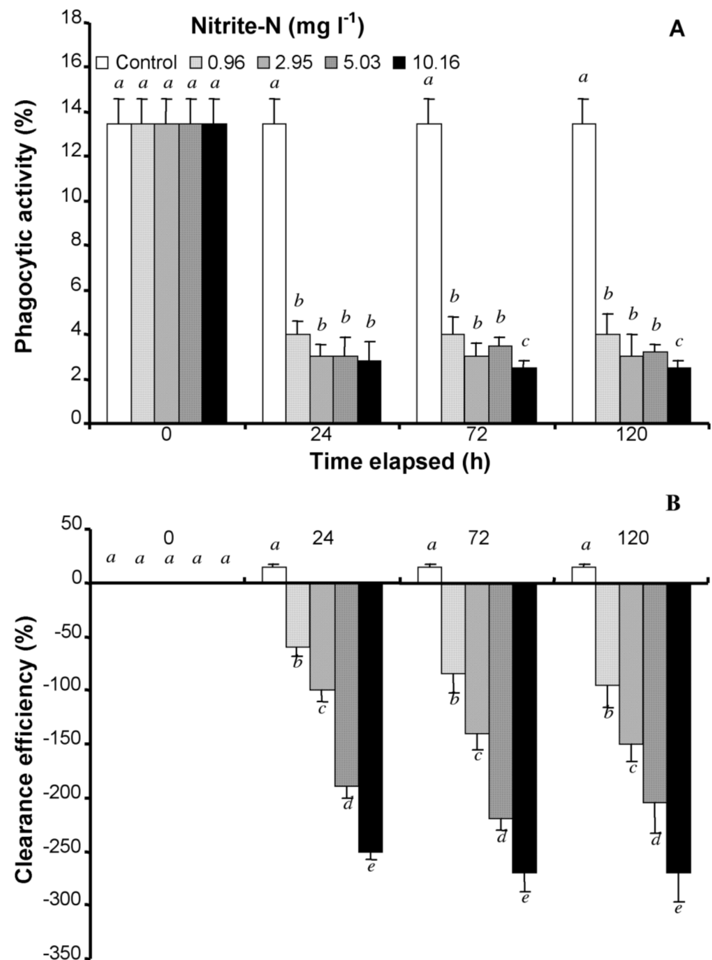

Fig. 2. *Haliotis diversicolor supertexta.* Mean (±SE) (A) phagocytic activity and (B) clearance efficiency after 0, 24, 72 and 120 h exposure to different concentrations of nitrite-N. Each bar represents mean (± SE) of 8 determinations. Values for same exposure time with different letters = significant difference ($p < 0.05$) among treatments

color supertexta was more susceptible to *V. parahaemolyticus* when abalones were transferred from 30‰ to 20, 25 or 35‰ (Cheng et al. 2004a) and also when reared in medium containing 2.05 mg l^{-1} dissolved oxygen (Cheng et al. 2004b). It can be concluded that salinity changes, hypoxia and the presence of nitrite-N can trigger an outbreak of the disease by weakening the immune response of *H. diversicolor supertexta*.

No significant difference in THC was observed in the freshwater prawn *Macrobrachium rosenbergii* exposed to ambient ammonia-N in the range 0 to 3.18 mg l^{-1} for 7 d (Cheng & Chen 2002), or exposed to ambient nitrite-N in the range 0 to 1.68 mg l^{-1} for 7 d (Cheng et al. 2002). No significant difference in THC was observed in the white shrimp *Litopenaeus vannamei* following 7 d exposure to ambient ammonia-N in the range 0.01 to 21.60 mg l^{-1} (Liu & Chen 2004). However, in the present study, THC increased in *Haliotis diversicolor supertexta* following 72 h exposure to 0.96 and 2.95 mg l^{-1} nitrite-N, but decreased following 24 h exposure to 5.03 and 10.16 mg l^{-1} nitrite-N. Phenoloxidase is stored in the secretory granules of semi-granular and granular hemocytes, whereas arganular hemocytes are involved in phgagocytoisis and the release of superoxide anion and other ROIs (Bachère et al. 1995). Further research is needed to clarify whether a change in DHC (differential hemocyte count) occurs in abalone under nitrite stress, and whether changes in THC and DHC result from cell proliferation, from leakage of cells from tissues into circulatory fluids, as a result of lysis, or through dilution of the hemolymph (Pipe & Coles 1995).

In the present study, *Haliotis diversicolor supertexta* exposed to 5.03 and 10.16 mg l^{-1} nitrite-N decreased its THC, but increased its phenoloxidase activity. The blue shrimp *Litopenaeus stylirostris* exposed to 3 mg l^{-1} ammonia decreased its THC by 51%, but increased its phenoloxidase activity by 33% (Le Moullac & Haffner 2000). A negative correlation between phenoloxidase activity and THC was observed in *L. stylirostris* following 24 h exposure to 1 mg l^{-1} DO (dissolved oxygen) (Le Moullac et al. 1998); in *H. diversicolor supertexta* following 24 h exposure to 2.05 mg l^{-1} DO (Cheng et al. 2004b); and in the common shrimp *Crangon crangon* following exposure to PCB (Smith & Johnston 1992). It is known that the prophenoloxidase system is activated by serine protease (the prophenoloxidase activating enzyme, ppA), a serine protease activated by the microbial cell walls. The ppA can be regulated by 2 protease inhibitors, α_2-macroglobulin (Hergenhahn & Söderhäll 1985) and trypsin inhibitors such as pacifastin (Hergenhahn et al. 1987). It would seem likely that, following exposure to ambient nitrite-N of ≥5 mg l^{-1}, the abalone would have less antiprotease activity available to regulate the ppA.

The phenoloxidase activity of *Litopenaeus vannamei* decreased significantly after 1 d exposure to 5.24 mg l^{-1} ammonia-N (Liu & Chen 2004), while that of *L. stylirostris* exposed to 3.0 mg l^{-1} ammonia increased significantly (Le Moullac & Haffner 2000). However, neither group of investigators reported the exposure times. In the present study, phenoloxidase activity of *Haliotis diversicolor supertexta* increased after 24, 72 and 120 h exposure to ≥0.96 mg l^{-1} nitrite-N. The transcript encoding prophenoloxidase and peroxinectin decreased by 60 and 50% when *L. stylirostris* were exposed to 1.5 and 3.0 mg l^{-1} ammonia, respectively, with a concomitant reduction in hemocyte count by 15 and 51% (Le Moullac & Haffner 2000). *L. vannamei* transferred from 26 to 34°C decreased its amount of peroxinectin mRNA in 2 d (Liu et al. 2004). Therefore, the defense responses related to phenoloxidase and peroxinection may also be weakened at the level of gene expression in *L. vannamei* under nitrite stress.

Exposure of *Macrobrachium rosenbergii* to 1.15 and 1.68 mg l^{-1} nitrite-N for 168 h stimulated the production of superoxide anion (Cheng et al. 2002). In the present study, *Haliotis diversicolor supertexta* exposed to ambient nitrite-N at ≥0.96 mg l^{-1} for 72 h increased the release of superoxide anion. In our experiments it was not possible to distinguish whether the increase in superoxide anion resulted from increased activity of NADPH oxidase (responsible for producing the superoxide anion), or from decreased activity of superoxide dismutase (SOD) (responsible for scavenging the superoxide anion). A small increase in the superoxide anion is considered to increase immunity. However, too great an increase may be toxic to the host (Cheng & Wang 2001). Further research is needed to examine the activities of enzymes such as SOD, catalase and peroxidase, and transport proteins such as ferritin and transferrin, which are capable of preventing production of hydroxyl radicals, as shown by Holmblad & Söderhäll (1999) for abalones during ammonia exposure.

Phagocytosis in invertebrates can be affected by environmental parameters (Bayne 1990, Pipe & Coles 1995). For example, elevated temperature has been reported to increase phagocytosis (Feng & Feng 1974) and hemocyte activity (determined as the ability of hemocytes to adhere to fluorescent beads) in the American oyster *Crassostrea virginica* (Foley & Cheng 1975). Phagocytic activity and clearance efficiency of *Vibrio parahaemolyticus* decreased in *Haliotis diversicolor supertexta* following 24 h exposure to 0.96, 2.95, 5.03 and 10.16 mg l^{-1} nitrite-N. This correlated well with the increased susceptibility of *H. diversicolor supertexta* to *V. parahaemolyticus* when the abalones were under nitrite stress. It would be interesting to determine whether nitrite stress affects the production of anti-microbial peptides, such as mytilin and

mytimicin observed in the mussels *Mytilus edulis* and *M. galloprovincialis* (Charlet et al. 1996) and Pacific oyster *Crassostrea gigas* (Hubert et al. 1996).

In conclusion, ambient nitrite decreased the resistance of *Haliotis diversicolor supertexta* to *Vibrio parahaemolyticus*. Ambient nitrite-N at a concentration of 0.96 mg l^{-1} increased the susceptibility of *H. diversicolor supertexta* by weakening its immune responses, e.g. by enhanced THC and phenoloxidase activity and a reduction in phagocytic activity and clearance efficiency of *V. parahaemolyticus*, together with an increase in the superoxide anion to levels possibly cytotoxic to the host.

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