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Tolerance of *Physocypria kraepelini* (Crustacean, Ostracoda) to water-borne ammonia, phosphate and pH value

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Abstract

This study evaluated the median lethal concentration (LC_{50}) and safe concentration of water-borne ammonia, phosphate and pH value on *Physocypria kraepelini*, a freshwater Ostracoda with a static renewal test system. The results indicated that the LC_{50} values of ammonia for *P. kraepelini* were 1026.71, 859.98, 771.79 and 583.82 mg/L at 24, 48, 72 and 96 h exposure, respectively, and the safe concentration range of ammonia for the long-term survival of *P. kraepelini* was less than 58.38 mg/L. The safe range of pH value for the survival of *P. kraepelini* was from 6.59 to 7.61. *P. kraepelini* has a high tolerance to ammonia, phosphate and pH value which are the main environmental factors in the serious eutrophication water.

Key words: ammonium; phosphate; *Physocypria kraepelini*

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Introduction

Since the species composition of Ostracoda correlated colosely to environmental factors, and the distribution of ostracod is sensitive to water environmental factors, the ostracod could be of significance as bio-marker for the environment (Stepanova *et al*., 2003; Bunbury and Gajewski, 2005; Yu *et al*., 2007; Külköylüoğlu *et al*., 2007). Preliteratures have showned that water pH value, nitrogen and phosphorus could pose signicant impact to the ostracod community composition (Yu *et al*., 2007). Nitrogen and phosphorus are the most essential nutrients for the growth of both algae and plankton (Ma, 2001). However, in recent years, human activities increased domestic sewage to freshwater ecosystems, including animal and human feces containing high levels of ammonia and phosphate. This results in excess nitrogen and phosphorus, which could be lethal to many species of the aquatic community. Nutrient loading also could change the water pH value, and further alter the community composition by decreasing or increasing unusually the survival and reproduction of many aquatic species. At present, this critical problem of eutrophication in freshwaters is ubiquitous (Pu *et al*., 2008), and high mortality of aquatic organisms in these polluted environments occurs quite frequently. *Physocypria kraepelini* is a common ostracod species found in many freshwaters, including those subject to eutrophication (Meisch, 2000; Kiss, 2002; Külköylüoğlu *et al*., 2007). Notably, it can survive in polluted ecosystem because *P. kraepelini* is specialized in swimming to expand its distribution range and escape from the adverse environment (Külköylüoğlu, 2005; Yu *et al*., 2007), and it also has an extremely strong tolerant to pollution environment (Meisch, 2000; Yılmaz and Külköylüoğlu, 2006; Yu *et al*., 2007). *P. kraepelini* is, therefore, one of the few ostracods that can live in the heavily polluted water (Shornikov and Trebukhova, 2001), for example, the Tai Lake and its associated rivers in the urban area of Shanghai with serious eutrophication (Yu *et al*., 2008).

This study tested the tolerance of *P. kraepelini* to ammonia, phosphate and pH value. The result could provide more information for understanding the underlying mechanism of the extensive distribution of *P. kraepelini*.

1 Materials and methods

1.1 Experimental animals

P. kraepelini used in this experiment were obtained from a shallow pool in Changfeng Park of the Putuo District in Shanghai, China. Organic matter is abundant in the pool. *P. kraepelini* were transferred under a dissecting microscope into a beaker of distilled water for culturing. After one week acclimation, adult ostracods with strong viability and of similar size were selected for the experiments.

1.2 Experimental methods

The concentrations of ammonia and phosphate were controlled by adding NH_4Cl or $NaH_2PO_4{\cdot}H_2O$. Working stock solutions of both NH_4^+ and $H_2PO_4^-$ were 2000 and

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20000 mg/L, respectively. The working stocks were diluted stepwise according to a set concentration gradient for the experiment. NaOH and HCl solutions were used to adjust pH value.

A static acute toxicity test system was applied in this study according to Wu and Fei (1999). Nutrient solutions could not be changed during the trial and no food was provided to avoid introducing confounds. To determine the concentration ranges for the test solutions in the experiment, ostracods were exposed to twenty solutions ranging in ammonia concentration from 0 to 2000 mg/L and phosphate concentration from 0 to 20000 mg/L for 24 h. For ammonia, 100 mg/L was the maximum concentration that allowed total survival and 1300 mg/L was determined to be the 100% lethal concentration for *P. kraepelini*. For phosphate, the maximum concentration that allowed total survival was 155 mg/L, and 15545 mg/L was found to be 100% lethal. The maximum pH value for total survival and 100% lethal pH value were 6.0 and 4.0 in the range of acidity, respectively. Those values for the range of alkality were 8.0 and 10.0, respectively.

Six concentration groups of ammonia and phosphate were set according to the determined maximum concentration for total survival and the 100% lethal. The ammonia concentrations used were 100.00, 167.00, 278.89, 465.75, 777.80, and 1300.00 mg/L. The phosphate concentrations used were 155.00, 390.48, 980.91, 2464.04, 6189.66, and 15545.00 mg/L. The corresponding pH values were 6.0, 5.5, 5.0, 4.5, 4.0; and 8.0, 8.5, 9.0, 9.5,10.0, respectively. Three treatment groups and one blank control group were exposed to each ammonia, phosphate, and pH treatment. For the ammonia and phosphate trials, pH value in the test solutions was maintained at 7.0 ± 0.5.

A 20-mL of each solution was put into a 50-mL beaker, and then 20 animals were put into the solution. The water temperature was controlled at (25±0.5)°C during the experiment and the environmental condition was kept constant during all trials. The duration of each trial was 24, 48, 72, or 96 h. At the end of each observation time, the dead animals was counted and removed.

1.3 Death identification

Naked-eye inspection was employed first to preliminarily judge the death of each *P. kraepelini*, as the living animal had a clearly visible transparent and lustrous shell. If the shell was white and lusterless, it was considered as dead. In some cases, a microscope was used for further observation. When the shell valves of *P. kraepelini* unfolded (Du, 1987), or no appendage movement was observed during an observation for 2 min, the animal was considered as dead.

1.4 Statistical methods

The percentage mortality was calculated according to the recorded mortality at 24, 48, 72 and 96 h for each experimental group. The resulting data were converted into probits (Hui, 2003). SPSS 14.0 statistical software was employed to perform the analysis. The log values of ammonia or phosphate concentration or pH value served as the horizontal coordinate and the probit of mortality served as the vertical coordinate to calculate a regression equation between the probit and concentrations or pH values of the experimental solutions. The LC_{50} of ammonia and phosphate, as well as their respective 95% confidence intervals, were calculated using the probit analysis in SPSS 14.0 (Reish and Oshida, 1987). The safe concentrations of ammonia, phosphate, H^+ and OH^- to *P. kraepelini* were calculated using empirical formulas. Safe concentrations were defined to be equal to 96 h-LC_{50} × 0.1 (Sprague, 1971). The calculated LC_{50} and safe concentrations for H^+ and OH^- were converted into pH values.

2 Results and analysis

For all the three treatments, the mortality of ostracod in the control groups was 0. This, therefore, removes the possibility that natural death of *P. kraepelini* or other external environment changes influenced the results. The regression equation between the probit corresponding to ostracod mortality at different exposure time and concentrations of the experimental solutions, the LC_{50} and the safe concentration were obtained with the analysis of SPSS 14.0 (Table 1).

2.1 Safe concentration of ammonia and phosphate for ostracod survival

The mortality of *P. kraepelini* increased with the increasing of ammonia (Fig. 1a) and phosphate (Fig. 1b), and significant dose-effect relationships were observed. A considerable linear positive correlation was found between the death probit of *P. kraepelini* and the log value of both the concentrations of ammonia and phosphate ($R^2 >$ 0.99) (Table 1). The LC_{50} of ammonia for acute toxicity testing of *P. kraepelini* survival at exposure time of 24, 48, 72 and 96 h were 1026.71, 859.98, 771.79 and 583.82 mg/L, respectively; and those of phosphate were 11840.97, 6421.32, 4105.19 and 3905.46 mg/L, respectively, suggesting that LC_{50} decreased with increasing exposure time, and the toxicity of ammonia and phosphate to *P. kraepelini* increases in a time-dependent manner. The observed safe concentration of ammonia and phosphate was 58.38 and 390.55 mg/L, respectively.

2.2 Safe range of pH value for ostracod survival

The mortality of *P. kraepelini* gradually increased with increasing H^+ concentration, from 1.0×10^{-6} to 1.0×10^{-4} mg/L (equal to the pH value from 6 to 4) (Fig. 2a), and it showed a marked dose-effect relationship. A significant linear positive correlation was found between the death probit of *P. kraepelini* and the log value of H^+ concentration ($R^2 > 0.99$). The LC_{50} of H^+ for *P. kraepelini* at exposure time of 24, 48, 72 and 96 h were 4.67×10^{-5}, 8.37×10^{-6}, 4.02×10^{-6} and 2.55×10^{-6} mg/L, respectively. The corresponding converted pH value was 4.33, 5.08, 5.40 and 5.59, respectively. The safe concentration of pH value for *P. kraepelini* was 6.59.

For OH^- concentrations, *P. kraepelini* mortality gradually increased with increasing pH value (Fig. 2b), and it also showed a marked dose-effect relationship. A considerable

Table 1 Three environmental factors to *Physocypria kraepelini* at different exposure time

Factor	Exposure time (h)	Regression equation	Correlation coefficient (R^2)	LC_{50} (mg/L)	Safe concentration (mg/L)
Ammonia	24	$y = 3.7911x - 6.4167$	0.9981	1026.71	58.38
	48	$y = 4.1154x - 7.0766$	0.9944	859.98	
	72	$y = 3.3839x - 4.7710$	0.9909	771.79	
	96	$y = 4.1173x - 6.3896$	0.9929	583.82	
Phosphate	24	$y = 3.3003x - 8.4434$	0.9891	11840.97	390.55
	48	$y = 2.9276x - 6.1472$	0.9944	6421.32	
	72	$y = 1.9575x - 2.0731$	0.9900	4105.19	
	96	$y = 1.6499x - 0.9259$	0.9921	3905.46	
H^+	24	$y = 1.8416x + 12.976$	0.9902	4.67×10^{-5} (pH 4.33)	2.55×10^{-7} (pH 6.59)
	48	$y = 1.012x + 10.138$	0.9921	8.37×10^{-6} (pH 5.08)	
	72	$y = 0.946x + 10.104$	0.9908	4.02×10^{-6} (pH 5.40)	
	96	$y = 0.9482x + 10.303$	0.9943	2.55×10^{-6} (pH 5.59)	
OH^-	24	$y = 1.3037x + 9.8779$	0.9913	1.81×10^{-4} (pH 10.26)	4.11×10^{-7} (pH 7.61)
	48	$y = 0.668x + 7.7022$	0.9916	9.01×10^{-5} (pH 9.95)	
	72	$y = 0.680x + 8.3581$	0.9989	1.15×10^{-5} (pH 9.06)	
	96	$y = 0.8262x + 9.4502$	0.9997	4.11×10^{-6} (pH 8.61)	

x: logarithmic concentration of environmental factors; *y*: probit mortality for *P. kraepelini*.

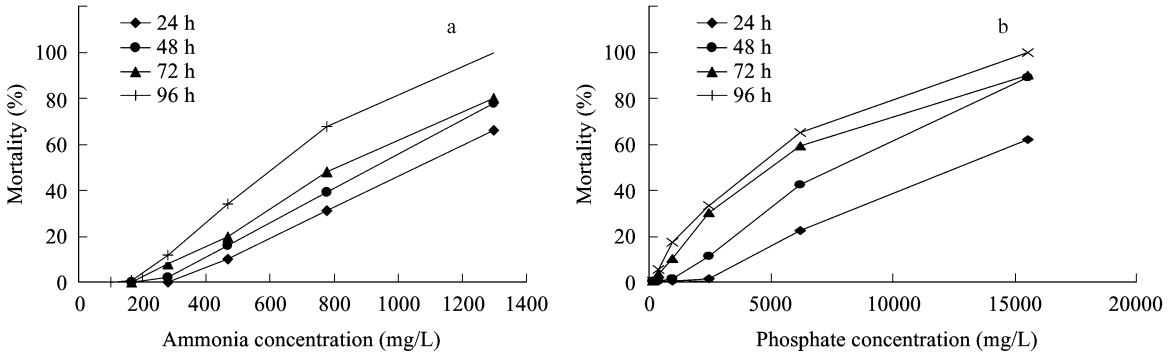

Fig. 1 Mortalities of *Physocypria kraepelini* at different ammonia (a) and phosphate (b) concentrations (the mortality rate in control was 0).

linear positive correlation was found between the death probit of *P. kraepelini* and the OH^- concentration (R^2 >0.99) (Table 1). The LC_{50} of OH^- concentrations at exposure time of 24, 48, 72 and 96 h were 1.81×10^{-4}, 9.01×10^{-5}, 1.15×10^{-5} and 3.96×10^{-6} mg/L, respectively. The corresponding converted pH value was 10.26, 9.95, 9.06 and 8.61, respectively. The safe concentration of OH^- for *P. kraepelini*, calculated using the empirical formula, was 4.11×10^{-7} mg/L (pH 7.61).

Consequently, the safe survival range of pH value to *P. kraepelini* was between 6.59 and 7.61.

2.3 Lethal threshold concentration (LC_{50}) of different agents to *P. kraepelini*

The change in the curve of the LC_{50} values for phosphate was gradually parallel to the time axis after 72 h (Fig. 3). The LC_{50} did not decrease with increasing time, and the mortality of experimental animals increased slowly, or even stopped increasing. The remaining living animals exhibited tolerance to the phosphate. A LC_{50} of 4105.19 mg/L at 72 h served as the lethal threshold concentration of phosphate to *P. kraepelini*. However, no lethal threshold concentration of ammonia or pH value was found during 96 h exposure time, which suggested that the mortality

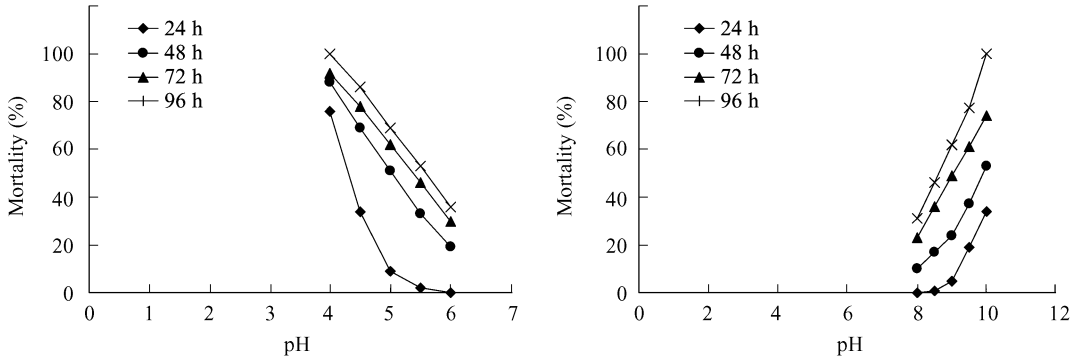

Fig. 2 Mortalities of *P. kraepelini* at different pH values (the mortality rate in control was 0).

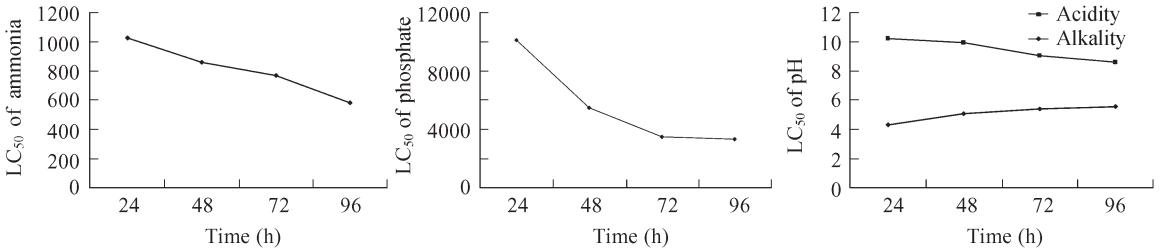

Fig. 3 Relationship of LC_{50} values and exposure time.

of *P. kraepelini* increased continuously with increasing exposure time, and further indicated that ammonia, H^+ and OH^- accumulated in the body of *P. kraepelini*.

3 Discussion

3.1 Tolerance of *P. kraepelini* to ammonia

With the increasing water pollution, the increasing nitrogen led to more and more economic losses for the aquaculture industry (Colt and Armstrong, 1981). The nitrogen in water contains organic nitrogen and inorganic nitrogen, of which, ammonia is one of the main existing forms of the inorganic nitrogen. In summer, the content of nitrogen can be reached 46 mg/L in aquaculture waters. Our study focuses on ammonia concentrations, but in fact, the ammonia in water includes NH_4^+ and NH_3, and NH_4^+ cannot permeate the cell membrane and, thus, it shows no obvious toxic effect on organisms. Nevertheless, there is a mutual transformation between NH_3 and NH_4^+ in water, and $NH_3{\cdot}H_2O$ can pass through the biological surface and permeate into the body. When the pH value of an organism tissue fluid is lower than that of the surrounding water, NH_3 can permeate into tissue fluid and result in ammonia intoxication (Qiao and Li, 2005). In this article, all ammonia referred was absolute concentration values which included $NH_4^+$and its transformed NH_3, unless otherwise noted.

The results showed that the safe concentration of waterborne ammonia to *P. kraepelini* was 58.38 mg/L, and the safe concentration of nitrogen was 45.41 mg N/L. Because the water temperature 25°C and pH value 7.0 were controlled in experiment, it could be calculated that NH_3 accounted for approximately 0.6% of the ammonia concentration assuming equilibrium conditions (Qiao and Li, 2005). The safe concentration of NH_3 to *P. kraepelini* was thus 0.27 mg N/L, which is higher than the permitted maximum NH_3 concentration (0.1 mg N/L) in aquaculture water (Qiao and Li, 2005). In addition, compared with related studies on crustaceans, the 96 h-LC_{50} of ammonia to *P. kraepelini* is much higher (Table 2). Among them, *Homarus americanus* (Young-Lai *et al*., 1991) and *Eriocheir sinensis* (Hong *et al*., 2007) have extremely strong adaptability to the environmental ammonia, suggesting that *P. kraepelini* also has a high tolerance to ambient ammonia, which implying that these two organisms probably have a related approach to counter the toxicity of ammonia nitrogen.

3.2 Tolerance of *P. kraepelini* to phosphate

$H_2PO_4^-$ is a weak acid and HPO_4^{2-} is a weak base in water. Because our experiment was conducted at neutrality, both $H_2PO_4^-$ and HPO_4^{2-} existed in the experimental solution. However, for convenience, the absolute phosphate concentration which included $H_2PO_4^-$ and its transformed HPO_4^{2-} was used in this study. Our results revealed that the safe concentration of phosphate in water for *P. kraepelini* was 390.55 mg/L, indicating that ostracods have an extremely high tolerance to phosphate, and that they probably have an optimized regulation mechanism. As shown in Fig. 3, only the phosphate solution manifested a lethal threshold concentration, which was 4105.19 mg/L.

Table 2 96 h-LC_{50} of ammonia for several species of crustaceans

Species	Ammonia (mg/L)	Life stage	Experiment type	Reference
Physocypria kraepelini	583.82	Adult	Static	This study
Ampelisca abdita	49.80	NR	Static	Kohn *et al*., 1994
Eohaustorius estuarius	125.50	NR	Static	Kohn *et al*., 1994
	126.70	NR	Static	Kohn *et al*., 1994
Penaeus chinensis	42.44	Juvenile (3.61 cm, 0.61 g)	Renewal	Chen and Lin, 1992
P. japonicus	52.65	Juvenile (10 g)	Not reported	Lin *et al*., 1991
P. paulensis	38.72	5.45 g	Static	Ostrensky and Wasielesky, 1995
P. penicillatus	29.77	0.40–0.69 g, 3.58–4.75 cm	Renewal	Chen and Lin, 1991
Rhepoxynius abronius	78.70	NR	Static	Kohn *et al*., 1994
Eriocheir sinensis	119.67	Juvenile (3.61 g)	Static	Hong *et al*., 2007
Homarus americanus	125.00	Larvae III	Static	Young-Lai *et al*., 1991
	144.00	Post-larvae	Static	Young-Lai *et al*., 1991
	219.00	Adult	Static	Young-Lai *et al*., 1991
Litopenaeus vannamei	39.54	22 mm	Static	Lin and Chen, 2001
	65.20	0.99 g	Static	Frias-Espericueta *et al*., 1999
	70.90	3.8 g	Static	Frias-Espericueta *et al*., 1999
Scylla serrata	95.35	Juveniles (0.373 g)	Static	Romano and Zeng, 2007a
Portunus pelagicus	50.65	Juveniles (0.732 g)	Static	Romano and Zeng, 2007b

In other words, the *P. kraepelini* that survived in the solution had adapted to the water environment having high phosphate by its regulation mechanism. Therefore, the mortality rate did not increase with the high levels of phosphate. Actually, phosphorous is an essential nutrient for all organisms, and phosphate, one of the buffering compounds for regulating the acid-base balance of body fluid in animals, plays a key role in the body. However, excessive phosphate can result in massive losses of calcium in the body. This is the main reason for the toxic effect of phosphate on animals. Studies on coral and calcified algae demonstrated that the most significant negative effect of excessive phosphate lies in its inhibition to their calcification process. Phosphate can cause the precipitation of calcium and eventually hinder the formation of calcium carbonate in coral. When the calcification process is interrupted, it will result in tissue atrophy (Holmes-Farley, 2002). From the above result, it is presumed that the extremely high solution concentration of phosphate in our study possibly results in calcium losses in the bodies of *P. kraepelini*.

The results of this study demonstrated that the safe concentration of phosphorous to *P. kraepelini* was up to 124.81 mg/L. The Organization for Economic Cooperation and Development (OECD) proposed that the average phosphorous concentration should not exceed 0.035 mg/L in lakes. According to this standard, both Tai lake and rivers in the urban area of Shanghai are classified as polluted with respect to phosphorous, and in these water bodies, the average phosphorous content is 0.492 mg/L (Wo *et al*., 2006), which is still in the safe concentration range of *P. kraepelini*. This could partially explain why *P. kraepelini* can thrive in those water bodies (Yu *et al*., 2007, 2008). Under natural conditions, effect of excessive phosphorous on animals in water is indirect. Microorganisms can transform phosphorous into ATP and ADP, which are the energy sources for biochemical reactions in organisms. Excessive phosphorous would allow organisms to obtain a large amount of energy, which can result in an abnormal multiplication. Gradual decrease of dissolved oxygen and the dramatic changes in pH that are associated with such massive growth and reproduction, however, often lead to the widespread death of aquatic animals. *P. kraepelini* thus likely has a high tolerance to these changes.

3.3 Tolerance and safe range of pH value to *P. kraepelini*

Külköylüoğlu (2000) reported the pH tolerance range of several globally distributed species, such as *Sarscypridopsis aculeate* (pH 7.21–7.56), *Heterocypris incongruens* (pH 6.00–9.83), *Cypridopsis vidua* (pH 5.20–12.0), *Herpetocypris reptans* (pH 7.30–9.00), *Ilyocypris gibba* (pH 6.64–9.80), *Cypria ophthalmica* (pH 5.00–13.00), *Potamocypris villosa* (pH 7.00–9.85), *Darwinula stevensoni* (pH 6.00–9.27), *Eucypris virens* (pH 7.60–9.27) and *Candona candida* (pH 4.60–13.00). Except for *S. aculeata*, these species had an extremely high tolerance to water pH. However, results from these species enumerated were not obtained in the laboratory under controlled conditions but field conditions (Külköylüoğlu, 2000), and it was not clear whether these animals existed under these conditions temporarily or permanently. As we know, the pH value of water can change temporarily due to anthropogenic factors (pollution discharge) or natural factors (typhoon). If the duration of this change is very short, it would induce only a stress reaction rather than a massive death of individuals. Nonetheless, under the acid conditions in this study at pH 4.0, living animals (8% on average) could be observed at the exposure time of 72 h, and the same was found under the basic conditions at pH 10.0, indicating that a few *P. kraepelini* could survive for 3 d under conditions of pH value at 4.0 or 10.0, suggesting a high tolerance to pH value changes. Therefor, it could be concluded that *P. kraepelini* has the ability to distribute widely. However, the obtained safe range of pH value to the long-term survival of *P. kraepelini* was from 6.6 to 7.6 by linear regression analysis, suggesting a preference for inhabiting a neutral water body.

3.4 Relationship between *P. kraepelini* and water eutrophication

The results in this study indicated that cosmopolitan ostracods *P. kraepelini*, a species that thrives in eutrophic conditions (Altınsaçlı *et al*., 2000; Altınsaçlı and Griffiths, 2001; Yu *et al*., 2008), has a high tolerance to ammonia, phosphate and pH value which are the main environmental factors in the serious eutrophication water. Ammonia and phosphate are usually limiting nutrients for the growth of phytoplankton and are therefore leading eutrophication, and in turn result in deterioration of water quality (Wu *et al*., 2006). Many previous results indicated that there was a close relationship between the increasing number of cosmopolitan ostracods and both the decreasing water quality and the increasing water eutrophication (Külköylüoğlu, 2004; Yu *et al*., 2007, 2008). Althought ostracod species has a wide range of tolerances to various environmental conditions may have limited usefulness as an indicator species (Külköylüoğlu, 2003), we can conclude that the increasing number of *P. kraepelini* may show an increasing severity of water eutrophication at the safe range of ammonia and phosphate concentration and pH value.

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