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Delayed Mortality of Benthic Amphipods *Monocorophium acherusicum* Exposed to Various Pollutants in Seawater (Cd, Cu, Hg, TBT, Ammonia and Phenanthrene)

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유해오염물질에 급성 노출된 단각류 *Monocorophium acherusicum*의 지연 사망률에 관한 연구

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요 약

다양한 유해오염물질에 급성 노출된 단각류 *Monocorophium acherusicum*의 노출 기간 이후에 발생하는 지연 사망(latent mortality)이 반수치사농도(LC50) 산출에 어떤 영향을 미치는 지를 규명하기 위한 일련의 실험이 수행되었다. 본 연구에서는 실험생물을 카드뮴, 구리, 수은과 같은 중금속, tributyltin (TBT), 암모니아 그리고 방향성탄화수소인 phenanthrene에 각각 96시간 동안 노출시킨 후 깨끗한 해수에 옮겨 다시 6일 동안 배양하면서 사망률을 조사하였다. 실험결과 구리, TBT, 암모니아, phenanthrene과 같은 물질에 노출된 *M. acherusicum*의 사망률은 노출이 끝난 이후에도 계속적으로 증가하는 지연 사망이 관찰되었으며, 이에 따라 기존의 방법으로 산출된 96-h LC50보다 지연 사망을 고려한 새로운 LC50이 크게 낮아지는 경향이 관찰되었다. 지연사망률을 고려하지 않은 기존의 독성시험 결과는 지연 사망의 영향을 반영하지 못하므로 실제 현장에서 발생할 수 있는 오염물질의 영향을 과소평가할 가능성이 있다. 따라서 지연 사망률에 대한 고려는 실제 현장 개체군에 대한 유해오염물질의 영향을 보다 정확하게 예측하는 데에 활용될 수 있을 것이다.

Key words : delayed mortality, amphipod, acute toxicity, metals, TBT, PAHs, LC50

INTRODUCTION

Lethal toxicity tests using various aquatic organisms is an essential part of the ecotoxicological studies to assess the impact of various hazardous pollutants entering into the aquatic environments on the biological systems (Rand, 1995). Traditionally, researchers conducting toxicity tests are quantifying the concentration (or dose) that killed 50% of exposed individuals, since such a median lethal concen-

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tration (LC50) was very convenient when comparing relative toxicities of chemicals or environmental samples and also estimates of median lethal concentrations generally tend to be less variable than those of other percentiles.

However, exposure duration in the traditional toxicity tests was not decided by biological or toxicokinetic attributes of test species and/or test chemicals, but by convenience for workers such as 4 day, which allows workers can begin a test on Monday and finish on Friday (Escher and Hermens, 2004). Four days can be too short for some organisms to exhibit lethal response, which can just maintain minimum physiological functions although they cannot be recovered afterward. Unfortunately, researchers often confronted the difficulty of selecting dead individuals from dying ones in many cases, because some severely damaged individuals could not respond as fast as healthy ones even though they were not dead, which might attribute to the large variability of bioassay results.

Therefore, the conventional test procedure of lethal tests may underestimate the actual impact of tested toxicants on population, if the health of some survivors at the end of the test period is severely damaged and they cannot be recovered even after transferred to uncontaminated water. There were some studies showed that various pollutants had delayed or latent toxic effect on organisms long time after the termination of short exposure. Abel and Garner (1986) reported that the pulse exposure for < 200 min to Cd, Cu, lindane, permethrin or cyanide significantly influenced on the survival rates of amphipod *Gammarus pulex* during the following 14-d incubation period in clean water. Similarly, pulse exposure of *G. pulex* to esfenvalerate at concentrations in the range of 0.1 to 0.6 $\mu g\ L^{-1}$ for as little as 1 h can have effects on the survival, pairing behaviour, and reproductive output that can still be detected at least 2 weeks following the pulse (Cold and Forbes, 2004).

Several factors may be related to the delayed or latent mortality, such as toxic mechanism and bioconcentration factor of the tested chemicals, exposure duration, life stage, experimental condition and so on (Zhao and Newman, 2004). Most importantly, even after the exposure of test animals to pollutants in external medium is stopped, the toxic action of the accumulated pollutants in animal tissues will persist in target sites, if the efflux or detoxification rate of pollutants in animal tissues is not too fast enough (Escher and Hermens, 2004).

In the present study, we exposed amphipod *Monocorophium acherusicum* to various concentrations of six representative pollutants in coastal environments (Cd, Cu, Hg, tributyltin, ammonia and phenanthrene) for 96 hours to decide the traditional LC50 values. Following the exposure, amphipods were transferred to uncontaminated seawater and incubated for 144 hours. We compared the traditional 96-h LC50s of six chemicals to the final LC50s after the additional 144-h incubation period following the pollutant exposure to evaluate the delayed effect of pollutants on the survival of test organisms. Finally, we tried to suggest the new toxic endpoint incorporating the near-future survivorship of the individuals stressed by the pollutant exposure.

MATERIALS AND METHODS

Test animals

Benthic amphipods *Monocorophium acherusicum* was used as test animals in the present study. They were reared in the laboratory of NeoEnBiz Co., of which the initial strain had been collected from a mudflat in Daebudo (37° 17′N; 126° 34′E), located in the Western coast of Korea. Corophiidae amphipods *M. acherusicum* is known as a tube-building deposit-feeder and they are globally distributed including Korean coastal waters (Kim, 1991; Jung, 2000). They were reared in the 20-L containers with clean sediment and overlying water with continuous aeration under static renewal condition with a biological filtering system, and fed ground fish meal (Tetra-Min®) and diatoms.

Various amphipod species have been used in vari-

ous bioassays to assess the bioavailability and toxicity of pollutants especially in sediments, because amphipods are widely distributed, easily collectable, tolerable to handling and culturing in laboratory, and one of the most sensitive taxa among various benthic animals. Amphipods were also used for water-only bioassay such as single chemical toxicity tests and whole effluent tests, since they responded to the dissolved phase of pollutants as well as to the particulate phase.

General experimental conditions

Filtered (GF/F) seawater provided by Incheon Fisheries Research Institute in Yeongheung-do was used as test media for all experiments. Water quality of test seawater or overlying water was checked at the beginning and end of each toxicity test. Salinity, temperature and dissolved oxygen were maintained at 30 psu, 20°C and over 80%. Exposure system was static-renewal and test solutions were exchanged everyday.

Test solutions were spiked by stock solutions prepared by established protocols (ASTM, 1999). Stock solutions of Cd, Cu, Hg and ammonia were prepared in deionized water by dissolving adequate amount of $CdCl_2$, $CuSO_4 \cdot 5H_2O$, $HgCl_2$ and NH_3Cl (reagent grade, Aldridge®) and those of TBT and phenanthrene in acetone by dissolving adequate amount of

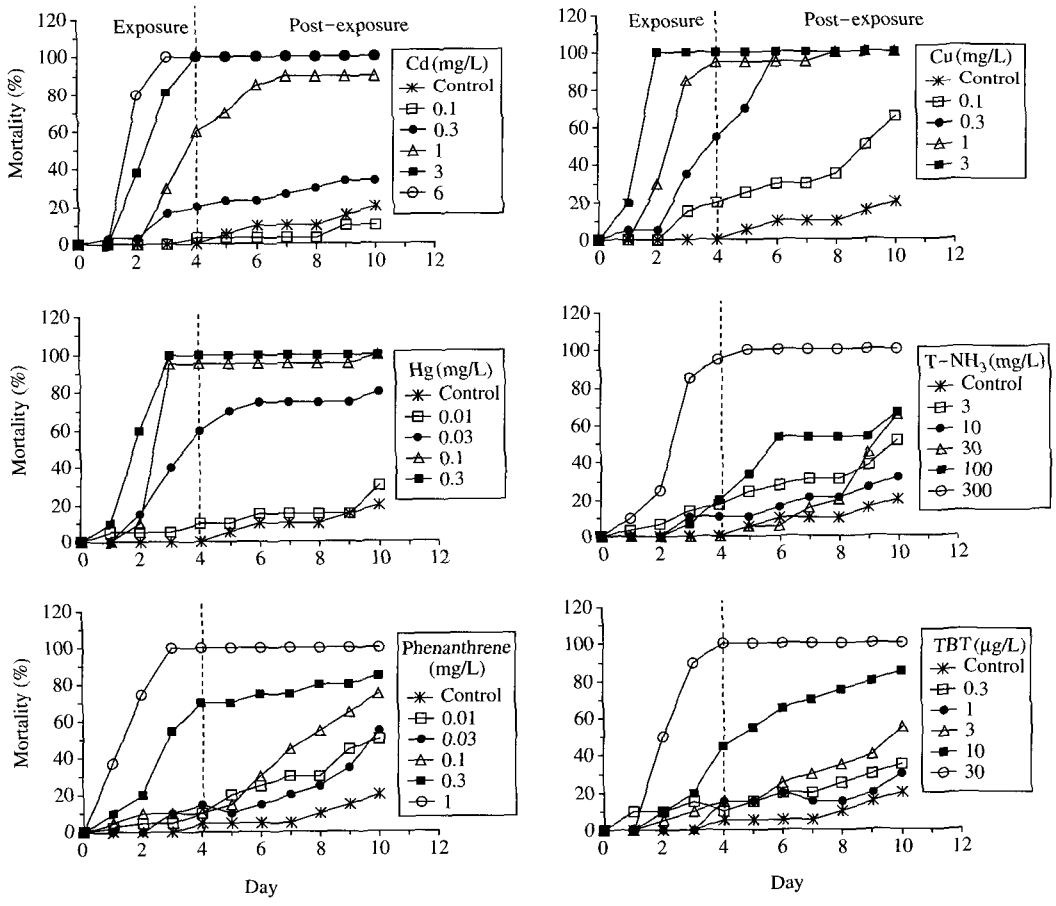

Fig. 1. Temporal variation of mortalities of *M. acherusicum* exposed to Cd, Cu, Hg, total ammonia (T-NH_3), phenanthrene and tributyltin (TBT). The vertical dashed lines at 96 hr separate the exposure and depuration period.

tributyltin chloride and phenanthrene solution (reagent grade, Aldridge®).

Exposure to pollutants

Individuals of *M. acherusicum* were exposed to control and 0.1, 0.3, 1, 3 and 6 mg/L of Cd, 0.1, 0.3, 1 and 3 mg/L of Cu, 0.01, 0.03, 0.1 and 0.3 mg/L of Hg, 0.3, 1, 3, 10, 30 and 100 μg/L of tributyltin, 3, 10, 30, 100 and 300 mg/L of total ammonia, and 0.01, 0.03, 0.1, 0.3 and 1 mg/L of phenanthrene, respectively. Twenty individuals allocated into four replicate 500-mL beakers were exposed to control and spiked media for 4 d. Following the 4-d exposure to pollutants, test animals were transferred to clean seawater and incubated for additional 6 d. During the pollutant exposure and depuration period, dead individuals were counted and removed everyday.

Data analysis

Student t-test and ANOVA tests were conducted using SPSS® to compare means of survival or avoidance data among treatments. LC50 values were estimated using Probit analysis or trimmed Spear-

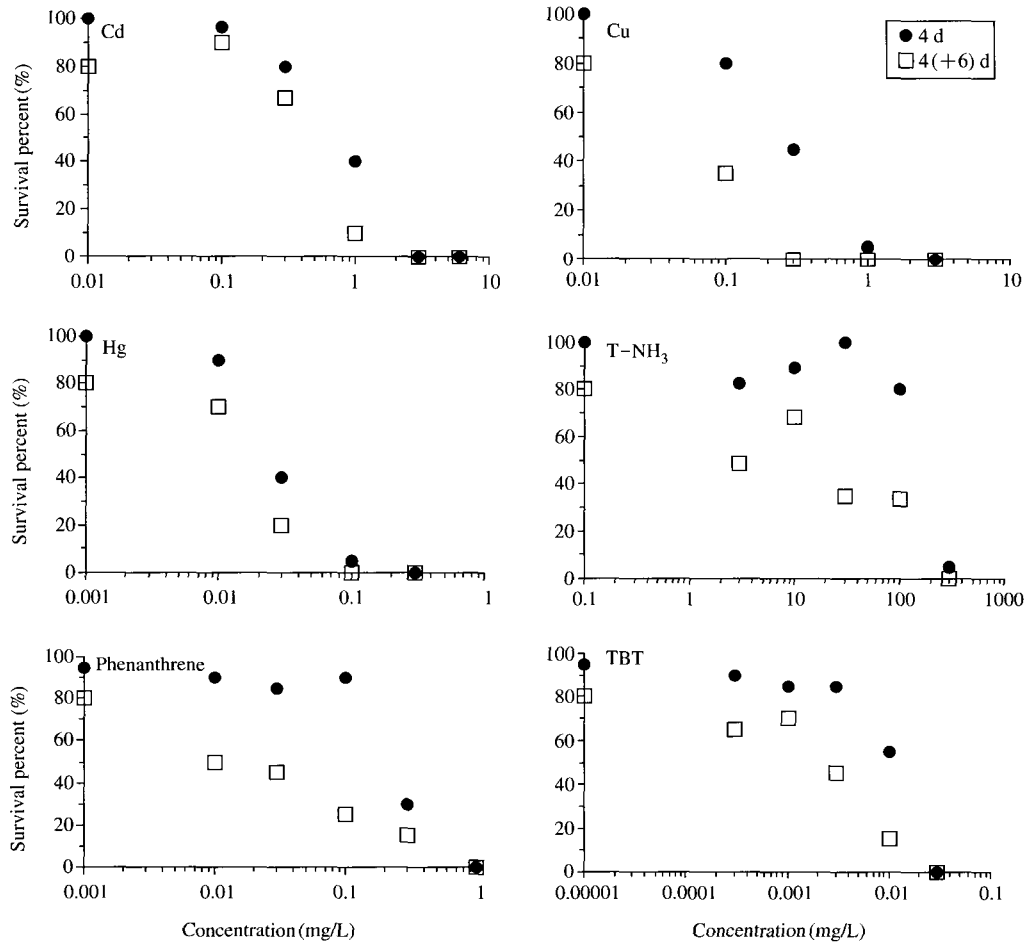

Fig. 2. Comparison of survival percent (%) of *M. acherusicum* at the end of 4-d exposure (●) to various concentrations of Cd, Cu, Hg, total ammonia, phenanthrene and tributyltin (TBT) with those at the end of 6-d post-exposure period (□).

man-Karber method depending on the data (USEPA, 1994). Unionized ammonia concentration was calculated using an equation from USEPA (1999).

RESULTS

Variation of survival rates during the 4-d exposure period

The mortality of *M. acherusicum* significantly varied as a function of concentrations of each pollutant and exposure time (Fig. 1). More than 50% of test animals were dead for the highest concentrations of most pollutants even after the 2-d exposure period except ammonia. Survival rates of amphipods incubated for 96 h in the seawater spiked with pollutant concentrations higher than 0.3, 0.1, 0.03, 100, 0.3 and 0.01 mg/L of Cd, Cu, Hg, total ammonia, phenanthrene and tributyltin (TBT), respectively, were significantly lower than in Control (Fig. 1).

Variation of survival rates during the 6-d post-exposure period

The survival rates of *M. acherusicum* in some treatments continuously decreased even after they were

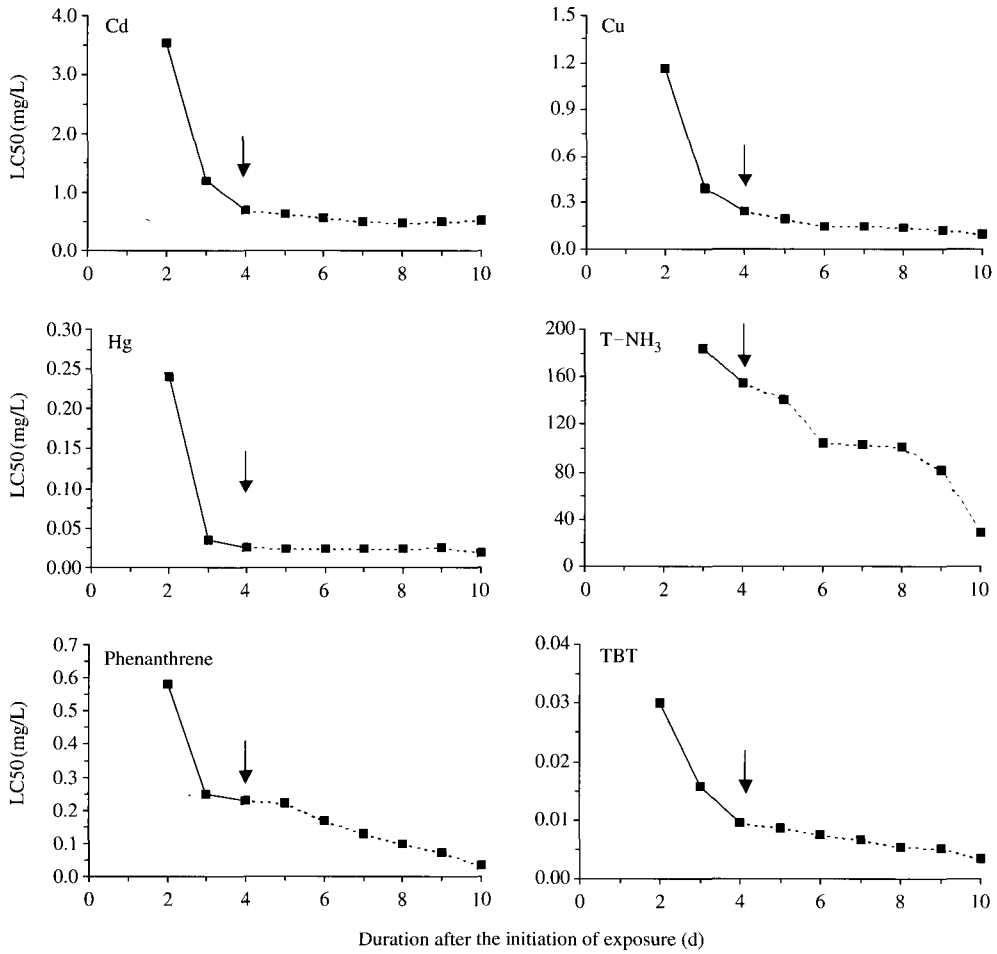

Fig. 3. Temporal variation of LC50s of Cd, Cu, Hg, total ammonia (T-NH_3), phenanthrene and TBT for *M. acherusicum*. Arrows and dashed lines indicate the cessation of exposure to pollutants and LC50 values during the post-exposure period, respectively.

transferred into the uncontaminated seawater (Fig. 1). The considerable mortality during the post-exposure period occurred when the test animals had been previously exposed to the 1 mg/L of Cd, 0.3 mg/L of Cu, 0.01, 0.03 and 0.1 mg/L of phenanthrene, 30 and 100 mg/L of total ammonia, and 3 and 10 μg/L of TBT. Mortality in Control also increased up to 20% during the post-exposure period.

The difference between mortalities at the end of the 4-d exposure period and at the end of the following 6-d post-exposure period was greatest for phenanthrene, followed by Cu, ammonia, TBT, Cd and Hg (Fig. 2). In the case of Cd and Hg, the difference of mortality before and after the depuration period was just comparable to control mortality (Fig. 2).

Temporal variation of LC50s during and after pollutant exposure

The median lethal concentrations (LC50s) sharply decreased during the exposure period (Fig. 3). The ratio of 2-d LC50 to 4-d LC50 was greatest for Hg (9.3X), and followed by Cd (5.1X), Cu (4.6X), TBT (3.2X) and phenanthrene (2.5X). The 2-d LC50 for total ammonia could not be estimated, since more than 50% of amphipods survived exposed to the highest concentration at t = 2 d (Fig. 1).

The LC50s of pollutants also decreased after the amphipods were transferred into the uncontaminated water (Fig. 3). The ratio of 4-d LC50 to LC50 after the 6-d depuration period was greatest for phenanthrene (6.7X), and followed by ammonia (5.3X), TBT (2.7X), Cu (2.5X), Cd (1.3X) and Hg (1.3X).

DISCUSSION

Comparing the sensitivity of *M. acherusicum* to chemicals with different amphipod species

The sensitivity of *Monocorophium acherusicum* to various inorganic and organic pollutants was first evaluated in the present study even though this species distributed worldwide. The sensitivity of *M. acherusicum* was generally comparable to that of other amphipod species when comparing LC50 values. The 96-h or 4-d LC50 of Cd ranged 0.1 to 3 mg/L for *Leptocheirus plumulosus* (McGee *et al.*, 1998), 0.3 to 1.3 mg/L for *Ampelisca abdita* (Kohn *et al.*, 1994), 0.5 to 1.9 mg/L for *Rhepoxynius abronius* (Kohn *et al.*, 1994), and 1 to 14 mg/L for *Corophium volutator* (Kater *et al.*, 2000), and all of these species are most commonly adopted in various ecotoxicity tests. These ranges explicitly enclose the 96-h LC50 of Cd for *M. acherusicum* (0.63 mg/L) from the present study and those from our previous study using same species (0.7 ~ 1.7 mg/L; Lee *et al.*, 2005). Not many studies evaluated the sensitivity of amphipods to Cu and Hg. The 96-h LC50s of Cu for freshwater amphipod *Echinogammarus tibaldii* (0.6 mg/L; Pantani *et al.*, 1997) and *Hyalella azteca* (0.06 ~ 0.7 mg/L; Suedel *et al.*, 1996) seemed to be comparable to Cu LC50 for *M. acherusicum*, while the 4-d LC50 of Hg for *E. tibaldii* (0.5 mg/L; Pantani *et al.*, 1997) was about 20X greater than that of *M. acherusicum* (0.026 mg/L).

The LC50s of total ammonia for *M. acherusicum* (155 mg/L) was comparable or slightly greater than those for other amphipods, including *L. plumulosus* (44 ~ 99 mg/L; Moore *et al.*, 1997), *A. abdita* (50 mg/L; Kohn *et al.*, 1994), *H. azteca* (22 ~ 204 mg/L; McDonald *et al.*, 1997), and *R. abronius* (79 mg/L; Kohn *et al.*, 1994). Higher tolerance of *M. acherusicum* to ammonia would be advantageous as sediment testing organisms, since ammonia is attributed as the important confounding factor severely interfering sediment bioassay results (Ankley *et al.*, 1994; U.S.EPA, 1994).

The 4-d LC50s of phenanthrene for *Eohaustorius estuaries* and *L. plumulosus* were 158 and 180 μg/L, respectively (Medador *et al.*, 1993; Boese *et al.*, 1997), which were slightly lower than that of *M. acherusicum* (230 μg/L). *E. estuaries* was most sensitive to TBT, which had the lowest LC50s (1.5 ~ 2.0 μg/L; Meador *et al.*, 1993), followed by *H. azteca* (5.3 μg/L; Bushong *et al.*, 1988), *M. acherusicum* (9.5 μg/L) and *R. abronius* (51 ~ 173 μg/L; Meador *et al.*, 1993).

Delayed mortality of *M. acherusicum* during the post-exposure period

The extent of delayed mortality depends on several factors including exposure concentration, exposure duration, mode of toxic action, and species-specific sensitivity.

The delayed mortality was evident for individuals previously exposed for 4 days to higher than 0.01 mg/L of phenanthrene, 0.1 mg/L of copper, 3 mg/L of total ammonia, and 0.3 μg/L of TBT. Among these ranges of test concentrations, most of all were all below 4-d LC50 of each chemical, however, the percent survival of *M. acherusicum* at these concentrations were mostly below 50% following the 6-d post-exposure period. In contrary, cadmium and mercury did not show a significant delayed mortality in all of treatment levels.

The extent of delayed mortality varied among chemicals in the present study. Consistently, Zhao and Newman (2004) found that the latent mortality was more evident when *Hyalella azteca* was exposed to Cu than pentachlorophenol (PCP), which might be due to the different mode of toxic action of two chemicals. They suggested that toxicity of PCP would be more reversible than that of Cu, since PCP is less cumulative and it is metabolized faster than Cu. However, Cu presumably attacks cells in gill epithelia and the damaged cells will not be easily recovered within a few days.

We also found that *M. acherusicum* showed the substantial latent mortality when exposed to Cu. However, we could not find the similar result for Cd and Hg, which probably have similar modes of toxic action with Cu. This inconsistency might be related to other factors such as exposure concentration, exposure duration and/or toxicokinetic parameters (e.g. uptake and elimination rate) of each element. Further studies need to be conducted to investigate whether *M. acherusicum* exposed to Cd, Hg or other metal pollutants will show the latent mortality in different exposure concentrations or duration.

Meanwhile, it was notable that test animals exposed to phenanthrene showed the most substantial latent mortality. The present results suggested that animals damaged by the exposure to a certain level of phenanthrene could not recover. The mode of acute toxicity of PAH compound is usually attributed as narcosis, which is known as very reversible (Di Toro *et al.*, 2000). Therefore, we can speculate that phenanthrene may have another irreversible mode of toxic actions or that damage due to narcotic effect (at least by phenanthrene) may not be so reversible as we thought. Our previous study also suggested that acute toxicity of PAH compounds including phenanthrene, fluoranthene and pyrene to puffer fish could not be explained by general narcotic model based on reversible response, indicating that there might be another important mode of toxic action for PAHs in addition to narcosis (Lee *et al.*, 2004).

Ammonia is toxic to organisms because it can disrupt biochemical reactions. It can combine with protons to elevate blood and intracellular pH, and also ammonium ion can disrupt normal transmembrane movements of other ions (Walsh, 1998). Most important toxic action of ammonia would be its deleterious effect on the nervous system (Randall and Tsui, 2002). The damage caused by ammonia or ammonium ion at the target sites of *M. acherusicum* exposed to 3~100 mg/L of total ammonia seemed not to be recovered after they were transferred into clean water.

The present study adopted only 6 days of depuration period, since the significant mortality occurred in Control, probably due to starvation. Test animals were not fed during the whole test period in order to maintain the experimental condition consistently during and after the exposure. Further studies adopting adequate feeding regime during post-exposure can elucidate how the delayed effect of pre-exposure to pollutants can occur for longer periods.

Predicting pollutant effects using the new LC50 incorporating delayed mortalities

Damage caused by toxicants is a function of exposure concentration and duration and the mortality is a result of accumulated damage in target cells (Lee *et*

al., 2002). Therefore, considerable mortality can occur latently after the end of exposure to pollutants. In the present study, mortality of *M. acherusicum* increased continuously after the end of exposure to some pollutants. However, most traditional toxicity tests did not incorporate the latent mortality probably because the ecotoxicity testing procedures were primarily derived from mammalian toxicity testing protocols, which are usually designed for the measurement of toxic effects on individuals, while ecotoxicological tests are often conducted to predict the effects on field population (Zhao and Newman, 2004). Even three decades ago, Linden (1976) pointed out some important shortcomings in standard testing procedures, including the latent effect following the short pollutant exposure, by observing the survival of *Gammarus oceanicus* for 30 days following the 48-h exposure to crude oil. Although most of *G. oceanicus* survived at the end of exposure period, they were dead about 2 weeks after the end of exposure, while all individuals survived in control for the 30-d experimental period. Consistently, the present study showed that the conventional LC50 might severely underestimate the deleterious effect of pollutants on the field populations of *M. acherusicum*, since the considerable number of individuals could not be recovered from damage by exposure to some pollutants. Therefore, the observation should be continued after exposure ends to incorporate the delayed mortality information for better assessing realistic lethal effects on the population of test species in field.

It is not certain how long the post-exposure observation should be extended. This study observed the apparent latent effect by adopting 6-d depuration period following the standard 96-h exposure. However, Cold and Forbes (2004) exposed *Gammarus pulex* to the organophosphate pesticide, esfenvalerate only for hours and observed the survival and reproduction of the pre-exposed organisms were significantly decreased during the depuration phase elongated up to 15 d. Post-exposure observation period can be determined depending on the test end point and toxicological attributes of pollutants. Practically, the post-exposure observation can be extended until control survival maintained above the test acceptability criteria. For *M. acherusicum*, control survival maintained over 90% until 4 days after the end of exposure, while it decreased to 80% at the end of post-exposure period. Therefore, 4 days of post-exposure observation would be preferable for estimating LC50 incorporating delayed mortality for *M. acherusicum*. Further understanding the influence of the toxicokinetic and toxicodynamic variables (e.g. elimination and damage-recovery rate) of the pollutants on the occurrence and extent of latent toxicity can help to modify the current bioassay protocols adequately integrating toxicity results during and after exposure.

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