Ocean Science Journal, 40(1), 17-24(2005)

Article

Ocean Science Journal

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Application of Indigenous Benthic Amphipods as Sediment Toxicity Testing Organisms

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Received 17 June 2004; Revised 27 November 2004; Accepted 18 March 2005

Abstract – A series of experiments were conducted to develop standard test organisms and test protocols for measuring sediment toxicity using candidate amphipods such as *Mandibulophoxus mai, Monocorophium acherusicum, Haustorioides indivisus,* and *Haustorioides koreanus,* which are indigenous to Korea. The relevant association of test species with sediment substrates was one of the important factors in sediment bioassay. The indigenous amphipods *M. mai* and *M. acherusicum* were well associated with test sediments when they were exposed to various sediment substrates from sand to mud. The tolerant limits to various physico-chemical factors affecting bioassay results such as temperature, salinity and ammonia, as well as sensitivities to reference toxicant and contaminated sediments, were investigated using *M. mai* and *M. acherusicum* in the present study. These amphipods were tolerant to relatively wide ranges of salinity (10~30 psu) and ammonia (<50 ppm), and displayed relevant sensitivity to temperature as well. They are more sensitive to Cd, the reference toxicant, when compared to the standard test species used in other countries. Field-sediment toxicity tests revealed that *M. mai* would be more sensitive to sediment-associated pollutants than *M. acherusicum*, while the sensitivity of *M. acherusicum* was comparable to that of *Leptocheirus plumulosus*, which has been used as a standard test species in the United States of America. Overall results of this first attempt to develop an amphipod sediment toxicity test protocol in Korea indicated that *M. mai* and *M. acherusicum* would be applicable in the toxicity assessment of contaminated sediments, following the further evaluation encompassing various ecological and toxicological studies in addition to test method standardization.

Key words – sediment toxicity test, amphipods, bioassay, *Mandibulophoxus mai, Monocorophium acherusicum*

1. Introduction

Sediment constitutes a repository of various pollutants such as heavy metals and persistent organic chemicals in most aquatic environments (Luoma 1989; Lee *et al.* 2000). Sediments can also include contaminant inputs from historic episodes into the aquatic environments. Contaminant concentrations in the water column have decreased through regulatory activities to reduce the input of contaminants from various anthropogenic sources in most developed countries. However, the contaminant concentrations in sediments would change relatively slowly and consequently contaminated sediments are often considered as a secondary source of pollution (Burton 1992).

The assessment of contaminated sediments has been carried out by various approaches such as chemical analysis of contaminant concentrations in environmental samples, ecological surveys using benthic community structure and sediment bioassay (USEPA 1992). Each approach provides complementary information in the integrative assessment of sediment quality. Among those approaches, only sediment bioassay can provide the direct evidence of toxicity exerted by contaminant mixtures in sediments. Therefore, sediment bioassay has been considered an essential component in the systematic assessment of contaminated sediments.

Recently, there has been a substantial increase in research and regulatory activity involving the contamination of estuarine and coastal environments in Korea. Consequently, concerns on the problem of contaminated sediments and their adverse effects on the aquatic ecosystem have increased. However, few studies have focused on the toxicity of contaminated sediments on the benthic animals and it is necessary to develop adequate standardized test organisms and methods for sediment toxicity tests.

Sediment toxicity tests are typically used to determine whether the tested sediment is toxic or non-toxic to benthic organisms by comparing a response in a test organism exposed to target sediments with control. Most standardized

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whole-sediment toxicity tests in other countries have adopted amphipods as test species because they are one of the most sensitive taxa among benthic animals, ecologically relevant, distributed worldwide, available in large numbers and easily culturable in the laboratory. Several amphipod species have been developed as standard test species for sediment bioassay in North America and Europe such as *Ampelisca abdita*, *Corophium volutator*, *Eohaustorius estuaries*, *Hyalella azteca*, *Leptocheirus plumulosus* and *Rhepoxynius abronius* (Stephenson and Mackie 1989; USEPA 1994, 2000, 2001; ASTM 1999; Kater *et al.* 2000). Unfortunately, most of these species are not found in Korean coastal environments (Kim 1986, 1991; Jo 1989; Jung 2000). Using indigenous species was usually recommended in the assessment of contaminated field sediments, if the adequate species and standardized test protocols were available, because the toxic responses of the indigenous species possibly represent the adverse effect of pollution on the regional benthic environment.

The objectives of the present study were to evaluate the applicability of indigenous amphipods as test organisms for sediment bioassay and also to discover the adequate test conditions would be for each species by investigating (1) the avoidance and survival in sediments according to various grain sizes, (2) tolerance to physico-chemical conditions such as temperature, salinity and ammonia concentration, (3) the sensitivity to reference toxicant, Cd and finally (4) the applicability of test species to the assessment of contaminated field sediments.

2. Materials and Methods

Test animals

Benthic indigenous amphipods *Mandibulophoxus mai, Haustorioides indivisus*, and *Haustorioides koreanus* were collected from Manripo beach (36°48'N; 126°11'E), and *Monocorophium acherusicum* from a mudflat in Daebudo (37°17'N; 126°34'E), located on the Western coast of Korea. Indigenous amphipods *M. mai, H. indivisus* and *H. koreanus* are free burrowing infauna, and *M. acherusicum* is tube-building deposit-feeders. All these amphipod species are widely distributed in the Western and Southern coastal environments in Korea (Jo 1989; Kim 1991; Jung 2000). Following the collection of amphipods, they were transported to the laboratory and acclimated to the experimental conditions (temperature, salinity, etc.) for 1-2 weeks. Benthic amphipods *Leptocheirus plumulosus*, which is a standard test species in North America, were purchased from Chesapeake Cultures, Inc. in VA, USA and acclimated in the laboratory. During the acclimation period, amphipods were reared in the 20-L containers with clean sediment and overlying water with continuous aeration, and fed diatom *Phaeodactylum tricornutum* and ground fish meal (TetraMin®).

General experimental condition

Filtered (GF/F) seawater provided by Incheon Fisheries Research Institute in Yeongheungdo was used as the test water media for all the experiments. Water quality of the test seawater or overlying water was checked at the beginning and end of water exchange. Salinity and temperature were maintained at 30 psu and 20°C, otherwise noted. Dissolved oxygen was always maintained at over 80%. Test media were exchanged everyday for water-only tests without aeration or exchanged every 5 d for sediment test with aeration. Negative and positive controls were always added to each experimental batch to confirm the test acceptability of test animals and the experimental procedure.

Avoidance and survival in sediments

Four amphipod species *M. mai, H. indivisus, H. koreanus* and *M. acherusicum* were incubated in sediments with 3 different sediment grain size compositions (sand only, mud only and a 1:1 mixture of sand and mud) for 10 days to test whether the survival and avoidance of each species were relevant as test organisms for sediment bioassay. Each species was incubated separately. Three replicate beakers were used for each sediment type and 20 individuals were introduced to each replicate. During the incubation, the number of individuals not burying in sediments was counted everyday to estimate the avoidance rates of animals. At the end of the incubation period, all the surviving individuals were recovered using a 0.5-mm sieve and counted. Avoidance or survival rate (%) was calculated by dividing the mean number of avoided animals or survived animals by the initial number of introduced animals (20 in the present study) and multiplied by 100.

Sand was collected from Manripo beach and mud was collected from mudflat in Yeongjongdo; particles with unnecessary grain size were removed by wet sieving (63-μm). Separate analysis of grain size of test sediments showed that mean sand content (%) for sand only and 1:1 mixed sediment of sand and mud was 99.9% and 54.1%, respectively, and mean mud content (%) for mud only sediment was 99.5% (N=3).

Tolerance to physico-chemical conditions

Amphipods *M. mai* and *M. acherusicum* were incubated under various temperatures, salinities or ammonia concentrations to test whether each species had the relevant tolerance range for these physico-chemical factors and also to determine the most adequate experimental condition for each species. *M. mai* was incubated at 13, 18, 20, 22 and 25°C and *M. acherusicum* at 5, 10, 15, 18, 20, 22, 25 and 30°C for 10 d to evaluate the effect of temperature on survival. To evaluate salinity effect, both *M. mai* and *M. acherusicum* were incubated in water media having 1, 5, 10, 15, 20, 30 and 35 psu (and additional 40 psu for *M.*

acherusicum) for 10 d. Both amphipods were exposed to 0~300 mg/l of total ammonia to determine the effect concentration of ammonia in water media.

Sensitivity to reference toxicant

Four amphipod species *M. mai, H. indivisus, H. koreanus* and *M. acherusicum* were exposed to control and 0.1, 0.3, 1, 3 and 10 mg/l of dissolved Cd in seawater (30 psu at 20°C) for 4 d and subsequent mortality at each concentration was determined by estimating median lethal concentrations (LC50) of Cd for each species. Preparation of Cd-spiked seawater was followed by established protocols (ASTM 1999). Thirty individuals of each species were introduced to each Cd concentration.

Sensitivity to field contaminated sediments

Amphipods *M. mai, M. acherusicum* and *L. plumulosus* were exposed to mixtures of uncontaminated and contaminated sediments in two separate bioassays. Amphipods *M. mai* and *M. acherusicum* were exposed to serial dilutions of sediment from a site near Sihwa industrial complex and subsequently *M. acherusicum* and *L. plumulosus* were exposed to serial dilutions of sediment from a site near Onsan industrial complex. Both sediment sampling sites were previously known as contaminated by heavy metals including Cd, Cu and Zn.

Muddy sediment from Yeongjongdo was used as reference sediment for all amphipods. The fraction of contaminated sediment varied from 2.1 or 2.5 to 100% for each bioassay. Each treatment had four replicate 1-l beakers. Twenty individuals of each species were added to each replicate. Following the 10-d incubation, survived individuals were recovered and LC50 were estimated for each species.

Data analysis

Statistical analysis procedures recommended by U.S.EPA (1994) were adopted in this study. Dunnett's test was used to compare means of survival or avoidance data among treatments when the data satisfied the normality and homogenous variance assumptions, otherwise nonparametric Steel's multi-task rank test was used. The LC50 values were estimated using Probit, trimmed Spearman-Karber or graphical methods depending on the results. Unionized ammonia concentration was calculated using an equation from USEPA (1999).

3. Results

Avoidance and survival in uncontaminated sediments

Survival rates of the *M. mai, H. koreanus, H. indivisus* and *M. acherusicum* were all higher than 90% when incubated in sediments with various grain sizes for 10 d except in the case of the survival rates of *H. koreanus* (80%) incubated in sand and mud mixture (Table 1). Avoidance was greatest for *H. koreanus* followed by H. indivisus and *M. mai*, while no avoidance was shown for *M. acherusicum* (Table 1). Avoidance rates of *M. mai*, *H. koreanus* and *H. indivisus* were generally lower in sand-only sediments and increased with the mud content in substrates.

Tolerance to physico-chemical conditions

Survival rates of *M. mai* were all 100% at the range of 13 to 20°C, while decreasing to 90.0±4.7 and 76.7±10.0% at 22 and 25°C during the 10-d incubation period, respectively (Fig. 1). The survival rates of *M. acherusicum* were >90% in the range of 15~20°C and >80% in the range of 10~22°C. However, the survival rate of *M. acherusicum* sharply decreased to ~20% at 5°C (Fig. 1).

The survival rates of *M. mai* were all >90% when the salinity of experimental media varied in the range of 15 ~ 35 psu, but decreased to 80, 43 and 0% when the salinity decreased to 10, 5 and 1 psu, respectively (Fig. 2). The survival rates of *M. acherusicum* were > 90% in the range of 15~30 psu, and decreased from 77 to 0% with the decrease of salinity from 10 to 1 psu.

The mortality rates of *M. mai* and *M. acherusicum* increased with the ammonia concentrations in water media (Fig. 3). The 96-h LC50s of total ammonia (and unionized ammonia as NH_3) for *M. mai* and *M. acherusicum* were 105 (2.6) and 155 (3.8) mg/L, respectively. The fraction of unionized ammonia was ~2.4% of total ammonium concentration under conditions of 20°C, 30 psu and pH 7.8.

Sensitivity to reference toxicant

The sensitivity of amphipods to Cd, the reference toxicant, in

Table 1. Mean survival and avoidance rate (%; mean ± SD) of four indigenous benthic amphipod species incubated for 10 d in seawater and sediment substrate (sand only [Sand], mud only [Mud] and 1:1 mixture of sand and mud [Sand+Mud]).

Species	Survival (%)			Avoidance (%)		
	Sand	Sand+Mud	Mud	Sand	Sand+Mud	Mud
Mandibulophoxus mai	95 ± 3	90 ± 0	93 ± 3	0	5.2 ± 3.2	6.3 ± 4.1
Monocorophium acherusicum	90 ± 0	93 ± 3	93 ± 4	0	0	0
Haustorioides indivisus	95 ± 7	95 ± 7	90 ± 7	6.3 ± 2.9	8.8 ± 0.6	9.0 ± 0.3
Haustorioides koreanus	95 ± 7	80 ± 14	95 ± 7	0.0	47 ± 4	86 ± 12

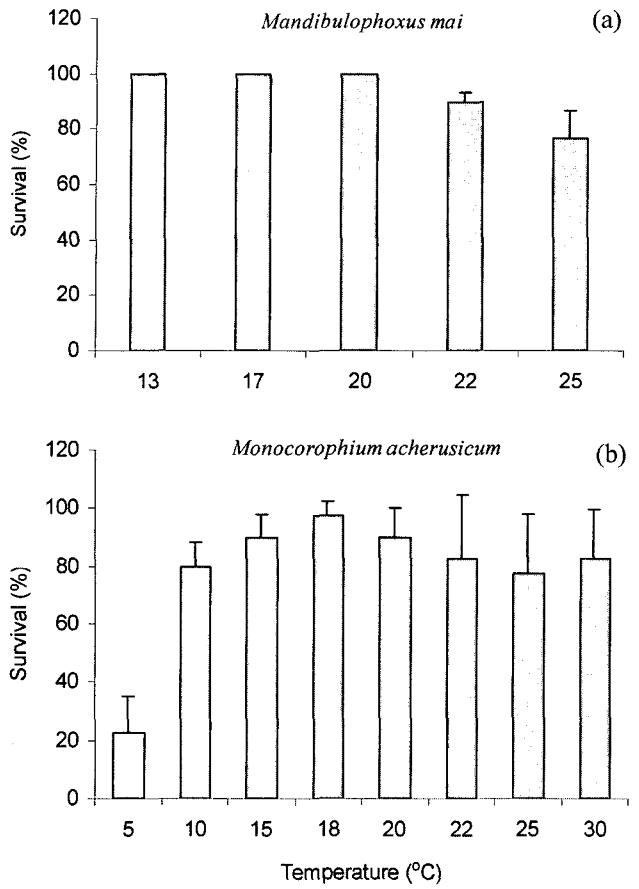


Fig. 1. Mean survival rates (±SD; %) of (a) *Mandibulophoxus mai* and (b) *Monocorophium acherusicum* incubated at varying temperature conditions for 10 d.

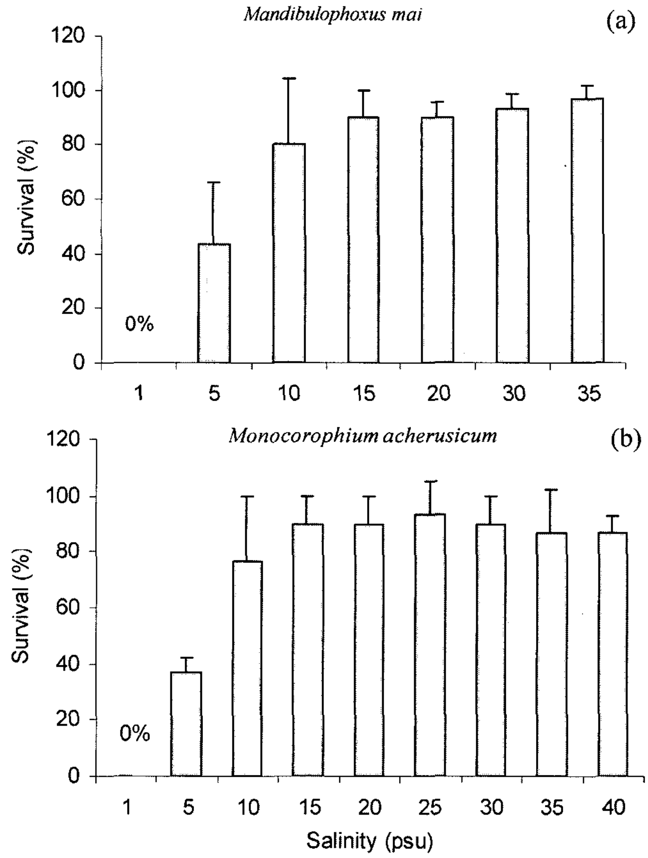


Fig. 2. Mean survivals rates (±SD; %) of (a) *Mandibulophoxus mai* and (b) *Monocorophium acherusicum* exposed to varying salinity conditions for 10 d.

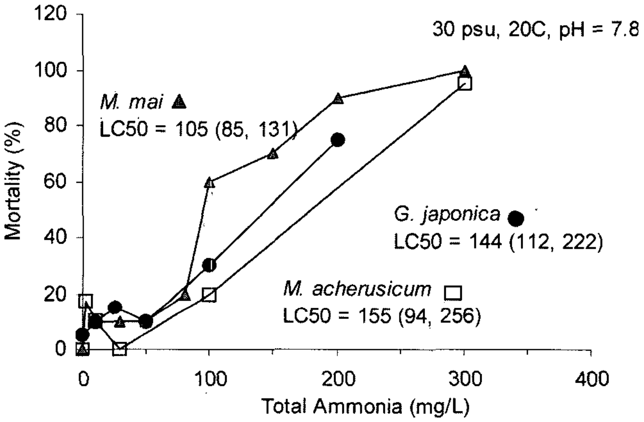


Fig. 3. Mortality (%) of *Mandibulophoxus mai* and *Monocorophium acherusicum* exposed to various ammonia concentrations in seawater for 96 h. LC50 values and 95% confidence intervals are indicated for each species.

water varied depending upon species and testing dates (Table 2). Mean 96-h LC50 of Cd was highest for *H. koreanus*, and the LC50 values for the other species (*M. mai, H. indivisus* and *M. acherusicum*) were generally similar (1.2~1.7 mg/L) in this study (Fig. 4). Intra-specific variation of LC50 was also greatest for *H. koreanus*, but the LC50 values of *M. mai* and *M. acherusicum* were relatively consistent among different test batches (Table 2). The LC50 values of Cd for the amphipod species tested here were usually comparable or considerably lower when compared to the LC50 values for the standard test species used in other countries (Fig. 4).

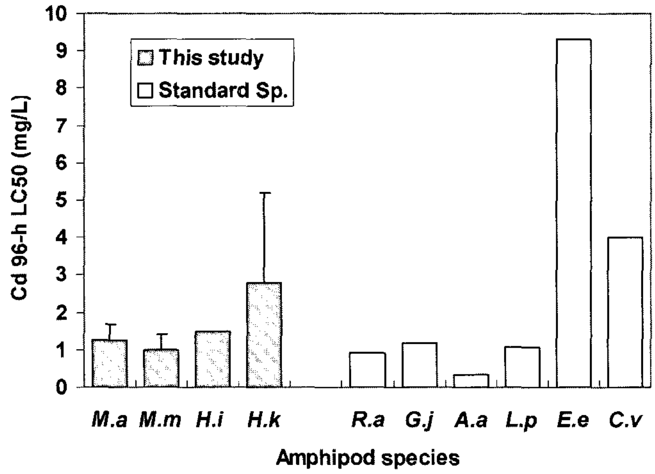
Sensitivity to field contaminated sediments

The relative sensitivity of *M. mai, M. acherusicum* and *L. plumulosus* to sediment-associated pollutants was compared by exposing the amphipods to the dilution of field-contaminated sediments (Fig. 5, 6). Significantly high mortality of *M. mai* compared to the reference sediment was observed even in sediment containing only 2.5% of the contaminated sediment from Sihwa, and the mortality of *M. mai* increased proportionally to the fraction of contaminated sediment (Fig. 5). All individuals of *M. mai* were dead when they were exposed to the sediments with

Table 2. Comparison of 48-h and 96-h median lethal concentrations (LC50; mg/L) of four indigenous amphipod species exposed to dissolved Cd.

Species	Test Date	48h LC50	96h LC50
Mandibulophoxus mai	'01.3.2	>3	1.2
	'02.9.2	>3	1.3
	'02.12.9	>3	1.4
	'03.5.15	5.5	0.5
	'03.11.24	>3	1.2
Haustorioides koreanus	'02.12.9	21	5.5
	'03.5.15	6.3	0.8
	'03.6.20	-*	2.0
Haustorioides indivisus	'03.5.15	5.1	1.5
Monocorophium acherusicum	'03.5.15	4.5	1.6
	'03.6.12	3.5	0.7
	'03.6.27	-	1.2
	'03.11.24	3.7	1.7
	'04.4.28	3.1	1.6
	'04.8.1	1.9	1.1
	'04.10.4	-	1.7

*No available data



M.m: *M. mai*
M.a: *M. acherusicum*
H.i: *H. indivisus*
H.k: *H. koreanus*
R.a: *Rhepoxynius abronius*
G.j: *Grandidierella japonica*
A.a: *Ampelisca abdita*
L.p: *Leptocheirus plumulosus*
E.e: *Eohaustorius estuarius*
C.v: *Corophium volutator*

Fig. 4. Comparison of 96-h Cd LC50 (mg/L) among various benthic amphipod species. Dark bars represent data from this study and light bars previous data using standard amphipod species found in other literature (USEPA 1994; Kater *et al.* 2000). Error bars represent standard deviation when multiple tests were conducted.

>50% of contaminated sediments. However, no significant mortality was observed for *M. acherusicum* exposed to the same contaminated sediments and a considerable number of *M. acherusicum* survived (~70%) even at the original contaminated sediment rates without dilution (Fig. 5). Mortalities of *M. acherusicum* exposed to >8.5% of Onsan sediment significantly increased compared to uncontaminated sediment. The LC50 of Onsan sediment for *M. acherusicum* (9.6%) was higher than that (4.0%) of *L. plumulosus* (Fig. 6).

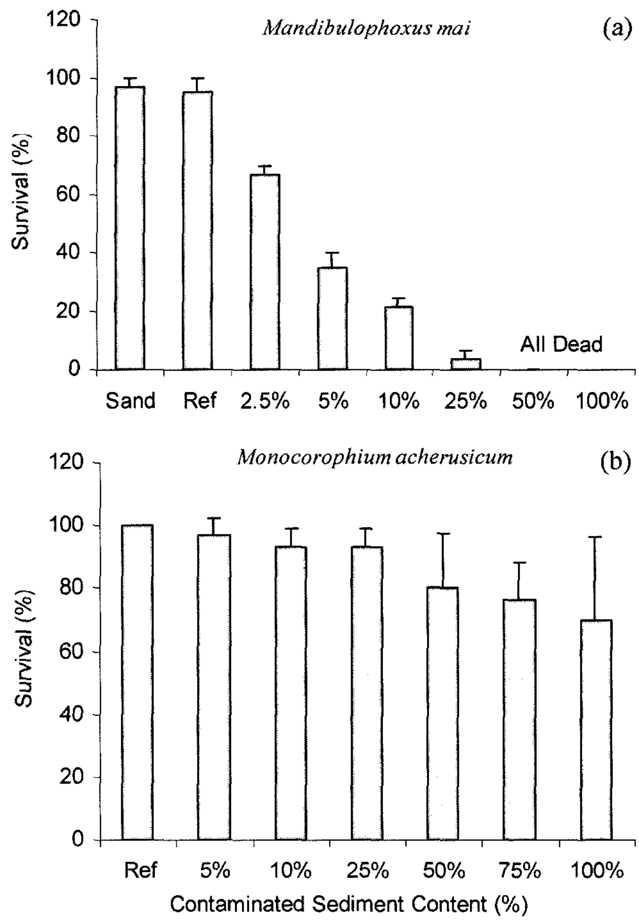


Fig. 5. Mean survivals rates (±SD; %) of (a) *Mandibulophoxus mai* and (b) *Monocorophium acherusicum* incubated in substrates composed of mixtures of contaminated sediments from a site near Sihwa industrial complexes and uncontaminated reference sediment (Ref) from a mudflat in Yeonjongdo for 10 d. Sand sediment was included only for *M. mai* as negative control.

4. Discussion

Selection of test species

Amphipods have been widely used in the biological assessment of contaminated sediments since the test method was developed in the 1970s (Swartz *et al.* 1985; USEPA 1994). However, only a few studies adopting sediment bioassay using amphipods were conducted in

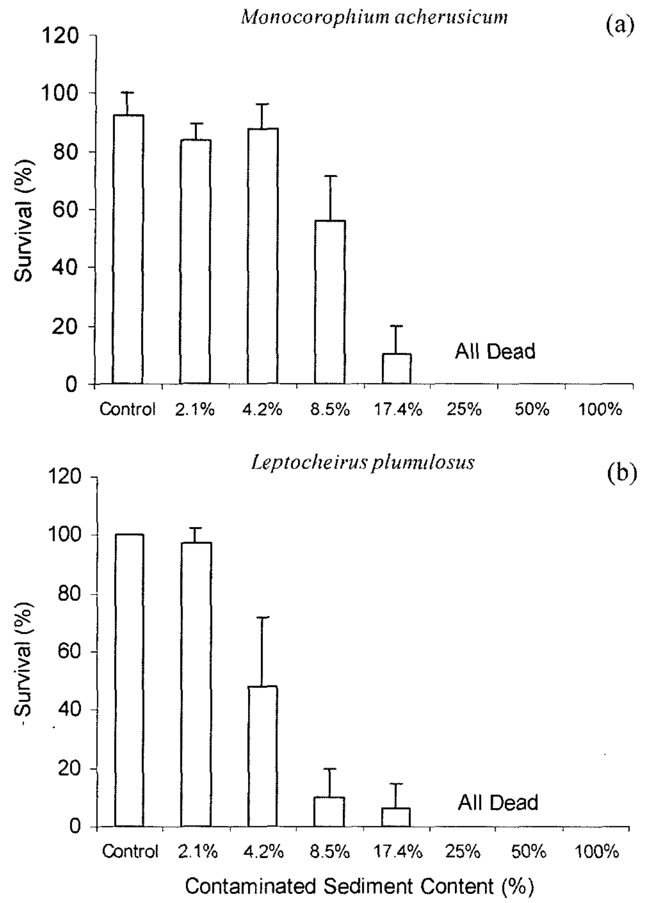


Fig. 6. Mean survivals rates (±SD; %) of (a) *Monocorophium acherusicum* and (b) *Leptocheirus plumulosus* incubated in substrates composed of mixtures of contaminated sediments from a site near Onsan industrial complexes and uncontaminated reference sediment (Ref) from a mudflat in Yeonjongdo for 10 d.

Korea so far (*e.g.* Song 2001), since adequate test species and standardized test methods have not been developed thoroughly.

To identify the adequate test species, we surveyed several locations along the Western coast of Korea and collected several amphipod species including *M. mai*, *H. koreanus*, *H. indivisus* and *M. acherusicum*, which were all benthic infauna, spending most of their life in sediments and also easily collectable.

Basically, test organisms for sediment toxicity tests should be able to inhabit various sediment types during test periods. However, 10-d sediment incubation experiments showed that *H. koreanus* guardedly avoided fine-grained sediments; therefore, this species was considered 'inadequate' for sediment bioassay. The avoidance rate of *H. indivisus* in mud only and sand+mud mixture sediments were slightly higher than those of *M. mai*. It is not certain which level of avoidance is acceptable in determining a legitimate sediment testing species. However, most of physiological and toxicological experiments were conducted using *M. mai* and *M. acherusicum* due to the logistical limitations of the present study. Further evaluation of *H. indivisus* as a test organism will be conducted.

Tolerance to environmental conditions

Temperature can be a very important experimental condition for the confident bioassay results since inadequate temperature can influence bioassay results by stressing test organisms. The tolerance range of *M. mai* and *M. acherusicum* for acute thermal change might be relatively wide. The upper tolerance limit of temperature for *M. acherusicum* was 22°C and the lower limit 10°C. Only the upper limit of temperature tolerance for *M. mai* could be determined as 25°C in the present study, since all individuals survived at 13°C. To determine the adequate temperature conditions for sediment bioassay, one should determine not only the tolerance range of temperature for the test species' survival, but also the influence of temperature on the sensitivity of pollutants. Our previous study reported that the adequate range of temperature for sediment bioassay would be 20°C for *M. mai* and 15-20°C for *M. acherusicum* considering the natural range of temperature in habitats, control performance and sensitivity to reference toxicants (Lee *et al.* 2005).

The tolerance range of *M. mai* and *M. acherusicum* for acute salinity change was relatively wide; most of *M. mai* and *M. acherusicum* could survive in a range of 15~35 psu for 10 d without acclimation. Because both species had been cultured in 30-psu media before the experiment, the sharp decrease of salinity in surrounding media might stress test animals exposed to the lower salinity. The osmotic stress due to the sharp decrease of salinity might begin to exceed the tolerance of both amphipods in media with salinity lower than 15 psu. Probably, more individuals of *M. mai* and *M. acherusicum* would be able to survive in <10 psu if we adopted long enough acclimation periods (Dexter 1993).

Ammonia is not only an important nutrient for some microbes and algal cells, but also acts as a toxicant for most aquatic organisms. The concentrations of ammonia in sediment porewater are generally thousands of times higher than in overlying water; therefore, ammonia can exert severe toxicity in some organically polluted sediments (Ferretti *et al.* 2000). Most polluted sediments contained enriched ammonia concentrations in porewater. However, the amphipod bioassay is not aimed at determining the ammonia toxicity in sediments, but mainly at determining the toxicity of heavy metals and/or organic compounds for the assessment of chemical pollution (Burton 1992). Therefore, test animals should not be too sensitive to ammonia toxicity and have enough tolerance to ammonia. The 96 h-LC50s of total ammonia (NH^3+NH^{4+}) for *M. mai* (105 mg/L) and *M. acherusicum* (155 mg/L) were within the range of those for standard test species such as *Ampelisca abdita* (50 mg/L), *Rhepoxinius abronius* (79 mg/L), *Leptocheirus plumulosus* (44~89 mg/L), *Eohaustorius estuaries* (126 mg/L) and *Grandidierella japonica* (148 mg/L), which were used in sediment bioassays in the USA and other countries (USEPA 1994; Kohn *et al.* 1994; Moore *et al.* 1997). *M. mai* and *M. acherusicum* are more tolerant to ammonia toxicity compared to most standard test species, indicating that the interference of ammonia on the sediment toxicity results might be relatively less important when using *M. mai* and *M. acherusicum*.

Sensitivity to reference toxicant

Various factors can influence the sensitivity of test animals to toxicants. The LC50 values of Cd for *M. mai* and *M. acherusicum* varied by a factor of 2 among different test batches probably because the sensitivity of test animals used in each experiment could be influenced by the difference of season, animal health and/or experimental conditions (McCahon and Pascoe 1988). The sensitivity of organisms in nature also varied according to the reproductive state, metabolic activity and/or nutritional state. For example, Cd LC50 values for both field-collected and laboratory-cultured *Corophium volutator* varied ~10 times seasonally (Kater *et al.* 2000) and *L. plumulosus* also showed large variability of Cd sensitivity according to sex, molt cycle, size and season (McGee *et al.* 1998). Therefore, it is important to include reference toxicant tests in every test batch as a positive control to check the consistency of test animals' sensitivity. For the amphipod test, Cd is the most frequently used as a referent toxicant for the positive control. The test acceptability criteria using positive control can be decided such as 'mean ± 2 SD of previously determined LC50 values' (USEPA 1994; McGee *et al.* 1998). Test acceptability criteria can be decided considering adequately both the variability of animal sensitivity and the assurance of consistent test results. More information is necessary to determine the acceptable test criteria of positive control data for *M. mai* and *M. acherusicum*.

Comparison of Cd LC50 data among species showed that sensitivity of *M. mai* and *M. acherusicum* was comparable to most standard test species and some species (*C. volutator* and *E. estuaries*) had much higher Cd LC50 values than *M. mai* and *M. acherusicum*. Although the Cd sensitivity may not represent the sensitivity to all other pollutants, the similarity or even better sensitivity of *M. mai* and *M. acherusicum* compared to other test species may indicate that these species can be potentially applicable in the assessment of sediment toxicity.

Field sediment toxicity test

The purpose of field-sediment toxicity tests conducted here was to evaluate the applicability of *M. mai* and *M. acherusicum* to sediment bioassay, and/or to compare the relative sensitivity with standard test species. The contaminated sediments used in the present study were known to be polluted by heavy metals including Cr, Cu and Zn due to the effluent from adjacent factories. Individuals of *M. mai* exposed to Sihwa sediments were much more sensitive than *M. acherusicum*, which could be partially explained by lower LC50 of Cd and ammonia for *M. mai*. The second field sediment bioassay using Onsan sediments showed that both *M. acherusicum* and *L. plumulosus* responded to the contaminants associated with sediments in monotonic dose-response relationships. Even though Cd LC50s were very similar for both species, *M. acherusicum* seemed to be less sensitive than *L. plumulosus* to Onsan sediments.

The indigenous amphipods *M. mai* and *M. acherusicum* seemed to be applicable to the assessment of field contaminated sediments because they are relatively tolerable to environmental factors and responded to the concentration of reference toxicant and also field-contaminated sediments. However, further studies including various spiked and filed sediment toxicity tests and elucidating the influence of environmental factors on the toxicological responses should be necessary to develop standardized sediment toxicity testing protocols using these amphipod species.

Acknowledgements

This study was conducted as a research project titled 'Development of sediment bioassay protocol with resident benthic species', which was partly funded by West Sea Fisheries Research Institute, NFRDI.

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