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Acute toxic concentrations of endocrine disrupting compounds on the copepod, the cladoceran, and the rotifer

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Reports have been increasing on the deleterious effect of endocrine disrupting compounds (EDCs) on marine inhabitants. Zooplankton, the primary consumer in the aquatic ecosystem are not spared on these effect. As a first step to investigate the chronic and reproductive effects of EDCs on marine zooplankton, we determined the acute toxic concentration of the known and suspected EDCs for vertebrates including two natural hormone (testosterone, 17β-estradiol), three estrogenic compounds (nonylphenol, octylphenol, bisphenol A), six pesticides (isoprothiolane, methoprene, fenitrothion, diazinon, iprofenfos, pyroquilon) and a herbicide (mefenacet) on the copepod *Tigriopus japonicus*, the cladoceran *Diaphanosoma celebensis* and the rotifer *Brachionus plicatilis*. Compound exposure was carried out in 6-well polysterene plates, and mortality was evaluated after 24 h. Although the three zooplankton depicted different sensitivities toward different compounds, a dose-response relationship was consistent in all cases. *B. plicatilis* was particularly resistant to most of the compounds tested, while *T. japonicus* and *D. celebensis* are comparatively sensitive. The data presented herein can be a basis for the determination of concentrations to be used in chronic and endocrine disruption experiments using marine zooplankton.

Key Words: acute toxicity, marine zooplankton, endocrine disrupting compounds

Introduction

Many environmental contaminants either of natural (*e.g.* phytoestrogens) or anthropogenic (*e.g.* industrial by-products) origin are known to affect the reproductive characteristics of both vertebrates and invertebrates by mimicking or antagonizing the action of hormones. These environmental contaminants are collectively called endocrine disrupting compounds (EDCs). For several decades, various reports on the deleterious effects of EDCS have been reported in aquatic inhabitants including fish[1,2] and invertebrates[3] including zooplankton. Among aquatic species, zooplankton presents distinctive characters that are useful in ecotoxicological studies which we have been discussed in our previous papers.[4-6] Since the number of chemicals that are suspected to have endocrine disrupting effects, therefore, information on the toxicities of different classes of EDCs is important.

The aim of this study is to provide information on the acute toxicity concentration of known and suspected EDCs for vertebrates, in marine zooplankton species including a copepod *Tigriopus japonicus*, a cladoceran *Diaphanosoma celebensis* and a rotifer *Brachionus plicatilis*. Compounds were selected to represent natural hormone (testosterone, 17 β -estradiol), estrogenic compounds (nonylphenol, octylphenol, bisphenol A), pesticides (isoprothiolane, methoprene, fenitrothion, diazinon, iprofenfos, pyroquilon) and herbicide (mefenacet). Because of the ubiquitous uses of these compounds, they are continuously discharged into the environment, adding to its environmental concentrations. Some of them are already in the list of endocrine disruptors and their uses have already been regulated. We used the standardized toxicity test developed by the American Society of Testing and Materials[7] and determined the LC_{50} of each compound for the test animals. Data obtained in this study can be a basis for the development of an experimental design to be used in chronic and endocrine disruption studies.

Materials and methods

Test animals

1) Copepod *Tigriopus japonicus*

T. japonicus is a harpactocoid copepod that thrives in wide temperature (below 0−31℃) and salinity (0−40 ppt) ranges.[8,9] *T. japonicus* used in this study originated from an outdoor pond of Fisheries Laboratory, The University of Tokyo, near Lake Hamana and has been maintained continuously in our laboratory.[9] They were cultured in a glass bottle containing GF/C (Whatman) filtered natural

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seawater diluted with sterilized distilled water to 25 ppt, fed *Nannochloropsis oculata* and kept under static-renewal conditions, where the culture water was changed twice a week. The culture was maintained in a photoperiod of 12L:12D at 25 ± 1℃.

2) Cladoceran *Diaphanosoma celebensis*

Cladoceran *D. celebensis* used in this study was obtained from Wang-Tak Yang of Mariculture Technology Laboratory, Korea, and has been continuously maintained in our laboratory. They were cultured in GF/C (Whatman) filtered natural seawater diluted with sterilized distilled water to 17 ppt, fed mixed microalgae (*N. oculata* and *Tetraselmis tetrathele*), and kept under static-renewal conditions with a 12L:12D photoperiod at 25 ± 1℃. Under these conditions, *D. celebensis* reaches sexual maturity and releases first neonate within 4-5 days. The neonate molts every day until sexual maturity, and the molting frequency is reduced thereafter. Only parthenogenetic reproduction by amictic female was observed under the culture conditions described above.

3) Rotifer *Brachionus plicatilis*

B. plicatilis is a monogonont rotifer that reproduces through cyclical parthenogenesis, incorporating both asexual (amictic) and sexual (mictic) reproduction. Most of the time, amictic females produce amictic eggs mitotically. Given appropriate environmental conditions, (*e.g.* high population density, ample food, optimum temperature and salinity), amictic females produce mictic daughters (called mixis). Mictic females subsequently produce eggs meiotically that develop into haploid males, or resting eggs if fertilized by males.

B. plicatilis NH1L strain was used in this study. This strain originated from The University of Tokyo and has been cultured in our laboratory with regular collection and storage of resting eggs.[10] Stock cultures were maintained in diluted seawater at 22 ppt, stored in 25 ± 1℃, and fed *N. oculata*.

Test Compounds

Compounds tested in this study were: bisphenol A, *p-t*-octylphenol, 4-nonylphenol, estradiol-17β, isoprothiolane, methoprene, testosterone, fenitrothion, diazinon, iprofenfos, pyroquilon and mefenacet. All chemicals were purchased from Kanto Kagaku, Japan, except testosterone and estradiol-17β which were purchased from Sigma Chemicals (St. Louis, MO, USA).

Because of the hydrophobicity of these chemicals, they were dissolved first in 100% dimethyl sulfoxide (DMSO), then in ultra-pure grade water (Milli Q). Test solutions were prepared by the addition of appropriate aliquots of aqueous stock solution to filter-sterilized seawater. In all chemicals, DMSO is present not more than 0.1% in the final test concentration. At this concentration of DMSO, no mortality in all test animals was observed throughout the experiment.

24-h acute toxicity test

1) Copepod *T. japonicus*

The acute toxic concentration of each chemical was determined using adult copepods. Treatment solutions were prepared by diluting specific volumes of aqueous stock solution in filter-sterilized seawater (22 ppt).

To start the bioassays, 10-20 adult male and female copepods were randomly selected from the stock culture and transferred to each well of 6-well polystyrene plate containing 10 mL of a toxicant solution and a control (filter-sterilized seawater). Seven concentrations were tested in each chemical. The plates were incubated at 25 ± 1℃ in darkness. Because of the short duration of the tests, the test organisms were not fed throughout the experiment. The number of live and dead animals was recorded after 24-h. Animals were considered dead if no movement was observed in 30 seconds of physical stimulation using a needle.

2) Cladoceran *D. celebensis*

The acute toxicity concentration of 12 chemicals was determined using adult cladocerans. Treatment solutions were prepared by diluting specific volume of chemical stock solution with filter-sterilized diluted seawater (17 ppt).

About 15-30 adult cladocerans were added to each well of 6-well polystyrene plates containing 10 ml of a toxicant solution as well as a control (filter-sterilized seawater). Seven concentrations were tested in each chemical. The plates were incubated at 25 ± 1℃ in darkness. Because of the short duration of the test, the test organisms were not fed throughout the experiment. The number of live and dead animals was recorded after 24h. Animals were considered dead if no movement was observed in 30 seconds of physical stimulation.

3) Rotifer *B. plicatilis*

Test animals were obtained by hatching resting eggs that were collected from the laboratory stock culture. Acute toxicity test was carried out according to the standard acute toxicity tests[7] for each chemical with ten concentrations. Twenty rotifers were transferred to individual wells of a 6-well polystyrene plate, each containing 10 ml of a test solution, or a control (filter-sterilized seawater). The plates were incubated at 25 ± 1℃ in darkness. Because of the short duration of the tests, the animals were not fed during the experiment. After 24h, the rotifers were observed under the stereomicroscope. Rotifers were considered dead if no movement of the cilia and mastax was observed over a period of 30 seconds. The dead and alive rotifers were counted.

Statistical Analysis

LC_{50} was calculated using Probit Analysis[11] and *t*-test was conducted to compare the data with the negative (sterilized water only) and DMSO controls.

RESULTS

1) Copepod *T. japonicus*

LC_{50} values, coefficients of variation, 95% confidence intervals, and sample sizes for the12 chemicals are presented in Table 1.

T. japonicus was not sensitive to androgen (testosterone) as high as 50 mg/l, (the solubility of most of the compounds in water was = 50 mg/l, thus, we set the limit to 50 mg/l. In some compounds, no mortality was observed at 50 mg/l (the highest concentration we tested), therefore, we could not show the regression analysis) but was relatively sensitive to estrogen (17β-estradiol). Among the estrogenic chemicals, *T. japonicus* was most vulnerable to nonylphenol and least vulnerable to bisphenol A. Among the pesticides and the herbicide, methoprene was the most toxic with an LC_{50} value of 2.47 mg/l. Pyroquilon and fenitrothion were not toxic to *T. japonicus* as high as 50 mg/l, and *T. japonicus* also tolerate isoprothiolane and mefenacet at relatively high concentrations (LC_{50} ranges from 30.39 to 45.02 mg/l). Diazinon and iprofenfos affected *T. japonicus* at almost the same concentrations.

2) Cladoceran *D. celebensis*

LC_{50} values, coefficients of variation, 95% confidence intervals, and sample sizes for 12 chemicals on adult *D. celebensis* are presented in Table 2.

Testosterone as high as 50 mg/ L was not toxic to *D. celebensis*. With respect to the estrogen and the estrogenic chemicals, *D. celebensis* was less sensitive to 17β-estradiol and bisphenol A (LC_{50} ranges from 10.37 to 10.42 mg/l), while they are sensitive to nonylphenol and octylphenol (LC_{50} ranges from 0.59 to 1.59 mg/l). For the pesticides and the herbicide, fenitrothion was the most toxic, followed by diazinon and methoprene. Isoprothiolane, pyroquilon, iprofenfos, and mefenacet were moderately toxic ("toxic" concentration refers to concentrations below 10 mg/l, "moderately toxic" refers to concentrations above 10 mg/l but below 50 mg/l, and "not toxic" refers to concentration = 50 mg/l) (LC_{50} ranges from 13.63 to 35.05 mg/l) to *D. celebensis*.

3) Rotifer *B. plicatilis*

LC_{50} values, coefficients of variation, 95% confidence intervals, and samples sizes for 12 chemicals on *B. plicatilis* are presented in Table 3.

Table 1. LC_{50} (mg/l), coefficients of variation (CV), 95% confidence intervals (CI) and sample sizes (N) of 12 chemicals on copepod, *T. japonicus* at 25 ± 1°C.

Chemical	LC_{50} (mg/ l)	CV (%)	95% CI	N
Natural hormone				
Testosterone	>50	-	-	103
17β-estradiol	3.54	0.21	3.14 – 3.95	82
Estrogenic compound				
Bisphenol A	4.83	0.14	4.55 – 5.11	105
4-nonylphenol	0.52	0.01	0.49 – 0.54	116
Octylphenol	0.64	0.27	0.58 – 0.69	109
Pesticide				
Methoprene	2.47	0.19	2.08 – 2.85	68
Diazinon	8.27	0.27	7.76 – 8.83	77
Isoprothiolane	45.02	1.14	42.79 – 47.25	85
Pyroquilon	>50	-	-	83
Fenitrothion	>50	-	-	116
Iprofenfos	8.69	0.71	7.29 – 10.08	78
Herbicide				
Mefenacet	30.39	1.69	27.09 – 33.70	62

Table 2. LC_{50} (mg/l), coefficients of variation (CV), 95% confidence intervals (CI) and sample sizes (N) of 12 chemicals on cladocerans *D. celebensis* at 25 ± 1℃.

Chemical	LC_{50} (mg/ l)	CV (%)	95% CI	N
Natural hormone				
Testosterone	>50	-	-	169
17β-estradiol	10.37	0.35	9.68 - 11.05	160
Estrogenic compound				
Bisphenol A	10.42	0.39	9.65 - 11.19	150
4-nonylphenol	0.59	0.76	0.54 - 1.44	116
Octylphenol	1.59	0.44	0.73 - 2.46	94
Pesticide				
Methoprene	5.05	0.45	4.43 - 6.22	100
Diazinon	0.03	0.04	0.02 - 0.04	68
Isoprothiolane	26.63	0.76	21.50 - 28.12	80
Pyroquilon	35.05	0.76	30.60 - 37.05	99
Fenitrothion	0.004	0.01	0.002 - 0.006	56
Iprofenfos	13.63	0.56	12.53 - 14.73	116
Herbicide				
Mefenacet	23.01	0.41	22.21 - 23.81	135

Table 3. LC_{50} (mg/l), coefficients of variation (CV), 95% confidence intervals (CI) and sample sizes (N) of 12 chemicals on rotifer *B. plicatilis* at 25 ± 1℃.

Chemical	LC_{50} (mg/ l)	CV (%)	95% CI	N
Natural hormone				
Testosterone	>50	-	-	180
17β-estradiol	>50	-	-	135
Estrogenic compound				
Bisphenol A	10.93	0.18	10.58 - 11.29	130
4-nonylphenol	0.67	0.005	0.66 – 0.68	130
Octylphenol	1.41	0.04	1.33 – 1.49	104
Pesticide				
Methoprene	>50	-	-	98
Diazinon	28.62	0.58	26.20 – 30.88	101
Isoprothiolane	>50	-	-	105
Pyroquilon	>50	-	-	105
Fenitrothion	>50	-	-	112
Iprofenfos	>50	-	-	123
Herbicide				
Mefenacet	>50	-	-	120

B. plicatilis can tolerate testosterone, 17β-estradiol, methoprene, isoprothiolane, pyroquilon, fenitrothion, iprofenfos and mefenacet at a concentration as high as 50 mg/l. Among the chemicals tested, nonylphenol was the most toxic to *B. plicatilis* with an LC_{50} value of 0.67 mg/l.

Discussion

Mortality in all controls and solvent controls was never above 10% and fulfilled required validity criteria.[7] Water and solvent controls were not significantly different (*t*-test, $p > 0.05$) in all tests, therefore, the influence of solvent residues on test results may be neglected. Our coefficient of variance was usually below 2% which is acceptable and common in toxicity testing. Thus, three replicates, which we used in this experiment are enough to obtain a conclusive result.

The results of the acute toxicity tests showed that rotifer *B. plicatilis* was the most tolerant among the three zooplankton species tested, because they can tolerate most of the chemicals (testosterone, 17β-estradiol, methoprene, isoprothiolane, pyroquilon and fenitrothion) at concentrations as high as 50 mg/l. No mortality was observed among the three zooplankton groups exposed to testosterone as high as 50 mg/ L. LC_{50} value of 17β-estradiol for *T. japonicus* (3.54 mg/l) was three times lower than that of *D. celebensis* (10.37 mg/ L), and more than 15 times lower than *B. plicatilis* (≥ 50 mg/l). Among the estrogenic chemicals, nonylphenol was the most toxic with an LC_{50} value ranging from 0.52 to 0.67 mg/l. The rotifer was the most tolerant to the pesticides and the herbicide followed by the copepod and the cladoceran. Fenitrothion was very toxic to the cladoceran with an LC_{50} value of 0.004 mg/l, while both copepod and rotifer can tolerate fenitrothion as high as 50 mg/l.

The acute toxicity values of 17β-estradiol among copepod species were comparable to each other, with LC_{50} values ranging from 0.045 mg/l to 3.54 mg/l. Estuarine copepod *Eurytemora affinis* was the most sensitive species.[12] Estuarine copepod *Acartia tonsa* and *T. japonicus* have comparable sensitivity to bisphenol A, while *E. affinis* was almost 10 times more sensitive compared to other copepod species. Using the same species (*T. japonicus*), Lee et al.[13] obtained more than ten times lower value (96 h LC_{50} = 0.2 mg/l) compared to our 24 h LC_{50} value (4.83 mg/l). The acute toxic concentrations of nonylphenol to various crustacean species were almost the same, except for the estuarine copepod *A. tonsa*, which has almost five times lower sensitivity to nonylphenol.[14] Meanwhile, 24 h LC_{50} is comparable with Lee et al.[13] using the same species (*T. japonicus*; 96 h LC_{50} = 0.5 mg/l). The acute toxic concentration of octylphenol to the copepod *T. japonicus* obtained in this study was also comparable to the concentration obtained by Lee et al.[13] using the same species (96 h LC_{50} = 0.3 mg/l) and the copepod *A. tonsa*.[14] The freshwater cladoceran *Daphnia magna* was more sensitive to octylphenol as compared to the euryhaline cladoceran *D. celebensis*. Meanwhile, the result of our study showed that the rotifer *B. plicatilis* has comparable sensitivity to the euryhaline cladoceran *D. celebensis*.

Among pesticides, *Artemia* sp. was the most tolerant among the crustacean species exposed to diazinon with a 24-h LC_{50} value of 19.0 mg/l.[15] Meanwhile, freshwater cladoceran *Ceriodaphnia dubia* and *D. magna* have comparable sensitivity to diazinon, while euryhaline cladoceran *D. celebensis* was more tolerant to the pesticide. Freshwater and marine rotifers have comparable tolerance to diazinon with an LC_{50} values ranging from 28.0 to 31.0 mg/l. High variation of LC_{50} values were also obtained in this study using copepod, cladoceran and rotifer exposed to fenitrothion. The lowest 24-h LC_{50} was reported in freshwater cladoceran *D. magna* (LC_{50} = 0.067 μg/l).[16] The cladoceran *D. celebensis* tested in this study was more tolerant to *D. Magna* exposed to fenitrothion (LC_{50} = 0.004 mg/l). Marine shrimp, copepod, and cladoceran have comparatively high tolerance to methoprene (LC_{50} range >1.0 mg/l - 5.05 mg/l), while the freshwater cladoceran *Moina macrocopa* was relatively sensitive.[17] Marine rotifer *B. plicatilis* can tolerate high concentrations of methoprene (LC_{50} was more than 50 mg/l).

In general, marine zooplanktons have higher tolerance to EDCs compared to freshwater zooplanktons. The tolerance of marine species to toxicants can be explained by their greater osmoregulatory capacity so that they can withstand wide temperature and salinity changes as well as toxicants present in their environment.

In farms in the United States the measured soil concentrations of up to 675 ng/ kg 17β-estradiol and 260 ng/kg testosterone was found and surface runoff concentration reached 50 to 2,300 ng/g 17β-estradiol and 10 to 1,830 ng/l testosterone.[18] For estrogenic compounds, a survey in German rivers, found bisphenol A concentration of 0.0005-0.7 μg/l in surface water and sewage effluents, and 0.01-1.4 mg/kg in sediment and sewage sludge.[19] In river waters of Japan and the United States, the concentration of bisphenol A was found to be less than 1 mg/ l.[20,21] Survey of several rivers in the United Kingdom, Japan, Switzerland and the United States found nonylphenol concentrations of 0.2-53 μg/ l,[21-23] and one river had up to 180 μg/ l.[23] Octylphenol concentrations are relatively lower than nonylphenol, because octylphenol comprised only 15-20% of the total alkylphenol polyethoxylates.[22] In sediment samples from the Great Lakes and northeast Canada, octylphenol ranged from 0.002-23.7 μg/g.[24] Pesticides diazinon, fenitrothion and isoprothiolane have been detected in several rivers in Japan at 0.02-20 μg/l.[25] In Korea the highest concentration of diazinon, fenitrothion and isoprothiolane were 0.912, 1.002, 1.367 μg/l, respectively.[26] Iprofenfos has been detected in Pal-dang reservoir, Korea ranging from 0.189-1.05 μg/l.[26] Following field application of Altosid Liquid Larvicide (with 5% (S) methoprene) the expected environmental concentration of methoprene is 0.03 μM.[27] The concentration of mefenacet in river water in Japan ranged from 23 to 87nM.[28]

Based on the above-mentioned concentrations, the toxic concentration found in this study was considerably higher than predicted environmental concentrations, hence natural copepod, cladoceran and rotifer populations are unlikely to be killed by the compounds at tested concentrations. The data obtained in this study had been applied to the experimental design of our past research, and could be used a a guide in future chronic and endocrine disruption studies involving marine zooplankton.

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カイアシ類，ミジンコ類及びワムシ類に対する内分泌かく乱化学物質の急性毒性濃度

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高等動物の内分泌をかく乱する物質が水生動物に与える影響について近年多くの報告がなされている。本研究では，水界の低次生産の重要な構成員であり，淡水種に比べて検討例が少ない海産の動物プランクトンを材料とし，化学物質12種の急性毒性を24時間LC_{50}によって調べた。実験に用いた海産動物プランクトンは，カイアシ類 *Tigriopus japonicus*，ミジンコ類 *Diaphanosoma celebensis*，ワムシ類 *Brachionus plicatilis* である。これら３種が属する分類群は水界生態系に広く分布して低次生態系の構成員として重要な位置を占めているのみならず，世代時間が短く比較的容易に培養できるため，生活環全体への影響や複数世代にまたがる影響を短時間で検討・評価できるという特徴がある。本研究に用いた化学物質は，天然ホルモン２種（エストラジオール17β，テストステロン），エストロジェン様物質３種（ビスフェノールＡ，ノニルフェノール，オクチルフェノール），殺虫剤６種（メトプレン，ダイアジノン，イソプロチオラン，ピロキロン，フェニトロチオン，イプロフェンフォス）および除草剤１種（メフェナセット）の計12種である。その結果，カイアシ類とミジンコ類に比較して，ワムシはいずれの化学物質に対しても強い耐性を示すことが分かった。多くの無脊椎動物では内分泌に関する知見が少なく，物質の影響が内分泌かく乱によるものか毒性によるものかの区別がつきにくい。したがって，内分泌系の制御下にあると想定される生殖関連の諸特性に対する作用を求めていく上で，本研究の結果は有用な基礎データとなる。