# 論 文 の 内 容 の 要 旨

論文題目　　Toxicity assessments of nickel and urban road dust by a novel chronic sediment toxicity test using a freshwater benthic ostracod *Heterocypris incongruens*

(淡水産底生カイミジンコ*Heterocypris incongruens*を用いた新規底質慢性毒性試験によるニッケルおよび都市道路塵埃の毒性評価)

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Sediment contamination poses significant environmental problems for aquatic ecosystems. Contaminated sediments contain a wide range of chemical substances such as heavy metals, polycyclic aromatic hydrocarbons, and polychlorinated biphenyls that degrade water quality and adversely affect aquatic organisms, particularly benthic organisms. Given that benthic organisms are the most likely to be in direct contact with and ingest the sediments, evaluating the effects of sediment-associated contaminants on benthic organisms is a direct measure of sediment quality and toxicity.

To test freshwater sediments, a cyst-based test using the benthic ostracod *Heterocypris incongruens* was successfully developed to reduce the burden of culturing and maintaining live stocks of organisms. The test was standardized as ISO 14371 in 2012. Toxicity effects on *H. incongruens* were assessed on the basis of their mortality as well as growth inhibition after a 6 d exposure to test sediments. The ISO 14371 endpoint is too short to observe the toxicity of contaminated sediments that may affect reproduction. Furthermore, in terms of aquatic conservation, the sublethal endpoint related to reproduction is required rather than the mortality rate of individual test organisms, which is still lacking in the conventional 6 d ostracod toxicity test.

This study aimed to develop and establish a new chronic sediment toxicity test using a freshwater benthic ostracod *H. incongruens*. The specific objectives include (1) To propose a new chronic sediment toxicity test using the benthic ostracod *H. incongruens* by intensive literature surveys, (2) To evaluate the repeatability of the test method based on the coefficient of variation in each endpoint of proposed chronic test and to determine the test acceptability criteria for control sediment. *H. incongruens* reproduction parameters such as egg production, first day of brooding, egg-laying ratio, and hatching ratio were examined in this study. The life history and reproduction characteristics of *H. incongruen*s in reference (RF) sediment (supplied from the Ostracodtoxkit) were investigated. The results from control tests would be necessary for future users of the chronic ostracod toxicity test by showing the test acceptability criteria of control sediment, (3) To provide application examples of the proposed chronic test using nickel and

urban road dust whose chronic toxicity to ostracod has never been reported yet. At present, fundamental studies for URD toxicity are based on 6 d mortality and growth inhibition of ostracods. In this study, the toxic effect of URD on ostracod reproduction was carried out in comparison with ISO 14371. The present study is expected to serve as the fundamental study on the chronic toxicity of URD to *H. incongruens*.

The chronic ostracod toxicity test was proposed based on the literature surveys for the suitability of freshwater ostracod *H. incongruens* as a cosmopolitan and representative benthic species including the advantage as cyst-based toxicity test. The proposed chronic test is composed of three consecutive phases as (1) a 14-day sediment exposure phase, (2) a reproduction phase, and (3) a hatching test. The 14-day sediment exposure phase was considered to expose ostracod in sediment until a few days before the release of the first brood. After that reproduction can be examined for all ostracod lifetime as well as the hatching test. The hatching test was simultaneously conducted in parallel with reproduction phase after ostracod laying the first brood of egg. The test and feeding conditions included a 24 h incubation of multi-well plates in the dark at 25°C, feeding the green algae (*Scenedesmus acutus*) at $3.0 \times 10^7$ cells/well during the first week, and feeding a TetraMin suspension for the remainder of the test. Previous studies examined the life history and reproduction characteristics of the ostracod *H. incongruens* that they can be used as reproductive endpoints. In this study, numerous endpoints were proposed for chronic ostracod toxicity test. Determinations of the 14 d mortality and growth inhibition compared with the controls are the effect criteria at the end of the 14-day sediment exposure period. Fecundity (e.g. life span, first day of brooding and egg-laying ratio etc.) are the measurement endpoints in the reproduction phase. Hatching rate and F2 generation rate of ostracod are the endpoint in the hatching test. However, to date, the repeatability and acceptability criteria of these endpoints have not been established for routine use for chronic ostracod toxicity test.

The test was first validated by determining the repeatability of the test method under seven control performances. The seven batches of the control tests (total 500 ostracods) were conducted using clean reference sediment as a part of the Ostracodtoxkit F (MicroBioTests Inc., Belgium). The results showed good test repeatability of most endpoints, with coefficient of variation (CV) results below 15%. However, lifetime egg production, hatching ratio, and the F2 generation rate were highly variable, with CVs ranging from 29.5% to 51.9%. The results from the control tests would be necessary for future users of the chronic ostracod toxicity test by setting the test acceptability criteria of control sediment. The test acceptability criteria of control sediment were first proposed and established in this study. The 20% mortality rate was decided as the acceptability threshold for 14 d mortality and at least 900 μm for the 14 d body length of *H. incongruens*. The reproduction of *H. incongruens* in control sediment was regarded acceptable if

the egg-laying ratio was in the range of 56.8–76.8%. The mean life span of all individuals was 20.1–29.3 d and 19.7–34.5 d for an egg-laying individual. First day of brooding and mean lifetime egg production were 18.9–22.9 d and 18.5–28.1 d, respectively. However, because the CVs for lifetime egg production, hatching ratio, and F2 generation rate were ≥30%, no acceptability criteria were defined for those parameters. The proposed test acceptability criteria for control sediment were used to determine the validity of the control test in the following chapters.

After the test validation, nickel was used as a reference toxicant to assess the toxicity effect on the reproduction of the benthic ostracod *H. incongruens*. The ostracod was exposed with a series of nickel concentrations diluted with standard freshwater (SFW) in 10 times interval. The mortality of ostracod after a 14-day exposure to a highest concentration of nickel (1200 µgNi/l) was 41.7%. Thus, the estimated 14-d $LC_{50}$ was $2.95 \times 10^3$ µgNi/l with a no-observed lethal concentration (14-d mortality) of 12 µgNi/l, and a 14-d $LC_{20}$ of 5.03 µgNi/l. Among the endpoint investigated in 14-day sediment exposure phase, the growth of ostracod was more sensitive than mortality with LOEC ≤ 0.012 µgNi/l for 14-d growth inhibition. Under sublethal concentration of nickel, it has an impact on ostracod reproduction. Mean life span decreased significantly ($p < 0.05$) in response to 120 and 1,200 µgNi/l. The egg-laying ratio was also significantly lower at these two concentrations. The no observed effect concentration (NOEC) was 12 µgNi/l for the egg-laying ratio and mean lifespan of all individuals. Furthermore, the statistically significant difference in hatching ratio ($p<0.05$, Chi-square test) was obtained in 1.2, 12, 120, 1200 µgNi/l. F2 generation rate became lowest in the highest concentration of nickel (C6=1200 µgNi/l) which the value ≤ 1 in 12, 120, 1200 µgNi/l indicates the possibility of extinction of the ostracod population. However, C1 (0.012 µgNi/l) cause the higher hatching ratio and statistically significant difference was obtained. Base on the results of this study, the hatching ratio as well as F2 generation rate showed high variation with nickel concentrations and may not be reliable endpoint. Thus, the egg-laying ratio and mean lifespan of all individuals was considered as sensitive, reliable endpoint and further calculate ACR of nickel. The benthic ostracod *H. incongruens* had the highest ACR among other test organisms. The high ACR demonstrated a great difference between the chronic and acute toxicities. From this view point, conducting only the acute toxicity test for *H. incongruens* may mislead to underestimate the nickel toxicity to ostracod. This indicates the importance of conducting the chronic toxicity test proposed in this study. However, it has to be considered for the wide-range of nickel concentrations as in 10 time interval. This would have a great effect on accuracy of ACRs in this study.

Urban road dust is one of the potential sources of sediment pollution as solid particles in urban road runoff. Next, an application example of the proposed chronic method was performed using a series of urban road dust (URD) samples diluted with reference sediment and compared to a 6 d

*H. incongruens* toxicity test. After the 6-day exposure with diluted URD samples, the results showed that the 6 d-LC50 and LC20 (with 95% UCL and LCL) of URD sample was 30% (26 - 37%) and 14% (11-17%), respectively with a TU50 of 3.3. Additionally, the results of the proposed chronic test showed that the 14 d-LC50 with 95%CI of URD samples was 15%(3–82%) with a TU50 of 6.67. There was a statistically significant difference ($p<0.05$) in 14 d mortality between the control and 12.5% URD, 25% URD, and 50% URD samples. A statistically significant difference ($p< 0.05$) in 14 d growth inhibition was found for all concentration of URD. Non-observed effect concentrations in 6 d mortality and growth inhibition were 12.5% and 6.25% URD, respectively. On the other hand, a 6.25% URD sample in the proposed chronic test impacted *H. incongruens* reproduction. There were statistically significant differences on the first day of brooding and lowest hatching ratio so that the F2 generation rate became lowest (<1 indicating the possibility of extinction). Overall results suggest that low concentrations of URD were toxic to *H. incongruens* reproduction, which was not previously identified as toxin by a standard 6 d toxicity test (ISO 14371).

The chronic and acute toxicity data of URD was obtained in this study and further calculated ACR of URD (ACR=6.8; from MATC of first day of brooding= 4.42). Considering chronic toxicity units (TUc = 100/MATC) as the safety threshold, this URD could become non-toxic when it gets mixed with 23 times more clean sediment. ACR of URD can be generalized to use as a factor for estimating chronic toxicity on the basis of acute toxicity to other URDs or dilution ratio required for clean RF sediment of URD to become non-toxic. This approach can be used in the effective management of URD in urban runoff and to estimate chronic thresholds of URD that protect the benthic community

The conclusion drawn from this study indicates the importance of conducting the chronic toxicity test as proposed in this study. Though, the application examples of chronic ostracod test were limited by using nickel and urban road dust. The study showed the first report of chronic nickel toxicity to *H. incongruens* as NOEC of 12 µgNi/l for the egg-laying ratio and mean lifespan of all individuals. However, it has to be mentioned for the wide-range of nickel concentrations in 10 time interval. This would have a great effect on accuracy of ACRs. The definitive toxicity test of nickel using the proposed chronic test is recommended. Furthermore, the results of the chronic test using URD demonstrated that low concentrations of URD were toxic to *H. incongruens* reproduction, which was not determined by standard 6 d toxicity test (ISO 14371). It suggested that there was chronic effect of URD when carried out by runoff to water environment. ACR of URD was calculated to estimate chronic thresholds of URD. This finding would be beneficial for effectively managing URD in urban runoff pollution that protective the benthic community.