博士論文

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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by

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A dissertation submitted to the Graduate School of Engineering,

The University of Tokyo in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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March 2017

ACKNOWLEDGEMENT

It would not be possible to conduct this research without the precious support of the various people involved during the research period. First and Foremost, I would like to express my sincere gratitude to my advisor Assoc. Prof. Fumiyuki Nakajima for the continuous support during my Ph.D. study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing the thesis. I could not have imagined having a better advisor and mentor in my Ph.D. study.

Besides my advisor, my great appreciation goes to the rest of my thesis committees: Assoc. Prof. Chihiro Yoshimura (Water Environment Lab, Department of Civil and Environmental Engineering, Graduate School of Science and Engineering, Tokyo Institute of Technology, Japan), Prof. Kazuo Yamamoto who also provided me a great opportunity to join his laboratory. Prof. Hiroaki Furumai, Assoc. Prof. Ikuro Kasuga. Special gratitude to all of them for their encouragement, insightful comments, and hard questions which incented me to widen my research from various perspectives. I would like to give special thank for Assist. Prof. Tomohiro Tobino for his comments and suggestions during the various research meetings.

I thank all my labmates in the Department of Urban Engineering for the stimulating discussions during the meetings. I would like to express special thanks to all the members in Yamamoto-Nakajima laboratory, especially Dr. Kyoshiro Hiki, Ms. Meena Yanagihara for their valuable comments and helpful during the laboratory works and meetings. In particular, I am grateful to Dr. Jenyuk Lohwacharin for all his supports and suggestions, Dr. Rajendra Khanal for enlightening me the first glance of research and used his sediment sample also his comments and suggestions on my first manuscript. In addition, I would say thank for Dr. Jatuwat Sangsanont for all his supports and Dr. Jason Torzewski for his help in English editing. Special thanks to my friend Ms. Kultip Suwanteep (Bo) for being supportive, encouragement and always beside me. Special thanks to all of Thai members in our department for their supports and inspiration.

Last but not the least, I would like to thank all my family members who always there for me: my dad, my mom, my aunt, my brother and sister for supporting me spiritually throughout my life. Their love and cheerful always help me overcome a hard time during my study. My grandfather as I wish you were here.

My sincere thanks go to all staff in the Department of Urban Engineering, The University of Tokyo who helps and gave access to the laboratory and research facilities.

To all relatives, friends and others who shared their support either morally and physically, thank you.

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ABSTRACT

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 3.0×10^7 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 × 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.

CHAPTER 1

INTRODUCTION

1.1. Background

The term of sediment refers to the particles which settle or deposit at the bottom of water bodies. Sediment is important in aquatic ecosystems which is a habitat and food sources for a wide variety of aquatic organisms. The components in sediment are a complex and heterogeneous matrix consisting of solid particles and pore water phase (Kalinowski et al., 2011; Diepens et al., 2014). Solid particles are composed of inorganic and organic materials. Solid particles differ in grain size, chemical compositions, and surface areas such as sand, silt and clay, which influence the adsorption and binding for a number of metals and organic chemical substances. These substances enter water bodies in many ways mostly by anthropogenic activities. They sink to the bottom and become part of the sediment (Mulligan et al., 2009). Therefore, sediments act as an important sink and source for various pollutants such as heavy metal, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Middleton et al., 2003; Christensen et al., 2006; Oliveira et al., 2014). Several studies have been reported the effect of sediment-associated contaminants in the aquatic ecosystems, which degrade the water qualities causing adverse effects on aquatic organisms, particularly benthic organisms (Jiang et al., 2007; Karlsson et al., 2008; Koike et al., 2012).

Toxicity of sediment can be associated with acute or chronic depending on the dose of a chemical and the exposure time. Standard method for sediment toxicity tests have developed for short-term and long-term tests (Burton et al., 1996; US EPA, 2000). Acute toxicity test results in mortality effects on the test organisms. In contrast, chronic toxicity represents the long-term exposure to certain contaminants and results in changes in reproduction, growth or other behaviors of biological biota. The chronic tests would be able to provide information for the toxic effect on the population level. In term of ecotoxicology, the decision making on the exposure periods refers to the goal of the

assessment. The selection of the toxicity endpoints is also varying in their sensitivity and ecosystem relevance. The chronic exposure shows more realistic in the environment and therefore more ecologically relevant to determine (Ingersoll and Nelson, 1990; Simpson et al., 2005). The chronic tests with sublethal and reproductive endpoints are usually more preference than a lethal one. These remark the need of chronic toxicity tests against acute toxicity test.

Several approaches are available for evaluating the sediment quality including chemical analysis, sediment toxicity tests, and benthic community structure studies. The chemical analysis may provide some information about the presence of toxicants in sediments, but it is obvious that chemical analysis alone is not sufficient in interpreting toxicity to biota (Mulligan et al., 2009). Benthic community structure is a help to identify the contaminants which cause degradation in the benthic ecosystem. This approach shows more long-term exposure effects of the sediment compared to conventional acute or chronic toxicity tests (Burton et al., 1992). Additionally, sediment toxicity testing provides a more direct means of assessing the potential adverse effects of contaminated sediment (Chapman et al., 2002; Fernandez-Alba et al., 2002; Hartl et al., 2005; Reinier et al., 2009). The sediment toxicity test does not require specific knowledge for pathways of interactions among sediments and test organisms. It is a directly-measured response between interactive toxic of complex contaminants in the sediment and exposed organisms (US EPA, 1994a). Sediment bioassays are used to evaluate the toxicity of sediments which test organisms exposed to whole sediment and or pore water. Whole sediment toxicity tests shows more realistic exposure predictions than pore water test and sediment physicochemical conditions include the different exposure routes (Liß and Ahlf, 1997; Bebon R., 2013; Diepens et al., 2014).

Previous studies proposed the diversity of organisms in the sediment toxicity test; for example, amphipod (*Melita plumulosa*) (Mann et al., 2009), midge larvae (*Chironomus tentans*) (Cui et al., 2010), marine bacteria (*Vibrio fischeri*) (Guzzella, 1998), green algae (*Pseudokirchneriella subcapitata*) (Nie et al., 2015). However, benthic organisms show more preferential selection as test organisms in solid phase tests on whole-sediment. They are important in the aquatic food web as low-level consumers and serve as a food source

for the higher trophic level of aquatic organisms. The benthic biotas inhabit in direct contact with sediment, expose to dissolved contaminant in sediment pore-water or ingest sediment which eventually poses a risk to them (Davoren et al., 2005). Considering these aspects, a whole–sediment test with benthic species is needed for a meaningful ecological evaluation to assess the overall effect of sediment on biological communities (Burton, 1992; Havel and Talbott, 1995; US EPA, 2000; Dekker et al., 2006; Turesson et al., 2007; Reinier et al., 2009).

A variety of benthic species such as midge larvae (*Chironomus riparius*, *Chironomus tentans*) and amphipods (*Hyalella azteca*) have been employed in whole sediment toxicity tests (US EPA, 2000). However, maintaining such test organisms is a burden to the testing laboratories. To overcome this problem, a cyst-based test using a benthic ostracod *Heterocypris incongruens* has been successfully developed by Chial and Persoone (2002a, b) to reduce the burden of culturing and maintaining live stocks of organisms. *H. incongruens* is small benthic species (freshly hatched neonates size 150-200 µm to 1.5 mm in adults) (Figure 1.1) that reproduces parthenogenetically (Cooman et al., 2015). It is capable of swimming in the water phase and is also found crawling on and digging into the sediments, thus representing a true benthic organism that is in direct contact with contaminated sediments (Chial and Persoone 2002a; Cooman et al., 2015). In 2012, a freshwater sediment toxicity test using *H. incongruens* (6-day toxicity test) was standardized as ISO 14371 (ISO, 2012). This bioassay has been widely applied to various solid samples such as sediments (Torokne and Toro, 2010), soils (Chial and Persoone, 2003; Santorufo et al., 2012) and road dust (Watanabe et al., 2011; Khanal et al., 2014). The sensitivity of *H. incongruens* was found to be comparable with amphipod *H. azteca* (Blaise et al., 2000; Cooman et al., 2015), midge larvae *C. riparius* (Belgis et al., 2003) and equal or greater than *Daphnia magna* (Torokne and Toro, 2010). However, 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 (ISO, 2012) endpoint is too short to observe the toxicity of contaminated sediments that may affect reproduction. Moreover, in terms of aquatic conservation, the sub-lethal 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.

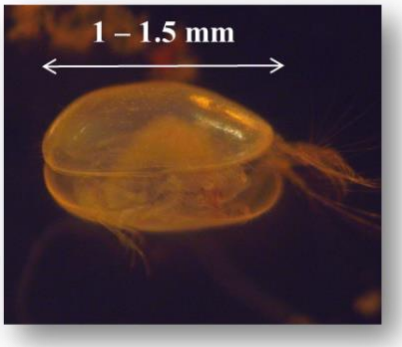


Figure 1.1. Ostracod species: *Heterocypris incongruens*

1.2. Statement of the problem

In aquatic ecosystems, sediments serve as an important sink of numerous substances which has been overlooked in ecotoxicology for many years. Contaminated sediments degrade water quality and adversely affect aquatic organisms, particularly benthic organisms due to their direct contact with and ingest the sediments. For a better understanding of ecological health impact, toxicity of sediment on benthic organisms should be assessed in addition to the water phase toxicity test. Sediment contamination can associate with acute and chronic effects on aquatic life. The chronic exposure is more realistic in the environment and therefore more ecologically relevant to determine (Ingersoll and Nelson, 1990; Simpson et al., 2005). In these aspects, a chronic sediment toxicity test with benthic organism is required. The test is a direct measure of sediment toxicity and to evaluate the effect of long-term sediment exposure to the organism.

Despite the wide variety of benthic species such as the midge *C. tentans* in the life-cycle test and amphipods *H. azteca* in the 42-d test have been employed as whole sediment toxicity testing (US EPA, 2000). However, they require spaces and costs to perform such tests. A new cyst-based 6-day ostracod toxicity test is one of the available direct-contact sediment bioassay which was standardized as ISO 14371 (ISO, 2012). The test using the benthic ostracod *H. incongruens* has successfully developed to reduce the burden of culturing and maintaining live stocks of organisms to the testing laboratories (Chial and Persoone 2002a, b). The cyst-based test has major advantages including the requirement of test organisms on demand and low-cost for routine monitoring the freshwater sediments (ISO, 2012). This bioassay has applied to different solid-phase of environmental samples; for

example sediments (Torokne and Toro, 2010), soils (Chial and Persoone, 2003; Santorufo et al., 2012) and road dust (Watanabe et al., 2011, 2013; Khanal et al., 2014). The ISO 14371 endpoint is assessed based on the 6 d- mortality and growth inhibition of ostracod; however, methods to test sublethal chronic effects such as reproduction as a result of long term exposure in sediment is necessary to allow for increased understanding of risk and increased ecological protection. Therefore, the current study is an intention for the development of a new chronic sediment toxicity test method using *H. incongruens*.

1.3. Research objectives

The main objective of this study is to develop and establish a new chronic sediment toxicity test method using a freshwater benthic ostracod *Heterocypris incongruens;* the following specific objectives are given.

The specific objectives,

- To propose a new chronic sediment toxicity test using the benthic ostracod *H. incongruens* by intensive literature surveys.
- 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.
- 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.

1.4. Structure of the research work

The research structure is divided into eight chapters. A brief outline of each chapter is given below. Figure 1.2 shows thesis structure and title given in each chapter.

Chapter 1. This chapter includes the background, objectives, statement of the problem and the structure of the thesis.

Chapter 2. This chapter reviews related literature and studies about the sediment toxicity test, types, and endpoint used in the toxicity tests and summarizing the toxicity of heavy metals in the aquatic environment including urban road dust.

Chapter 3. This chapter summarizes chemicals and reagents used, test organisms, food cultivation and preparation, brief procedures of 6-day ostracod toxicity and statistical analyses used in this study.

Chapter 4. This chapter proposes the detailed procedures of a chronic sediment toxicity test using the benthic ostracod *H. incongruens* and summarizes the test conditions and measurement endpoint used in this study.

Chapter 5. This chapter reports the repeatability of chronic ostracod toxicity test and proposes test acceptability criteria for control sediment

Chapter 6. In this chapter, the aim was to assess the toxicity of nickel on the reproduction of the benthic ostracod *H. incongruens* and to compare the sensitivity of ostracod to nickel with other test species

Chapter 7. The application example of proposed chronic test is shown in this chapter. The toxicity assessments of urban road dust on ostracod reproduction were conducted alongside the ISO 14371. The present study is expected to serve as the fundamental study on the chronic toxicity of URD to *H. incongruens*.

Chapter 8. This chapter shows the overall summary and conclusions of this study and recommendation for further studies and future development.

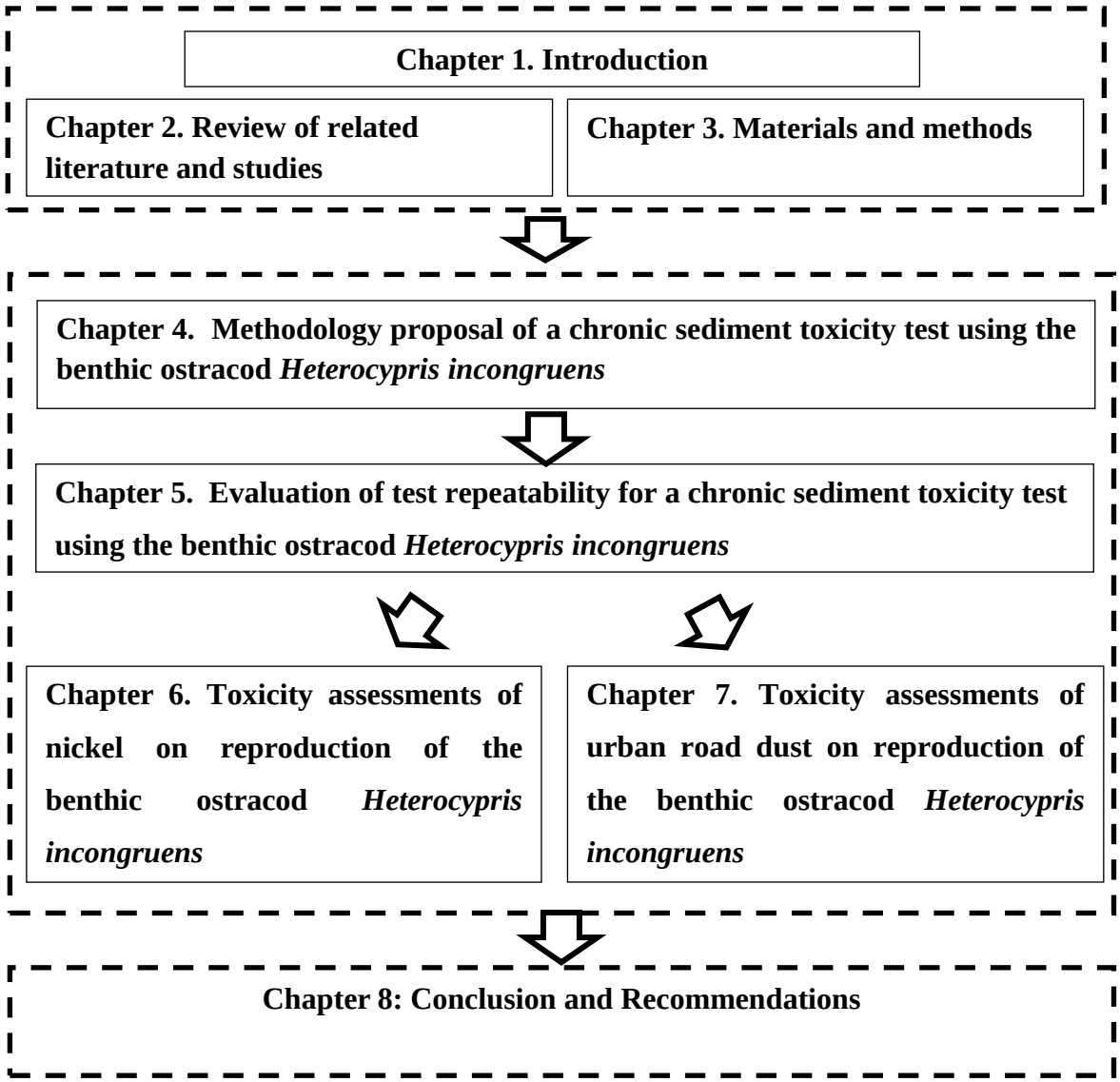


Figure 1.2. Thesis structure

CHAPTER 2

REVIEW OF RELATED LITERATURE AND STUDIES

2.1. Toxicity testing

2.1.1. Acute and chronic toxicity testing

Toxicity test is one of an essential tool for assessing the effects of toxicants in aquatic ecosystems. It has been widely used as a method to identify suitable bioindicator organisms and to derive water quality standards for certain chemicals (Luoma et al., 2008). Toxicity tests generally assess the ability of a substance to negatively affect morphological, physical, behavioral, and/or reproductive characteristics or death of a test organism (Stankovic et al., 2014; Hering et al., 2015). Toxicity tests are generally divided into acute and chronic toxicity tests, depending on the duration of exposure time and endpoints (US EPA, 2000).

Acute toxicity tests are short-term tests usually at a high concentration of the toxicant in which an immediate response of the test organism is observed. Death of the organism is the measurement endpoint for an acute toxicity test. These tests are generally performed with organisms during a partial life cycle or specific time period (Nikinmaa, 2014). The effect of the chemical is observed within a specific period of time.

In contrast, chronic tests assess the prolonged response of a test organism to a moderate to low dose of the contaminant. Chronic tests last for weeks, months, or years. A test is considered chronic if it encompasses more than 10% of the lifespan of the organism (Radix et al, 2000; Nikinmaa, 2014). Sublethal endpoints are generally evaluated during continuous chronic exposure (Burton et al., 1992). A full lifecycle, multiple generations, and the reproductive cycle of an organism are considered for chronic tests (Sancho et al., 2016). Standardized chronic tests conducted by the US Environmental Protection Agency (EPA), the American Society for Testing and Materials (ASTM), and the Organization for Economic Cooperation and Development are not considered valid if mortality of the

control is > 20%. Results are generally reported in sublethal concentrations of the toxicant and sublethal endpoints of the chronic effects. Sublethal effects include reduced growth, reproduction or population size of a test organism (Palma et al., 2016).

2.1.2. Endpoints of toxicity testing

Toxicity endpoints are acute or chronic and are established to observe the effect or degree of harm to organisms.

Acute toxicity endpoints: Lethality is generally used as an endpoint for acute toxicity tests and as the lethal endpoint that is estimated to kill 50% of the test organisms. The half maximal effective concentration (EC50) is often measured in acute tests (Ahlers et al., 2006). The duration of acute toxicity testing is typically 24 or 48 h of exposure time for invertebrates and 96 h for vertebrates. A dose response curve is the most common way to estimate the LC50 and EC50 values.

Chronic toxicity and partial life cycle endpoints: The endpoints used for lifecycle and chronic toxicity tests include the hatching ratio, growth (body length or weight), and long-term survival (%). Other parameters of interest in chronic testing are reproduction, behavior, the total number of eggs produced, and the number of broods released (Ringenary et al., 2007). The no observed effect concentration (NOEC) is the highest concentration in which there is no significant effect from the control test. The lowest observed effect concentration is the lowest concentrations in which there is a significant difference effect from the control test. The *t*-test and analysis of variance (ANOVA) are usually used to determine these chronic test endpoints (Keithly et al., 2004). The approach for assessing such endpoints is to divide the data into two parts or classifications (hatching success, number dead or alive organisms) to use the chi-square or Fisher's exact test. Dunnett's test, which is based on ANOVA for continuous data, can be used for growth variables, reproductive variables, or hatching success and survival data expressed as percentages (Radix et al., 2000).

2.1.3. Acute to chronic ratio (ACR)

The ACR expresses the acute toxicity of an effluent or a toxicant to its chronic toxicity (Raimondo et al., 2007). It is used to determine chronic criteria that would not result to show any adverse effects to population of aquatic organisms (Mebane et al., 2008). In the USA, the ACR is usually used to estimate chronic threshold concentration of an untested species (Raimondo et al., 2007). The ACR is calculated as the ratio of the LC50 to the NOEC or the maximum acceptable toxicant concentration (MATC). The ACR is used if the chronic toxicity mechanisms are the same as those used for acute toxicity. The ACR has been used to derive the U.S. ambient water quality criteria, water quality standards, and risk assessments, which are calculated for species and chemicals that have available acute and chronic test data. The ACR is used to estimate chronic values to different species by the same chemical (Barron et al., 2005) and to estimate chronic toxicity of chemicals with known acute toxicity, but with no chronic toxicity data. Variability in the ACR has been emphasized in aquatic toxicology. The large variation in ACR is related to differences in species, chemical class, chemical mode of action (MOA), and test conditions (Ahlers et al., 2006). The most important determinants of variations in the ACR are chemical MOA and class. For example, higher ACRs are found in heavy metals pesticides, and other chemicals with more specific modes of action than industrial narcotic chemicals (Kenaga et al., 1982; Ahlers et al, 2006).

2.2. Sediment toxicity tests

Sediment toxicity testing is designed to determine whether toxicants or chemical substances in sediment have any effect on living organisms. The main advantage of sediment toxicity testing is that no specific knowledge is required to identify the toxicants, unlike chemical analyses. The tests offer a direct method for assessing toxicity and measuring the potential toxic effects of complex contaminant mixtures in sediments (ASTM, 2005; Palma et al., 2016). The level of toxicity is based on the biological responses and sensitivity of the test organisms (Mann et al., 2009). Dissolved toxicants may have a greater effect on organisms exposed in an aquatic environment, but organisms

can also be susceptible to contaminants adsorbed to suspended solids, particularly benthic organisms (Burton et al., 1992; De Serres and Bloom, 1996).

Sediment toxicity testing was developed during the past few decades because sediment associated contaminants are now acknowledged as a long-term widespread pollutant source. Pore water (interstitial water) toxicity testing is used to identify contaminants in sediment pore water, which can be transported to overlying water by a variety of processes. However, chemical contaminants are disrupted during the pore water toxicity test; thus, bioavailability may be significantly altered. In addition, results of the pore water toxicity test do not represent the effect of the entire sediment, which is caused by ingestion and is a significant exposure route in benthic organisms (Doe et al., 2001). Whole-sediment toxicity tests on field-collected and laboratory-dosed sediments are essential to evaluate the adverse effects of contaminated sediments on aquatic organisms, particularly benthic organisms, due to their direct contact with sediment (Burton and Landrum, 2003). Several studies have developed whole-sediment toxicity tests using a variety of benthic organisms, such as the cladoceran *Chydorus sphaericus* (Dekker et al., 2006) and the copepod *Attheyella crassa* (Turesson et al., 2007). Additionally, a number of standardized methods for whole-sediment toxicity testing were established within the US EPA and the ASTM by the mid-1990s. Unfortunately, most sediment toxicity test methods involve acute and not chronic studies, such as toxicity in benthic macroinvertebrate using the amphipods *Hyalella azteca* and *Rhepoxynius abronius* or the midges *Chironomus tentans* and *Chironomus riparius* (US EPA, 1994a,b; ASTM, 200). Chronic effects such as changes or decreased reproduction are of greater concern. Recently developed sediment toxicity test methods (US EPA, 2000, 2001a) focus on long-term chronic studies using sensitive organisms and/or sensitive life stages of test organisms. The guidelines include use of the freshwater amphipods *H. azteca* and the midges *C. tentans* and *C. riparius* as test organisms. These are the most prominent and widely used organisms in whole-sediment testing. A list of standardized solid-phase sediment toxicity tests for freshwater invertebrates is shown in Table 2.1.

Table 2.1 List of standard sediment toxicity test for freshwater invertebrates**.**

Species	Duration	Endpoints	References
Heterocypris incongruens	6-day	Mortality and growth inhibition	ISO, 2012 (ISO14371)
Hyalella azteca	10-day	Survival and growth	US EPA, 2002 (Test Method 100.1)
Chironomus dilutus formerly known as *C. tentans*	10-day	Survival and growth	US EPA, 2002 (Test Method 100.2), ASTM-E1706-05 (2005)
Chironomus riparius	Up to 10 to 14 days if larval survival or growths are monitored. Up to 30 days if emergence of adults is monitored.	Larval survival, growth, and head capsule width, emergence of adults	ASTM-E1706-05 (2005)
Hexagenia spp.	21 day	Nymphal survival and growth (weight or length), molting frequency and behavior (optional)	ASTM-E1706-05 (2005)
Tubifex tubifex	28 day	Survival and reproduction	ASTM-E1706-05 (2005)
Diporeia spp.	28 day	Survival and behavior	ASTM-E1706-05 (2005)
Hyalella azteca	42-day	Survival, growth, and reproduction	US EPA, 2002 (Test Method 100.4)
Chironomus dilutus formerly known as *C. tentans*	Life-cycle test for measuring the effects of sediment-associated contaminants	Survival (20-day), growth (20-day), emergence (23-day and on), and reproductions as number of eggs per female (23-day and on), and percent hatching success (23-day and on).	US EPA, 2002 (Test Method 100.5) ASTM-E1706-05 (2005)

Ideally, sediment toxicity tests should be rapid, simple, reproducible, standardized, sensitive, and ecologically relevant (Bebon R., 2013). Toxicity endpoints vary in their sensitivity and ecosystem relevance. Chronic rather than acute endpoints are usually preferable to understand how chemicals and toxic sediments affect organisms over long periods of time. Chronic whole-sediment toxicity testing can be used to determine the long-term impact of contaminated sediment (Gale et al., 2006). Reproduction is more likely to be linked to an ecotoxicological assessment than growth. Therefore, a test evaluating only growth and mortality may not be sensitive enough to detect loss of a population. Chronic toxicity tests are recommended to evaluate the long-term effects of sediment contamination. "No significant risk" of potentially contaminated sediment can only be demonstrated using chronic tests; however, their long effort and high cost may obstruct their widespread use (MADEP, 2006). Although chronic toxicity tests are often preferable, they do have certain drawbacks. Longer tests allow more time for unknown biotic and abiotic factors to affect the results. Therefore, the results may be expected to be more variable than those of acute tests. However, chronic exposure is more realistic and more relevant to study than acute exposure (Bebon R., 2013).

Numerous studies have proposed the significant importance of using various test species in evaluation of contaminants in aquatic ecosystems because sensitivity of the species varies among toxicants. Given that benthic organisms are the most likely to be in direct contact with and ingest sediments, evaluating the impact of sediment-associated contaminants on benthic organisms is a direct measure of sediment quality and toxicity (US EPA, 2000; Dekker et al., 2006). Many test species, such as *Daphnia magna*, *Ceriodaphnia dubia*, *Tubifex tubifex*, *Chironomus tentans*, and *Chironomus ripanus*, and endpoints have been determined as useful surrogates in water, effluent, and sediment toxicity studies (Burton, 1991). Differences in sensitivities to contaminants among benthic organisms have been considered based on differences in organism behavior (burrowing and feeding), exposure pathways, and the properties of the contaminants (partitioning and bioavailability). A benthic ostracod has been used in ecotoxicological studies as a freshwater sediment model organism (Chial and Persoone, 2002a,b; Ruiz et al., 2004; Khangarot and Das, 2009). Previous studies have reported that ostracods are highly sensitive to heavy metal, oils, and anoxic conditions (Ruiz et al., 2004) and one study

indicated the need to include ostracods in assessments of solid-phase toxicity, such as soils, sewage sludge, sediments, and aquatic ecosystems (Khangarot and Das, 2009). Khangarot and Das (2009) reported that the ostracod *C. subglobosa* is highly sensitive and accurate species to assess the toxicity of metals and reference toxicants.

2.3. Heavy metals in the aquatic environment and their ecotoxicity

Heavy metals are a significant contaminant in the aquatic environment due to their persistence and risk to aquatic organisms. Metals in receiving water originate from natural processes and anthropogenic activities, such as agriculture, mining, fuel combustion, and manufacturing (Luoma et al., 2008). The concentrations of metals in the environment can be very low (non-significant toxicity) to high (high toxicity), which are attributed to the negative effects on receiving water including sediment contamination. The toxicity and exposure routes by which aquatic animals respond to heavy metal exposure have been studied. The toxic responses to metals by aquatic organisms are generally complex and are affected by various factors and/or species sensitivity. The toxicity and bioavailability of metals in the aquatic environment depend on many factors, such as salinity, dissolved organics, pH, hardness, and sedimentation. Metal uptake by aquatic organisms generally involves absorption or surface binding with slow transport into cells (Crist et al., 1988). Metal uptake differs in vertebrates and invertebrates. Invertebrates are exposed to metals via extracellular and intracellular digestion. The metal uptake pathways in benthic invertebrates include ingestion of sediment particles and water.

Sediment is a recognized sink for metals, metalloids, and other contaminants (US EPA, 1998). Heavy metals persist in water and accumulate in sediment after release into the aquatic environment. Therefore, they cause adverse effects on aquatic organisms, particularly in benthic organisms via direct absorption through ingesting water or sediment (Iwasaki et al., 2009; Stankovic et al., 2014; Magalhães et al., 2015). Metals are partitioned in sediments in many soluble, insoluble, and bound complexes with other contaminants, such as soluble-free ions, soluble organic and inorganic complexes, easily exchangeable ions, and precipitates of metal hydroxides with colloidal ferric and manganic oxyhydroxides (Burton, 1991). Additionally, the bioavailability of toxicants that

affect metal speciation in sediment is influenced by many factors, such as the functions of particulate organic carbon and dissolved organic carbon content. Water quality and conditions can also reduce or increase metal bioavailability and toxicity in sediment, such as hardness, pH, and salinity. These characteristics must be considered when assessing the toxicity of sediment contamination.

2.3.1. Toxicity of nickel in the aquatic environment

Nickel is released into the aquatic environment from mining-related activities or anthropogenic sources including burning of coal and other fossil fuels (Eisler, 1998). Concentrations of nickel in the environment range from 1 to 10 µg/l in uncontaminated freshwater and reach 1,000 µg/l in highly contaminated waters (Pane et al., 2004). Nickel is essential to aquatic organisms, particularly plants and bacteria; however, nickel can cause respiratory disorders, inhibit magnesium uptake, and cause kidney lesions in fish and other aquatic animals (Wogram et al., 2011; Shuhaimi-Othman et al., 2012). The gut and kidneys play important roles regulating nickel levels in adult rainbow trout (Chowdhury et al., 2008).

Nickel is toxic to aquatic ecosystems and has been defined as a priority pollutant on EPA's Aquatic Life Criteria Table (US EPA, 1986). The sensitivity and chronic toxicity of nickel have been investigated in a number of invertebrate species, such as larvae of *Chironomus riparius* (Powlesland and George, 1986), the cladoceran *Ceriodaphnia Dubia* (Kszos et al., 1992; Keithly et al., 2004), and the amphipod *Hyalella azteca* (Borgmann et al., 2001; Keithly et al., 2004). The chronic effects of nickel vary among aquatic species. The values from chronic tests are essential for establishing aquatic life criteria guidelines and deriving chemical concentration criteria; however, no toxicity data are available on chronic nickel exposure or sensitivity to benthic ostracod species.

2.3.2. Toxicity of chromium in the aquatic environment

Chromium is an essential trace nutrient that is required in small amounts for carbohydrate metabolism, but it becomes toxic at higher concentrations (Czapla, 2015). The most bioavailable and toxic form of chromium is hexavalent Cr (VI) (Kim et al., 2002; Hosseini and Belador, 2009). Therefore, aquatic life must be protected more closely from Cr (VI) than from trivalent chromium Cr (III). Toxicity of chromium to aquatic organisms increases as water temperature increases and as pH and salinity decrease. Additionally, chromium is more toxic in soft water than hard water. The chromium LC50 is 3 mg/l in soft water and hard water of 72 mg/l for fish fathead minnows and for goldfish of 18 mg/l in soft water and 133 mg/l in hard water (Taub, 2004).

Chromium (10 µg/l) reduces survival and fecundity of *Daphnia magna* after a 32 day exposure (US EPA, 1980) and causes behavioral changes in the midge *Chironomus tentans* at 100 µg/l in a 48 h test (Catalan, 1982). Trivalent chromium is usually less effective than Cr (VI) for reducing fecundity in *Daphnia magna* [44 µg/l Cr (III) and 10 µg/l Cr (VI) (US EPA, 1980)]. Benthic invertebrates show limited ability to accumulate chromium from clays or in sediments (Neff et al., 1978). The insoluble oxides, hydroxides, and phosphates form of Cr (III) is generally found which are associated with organic matter in sediments (Cervantes et al., 2001). Cr (VI) is usually associated with oxygen to produce oxyacids, such as chromate ions or dichromate (Singh et al., 2013). These forms are more soluble and more toxic to the aquatic organisms than Cr (III). It is widely accepted that dissolve forms of metals have significant effect and cause toxicity because they are more bioavailable (Magalhaes et al., 2015). One study reported the environmental fate and partitioning of chromium in the environment. Absorption of Cr (III) increases on suspended solids and sediment as pH increases (EU RAR, 2005). In contrast, Cr (VI) adsorption decreases with increasing pH. The acute toxicity of Cr (VI) to invertebrates appears to depend on media properties, such as hardness, pH, and temperature. Persoone et al. (1989) reported a decrease in the EC50 values of *Daphnia magna* as hardness decreased and temperature increased. Chronic tests appear to show less of an effect of these properties on toxicity (EU RAR, 2005).

2.3.3. Toxicity of zinc in the aquatic environment

Zinc is an essential element of all living organisms. However, it is toxic at high concentrations. Zinc is a common metal in freshwater and saltwater environments. Zinc occurs as dissolved, suspended, or in solid binding with other chemicals. Zinc forms and complexes with hydrated, oxyions or organic ligands (humic and fulvic acids) in freshwater. Adsorption of zinc on soil, sludge, and suspended solids affects the speciation and bioavailability of zinc (Gillis et al., 2006). Speciation of zinc is influenced by water characteristics, such as pH, organic matter content, and redox potential. In 2003, the Environmental Quality Standard for zinc was established in Japan as 30 μg/l to protect living organisms (Tsushima et al., 2010).

Sensitivity to zinc varies between species and the different endpoints investigated. Muyssen et al. (2006) studied the response of *D. magna* to chronic zinc exposure. They revealed the mechanisms of chronic zinc toxicity to *D. magna*, as zinc inhibits uptake of calcium, which causes hypocalcemia. Zinc freshwater toxicity data are available for algae, crustaceans, and fish, but few reports were found on ostracod species. The LC50 of zinc ranges from 0.070 mg/l to 7,800 mg/l. The lowest LC50 of zinc is found in crustaceans and the highest occurs in fish (SCHER, 2007). Chronic zinc data have been reported for freshwater algae, invertebrates, and fish. Based on these studies, the NOEC values were 17 for algae to 660 μg/l for fish (Bodar, 2007). The NOEC values for invertebrates range from 37 μg/l in *Ceriodaphnia dubia* (reproductive endpoint) to 400 μg/l in the zebra mussel *Dreissena polymorpha* (survival endpoint). Most of the data on freshwater invertebrates include the water flea species *D. magna* and *Ceriodaphnia dubia* (crustaceans) (Bodar, 2007).

2.4. Urban road dust and its toxicity

Urban road dust is a potential source of sediment contamination and may cause adverse effects to benthic organisms and deteriorate the water quality of urban road runoff. Previous studies have reported that urban road dust contains high concentrations of many potentially toxic chemicals, such as hydrophobic organic compounds, heavy metals, polycyclic aromatic hydrocarbons, and perfluorinated surfactants (PFSs) (Fang et al., 2004; Pengchai et al., 2004; Murakami et al., 2005; Murakami and Takada, 2008; Atiemo et al., 2011). The main sources of these pollutants are tire dust, brake dust, road surface wear, and vehicle exhaust (Khanal et al., 2014; Mummullage et al., 2014).

Urban road dust is a particulate source of urban runoff that is toxic to aquatic organisms (Boxall and Maltby et al., 1995; Watanabe et al., 2013; Khanal et al., 2015). The toxicity of urban road dust is attributed to the site-specific nature of the toxicant distribution (Watanabe et al., 2011, 2013; Khanal et al., 2013, 2014). Various authors have reported that traffic characteristics, such as the amount of traffic (annual average daily traffic), type of traffic (commuter, industrial, or construction), and traffic congestion (resulting in brake use) may affect the toxicity of road dust (Grant et al., 2003; NIWA, 2008). The heterogeneity of urban road rust causes wide variability in its toxicity. Mummullage et al. (2014) studied the variability in metal composition and concentrations in four different urban land use areas in Australia. The most abundant heavy metals in road dust from urban land were iron, zinc, aluminum, and magnesium. High concentrations of nickel, titanium, copper, and zinc are found on industrial sites. A factor analysis study revealed that soil and related traffic are key sources of metals deposited on road surfaces in urban areas (industrial, residential, commercial, and mixed areas).

Petroleum hydrocarbons are a major organic contaminant of interest in urban road dust samples. Petroleum hydrocarbons and their derived compounds are referred to as total petroleum hydrocarbons (TPHs). In addition, polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in the urban environment, including urban road dust. PAHs are an important subset of compounds included in TPH and are both environmentally persistent and potentially toxic to the environment. Sixteen PAHs have been listed as priority

pollutants by the US EPA (US EPA, 1982). In this report, TPHs refer to organic contaminants and, in particular, a subgroup of hydrocarbons called PAHs. The significant source of TPHs and PAHs are from vehicle fuels and emission, such as oil and grease. PAHs have been reported in road dust from automobile exhausts, asphalt particles, tire particles, gasoline, and diesel fuel in many studies (Takada et al., 1991; Murakami et al., 2005; Boonyatumanond et al., 2007).

The median TPH concentration is 1000–2000 µg/g (NIWA, 2008). The highest concentration of TPH in road dust was found in an industrial area with crankcase oil and a small amount of fuel oil. High volumes and the type of road use activities result in high concentrations of TPHs. Thus, TPH concentration appears to be dependent on traffic characteristics. For example, TPH concentrations of 353, 1,680, and 3,490 µg/g were reported in urban road dust from residential, highway, and industrial sites, respectively (Latimer et al., 1990).

The concentration of PAHs in road dust varies considerably (2–200 mg/kg) (Yang et al., 1995), Interestingly, traffic volume does not appear to be related to PAH concentration, because a Tokyo road with 100,000 vehicles per day contained only 4 mg/kg of PAHs (Takada et al., 1991), whereas a study by Lee and Dong (2010) reported that many road and traffic related characteristics such as pavement type, traffic volume, vehicle speed greatly affected PAH concentrations in road dust. Wang et al. (2009) determined the concentrations, distribution, and sources of PAHs in urban street dust in China. Total PAH concentrations in street dust were 1,890–17,070 ng/g (dry weight) with an average of 7,460 ng/g. A principal component analysis was used to apportion the sources of PAHs in urban street dust. The results showed that pyrogenic and petrogenic sources contributed 70% and 22.4% of total PAHs in street dust and fossil fuels (coal and petroleum); biomass combustion accounted for 64.4%, and 5.6% of total PAHs were from pyrogenic sources (Wang et al., 2009).

Several studies have investigated and identified PCBs, PFSs, herbicides, and pesticides in road dust and road runoff. Murakami et al., (2005) reported that urban highway runoff contains these organic compounds but that they are not directly related to vehicles. PFSs

are at significantly higher concentrations in size-fractioned street dust from a heavily trafficked area than from a residential area (Murakami et al., 2008).

As the sources of urban road dust and it composition are diverse and many factors affect concentrations of toxicants, toxicity assessments of urban road dust must be further investigated. The high variability of potential toxicants in urban road dust makes it difficult to generalize the primary toxicants. Most studies have focused on chemically characterizing urban road dust (Fang et al., 2004; Pengchai et al., 2004; Murakami et al., 2005; Murakami and Takada, 2008; Atiemo et al., 2011; Mummullage et al., 2014). However, a chemical analysis alone cannot predict the biological effects of urban road dust. Toxicity testing is necessary to determine the potential impact of urban road dust on benthic biota. Sediment bioassays with benthic species would be helpful to evaluate the overall effect of urban road dust as a potential source of sediment contamination in biological communities. Toxicity assessments of urban road dust on benthic organisms have been applied to the ostracod direct contact test (Watanabe et al., 2011, 2013; Khanal et al., 2014, 2015). The adverse effects of urban road dust were only assessed based on 6-day mortality and growth inhibition, although organisms could be exposed to these contaminants for a longer period of time. However, the long-term effects of urban road dust on the benthic ostracod *H. incongruens* have not been reported.

2.5. Summary

Sediment contamination caused by many chemicals and pollutants binds to organic and inorganic particles that eventually settle to the bottom of the aquatic environment to degrade water quality and adversely affect benthic organisms. Whole-sediment toxicity testing is an essential tool to evaluate the overall effect of contaminated sediment on benthic species. Once contaminants bind to particulate matter, biotransformation is rarely occur and desorption is usually very slow. Therefore, contaminants embed or sorb for long periods in sediments. Most sediment toxicity testing has been developed to assess short-term exposure as an acute toxicity test. However, long-term chronic toxicity testing should be considered for an ecologically meaningful evaluation.

CHAPTER 3

MATERIALS AND METHODS

3.1. Introduction

This chapter presents the chemical reagents, test organism, test chemicals, urban road dust sample, algae cultivation, preparation of algae suspension including briefly procedure of standard 6 d ostracod toxicity test (ISO, 2012) and statistical analysis used in this study.

3.2. Test medium

3.2.1. Standard freshwater (SFW)

Standard freshwater was commonly used for culturing ostracod. Table 3.1 shows the composition of SFW according to the formula of moderately hard water in manual's instructions (hardness: 80-100 mg $CaCO_3$/l and alkalinity 57-64 mg $CaCO_3$/l) (US EPA, 2002). Thus prepared, the SFW medium has a pH of 7.6 ± 0.3 (ISO, 2012). The SFW was aerated for 15-20 minutes prior to use.

Table 3.1. Composition of standard freshwater (SFW) (US EPA, 2002).

Component	Weight per 1 l of medium
$NaHCO_3$	96 mg
$CaSO_4 \cdot 2H_2O$	60 mg
$MgSO_4$	60 mg
KCl	4 mg

3.2.2. C medium

Green algae *Scenedesmus acutus* Meyen (NIES-94) used as food for ostracod were cultured in the C medium. Tables 3.2 and 3.3 show the composition of the culture medium, which was prepared according to the instruction of the culture collection (NIES, 2004). The pH of the culture medium was adjusted to 7.5 by 2N hydrochloric acid.

Table 3.2. Composition of C medium (NIES, 2004).

Component (concentration in stock solutions)	**Concentration (per l)**	**Amount of stock solution or chemical in 1 l of medium**
H_2O (Milli-Q water)	-	962.7 ml
TRIS	500 mg	500 mg
$Ca(NO_3)_2 \cdot 4H_2O$ (10 mg/ml)	150 mg	15 ml
KNO_3 (10 mg/ml)	100 mg	10 ml
$Na \cdot glyceroPO_4 \cdot 5H_2O$ (10 mg/ml)	50 mg	5 ml
$MgSO_4 \cdot 7H_2O$ (10 mg/ml)	40 mg	4 ml
Vitamin B_{12}* (1 mg/ml)	0.1 mg	0.1 ml
Biotin (Vitamin B_7) * (1 mg/ml)	0.1 mg	0.1 ml
Thiamin (Vitamin B_1)* (100 mg/ml)	10 mg	0.1 ml
P_{IV} multi metals**		3 ml

*Three vitamins are added after sterilized C medium by autoclaving under the clean bench

Contents of P_{IV} metals stock solution is given in **Table 3.3.

Table 3.3. Contents of P_{IV} multi metals solution (NIES, 2004).

Component	**Weight per 100 ml**
$FeCl_3 \cdot 6H_2O$	19.6 mg
$MnCl_2 \cdot 4H_2O$	3.6 mg
$ZnCl_2$	1.05 mg
$CoCl_2 \cdot 6H_2O$	0.4 mg
$Na_2MoO_4 \cdot 2H_2O$	0.25 mg
$Na_2EDTA \cdot 2H_2O$	100 mg

3.3. Test organisms

Heterocypris incongruens were purchased as dormant eggs (cysts) from MicroBioTests Inc., Belgium. The cysts were hatched in standard freshwater (SFW) according to the formula of moderately hard water in manual instructions (US EPA, 2002) followed by

incubation at 25°C under fluorescent light (3000–4000 lux) for 52 h. The hatched ostracod neonates were pre-fed with dried Spirulina powder 4 h prior to their use as test organisms. The body length of ostracod neonates ranged from 169 to 223 µm.

3.4. Food for ostracod

Laboratory-cultured green algae *Scenedesmus acutus* Meyen (NIES-94) and commercial fish food TetraMin (Spectrum brands, Japan) were used for feeding ostracods in both of the acute and proposed chronic ostracod toxicity tests.

3.4.1. Algae cultivation

C medium (section 3.2.) was used for culturing the algae in the laboratory. The pH of medium was adjusted to 7.5 before autoclaving (120^{o}C, 15 minutes). After transferring 100 ml of autoclaved C medium to 300 ml sterilized Erlenmeyer flask (at 135^{o}C for 2 h), 10 ml of algae stock suspension was inoculated to each flask using a sterile pipette. The culturing flasks were put in the incubator of the following culture conditions; temperature: 25±2°C, light: fluorescent light, photoperiod (12h light: 12h dark), light intensity (5000 lux). The above procedure was conducted every 2 weeks to keep the algae stock fresh.

3.4.2. Preparation of algae suspension for ostracod food

The algae stock suspension was centrifuged at 3000 rpm for 15 min to harvest the cells. And then, fresh SFW was added to re-suspend the algae cells by hand-shaking. The rinsing procedure was repeated 2-3 times to remove C medium remaining in the algae suspension. Finally, the settled algae pellets were transferred to a volumetric flask and re-suspended in the designated volume of SFW to make the algae cell density of 3.0×10^{7} cells/well. Algae cell density was calculated from the absorbance at wavelength 560 nm using Hitachi U-2010 UV spectrophotometer by the equation below.

Algae concentration (cells/ml) = 4.616×10^{6} × Dilution rate × Absorbance

(This equation was determined by measuring absorbance and counting of cells of algae directly using flow cytometer).

3.4.3. TetraMin

A commercial fish food TetraMin (Spectrum brands, Japan) was also used as ostracod food in this study. Flakes of TetraMin were ground in a mortar and pestle and sieved through 250 µm screen before feeding to ostracod.

3.5. Standard ostracod toxicity test (ISO14371)

The method and test materials followed ISO 14371 (ISO, 2012) as show in Figure 3.1; the method is hereafter referred to as the 6-day ostracod toxicity test in this study. The hatching of the ostracod cysts was initiated 52 h prior to the toxicity test as described above (section 3.3). The test was started by inoculating 10 hatched neonate ostracods to each well of a 6-well microplate (IWAKI) containing a mixture of 1 ml sediment, 2 ml SFW, and a 2 ml green algae suspension. Laboratory-cultured green algae *S. acutus* Meyen (NIES-94) were used to feed ostracods at a concentration of 3.0×10^7 cells/well. The algal cultivation and preparation process was described above. Salinity, pH, and conductivity in the overlying water were measured at the beginning and end of the toxicity test using Horiba compact pH meter B212 and Twin compact conductivity meter Horiba B-173. The 6 d ostracod toxicity test was regarded as acceptable when the following two criteria were fulfilled in the control sediment: a) ostracod percentage mortality did not exceed 20%, and b) the mean ostracod length increased by a factor 1.5 compared to the initial mean length, in accordance with the ISO 14371 guidelines (ISO, 2012). The ostracod bioassays endpoints were 6 d mortality and growth inhibition. 6 d mortality is calculated based on the total number of dead ostracods and the total number of ostracod inoculated at the start of the test. 6 d growth inhibition is the second effect criterion of the sublethal toxicity of sediments. Growth inhibition was determined by comparing the length increments of the surviving ostracods in the test sediment with that in the control sediment at the end of the test.

Determination of mortality

% mortality = (B/C) × 100

B = C – A

Where; A: number of surviving ostracods at the end of the test

B: number of dead ostracods

C: number of ostracods at the start of the test (=10)

Determination of growth inhibition

Growth inhibition is usually used when the mortality is below 30% (ISO, 2012). The body length of the ostracod was measured on a digital picture of the ostracods taken under the microscope, using image analysis tool (Image J software, NIH).

% growth inhibition = 100 – ($L_{increment}$ in the test sediment / $L_{increment}$ in control) ×100

Where;$L_{increment} = L_{end} – L_{start}$

L_{end} : mean body length of surviving ostracods at the end of test in the well

L_{start} : mean body length of ostracod neonates at the start of the test (n=10)

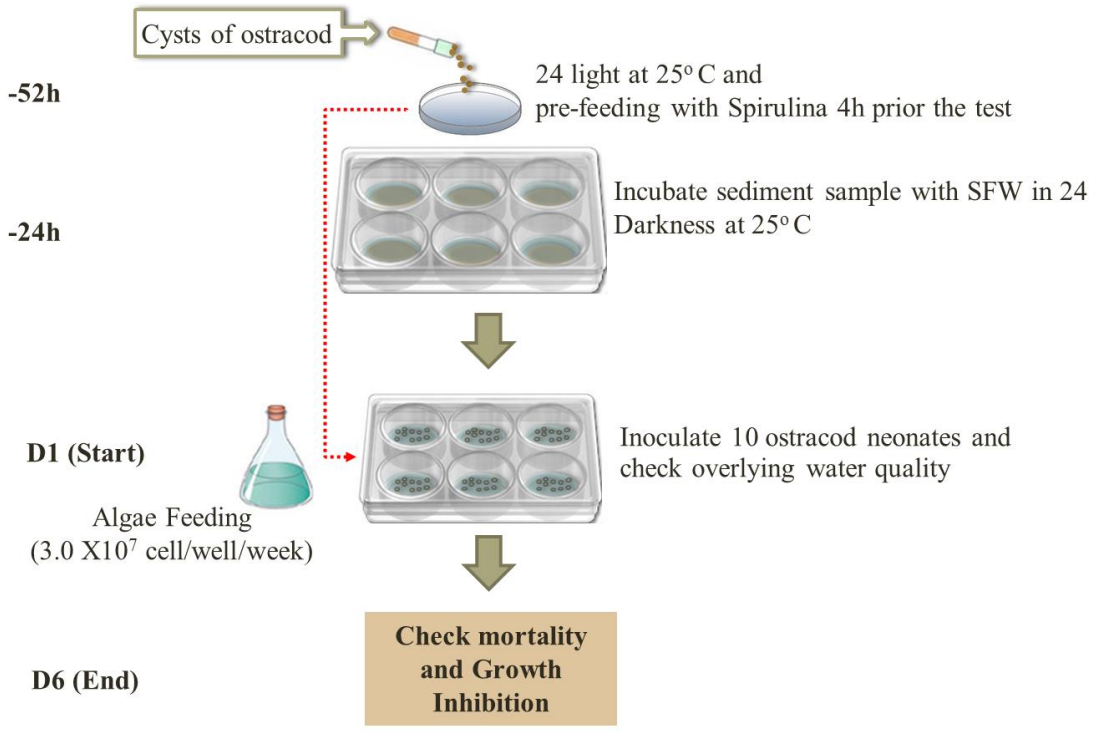


Figure 3.1 Procedure of 6-day ostracod toxicity test (ISO, 2012)

3.6. Test chemicals

Analytical grade of nickel chloride hexahydrate ($NiCl_2.6H_2O$) was used as the reference toxicants. The 100 mg/l stock solution was prepared by dissolving 10 mg $NiCl_2.6H_2O$, in 100 ml Milli-Q water. Exposure concentrations to 0.012, 0.12, 1.2, 12, 120 and 1200 µg/l of Ni were used for the ostracod toxicity test. The exposure concentrations were half as much as measured values because of the subsequent dilution by algal food suspension.

3.7. Urban road dust sample

An urban road dust (URD) (<2000 µm) sample that was previously collected by Khanal et al., (2014) (detail descriptions in Appendix B) from a roadside in Tokyo in 2012, was used in this study, and the clean reference sediment (RF) sediment was used as the control. URD was diluted with the RF sediment to obtain concentrations of 3.125%, 6.25%, 12.5%, 25%, and 50% (v/v). The clean RF was obtained as a part of Ostracodtoxkit F (MicroBioTests Inc., Belgium).

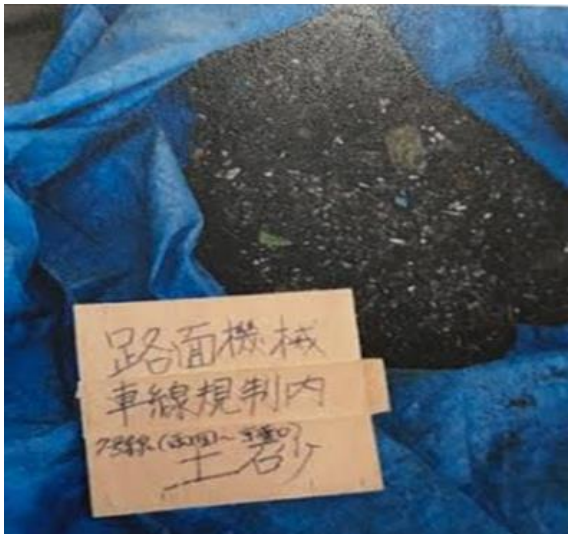


Figure 3.2 Road dust collected by cleaning vehicle (Hiki, 2013)

3.8. Statistical analysis

All data and results were reported with standard deviation. The Grubb's test was applied to test outliers of the control data using Minitab 17 (State College, PA: Minitab Inc.). The CV was calculated as CV = standard deviation/mean × 100% for each endpoint to determine test repeatability. Dunnett's test was used to determine the significant difference ($p < 0.05$) between URD samples and control sediment using Minitab 17. Dunnett's test was used in ANOVA to create confidence intervals for differences between the means of control and treatment groups. A chi-square analysis was applied to the hatching ratio. The median lethal concentration (LC50) and/or LC20 of nickel and URD were calculated by using Probit analysis. The NOEC (non-observed effect concentrations) and LOEC (lowest-observed effect concentrations) were estimated by Dunnett's test.

CHAPTER 4

METHODOLOGY PROPOSAL OF A CHRONIC SEDIMENT TOXCITY TEST USING THE BENTHIC OSTRACOD *HETEROCYPRIS INCONGRUENS*

4.1. Introduction

Sediment is the important compartment in aquatic ecosystems. Taking into account that sediment is the habitat for a wide variety of aquatic biota and feeding ground for all benthic organisms. To determine the potential impact of contaminated sediment on aquatic organisms, the pore water bioassays were originally performed based on the concept of soluble toxicants or called the bioavailability parts. For an ecologically meaningful evaluation, the direct contact tests with benthic organisms are needed. The attention was gradually paid to the evaluation of contaminated sediments on biological communities as a whole-sediment toxicity test (Burton, 1991, 1996; Chial and Persoone 2002a).

The freshwater sediment toxicity test using *H. incongruens* (6 d ostracod toxicity test) is one of the available solid-phase tests and standardized as ISO 14371 (ISO, 2012). The test was developed as a cyst-based test to reduce the burden of culture and maintenance the stocks of test organisms in the laboratory (Chial and Persoone, 2002a, b). The sensitivity of *H. incongruens* has been compared with *H. azteca and C. riparius* in the toxicity testing of rivers sediments (Chial et al., 2003a). The result showed that the sensitivities between the species are quite similar. However, the toxicity criteria of the 6-day ostracod test based on two endpoints: mortality and growth inhibition. For a better understanding of the impact on long-term exposure to contaminated sediment, chronic endpoints are more realistic and relevant to determine which still lack in the conventional 6 d ostracod toxicity test.

Previously, the life history and reproduction characteristics of ostracod *H. incongruens* (previously name is *Cyprinotus incongruens*, Karuthapandi et al., 2014). Lifetime fecundity and egg development time were further examined by Havel and Talbott (1995); results showed that these factors were highly variable in contrast to lesser variations in 10 d ostracod body lengths. Angell and Hancock (1989) studied the hatching characteristics of ostracod eggs under different experimental stresses. The eggs of *H. incongruens* could withstand drying and freezing at various temperatures and could be hatched afterwards. In addition, the further study of Tsukahara (2012) investigated the relationship between the concentration of zinc and the life history of ostracod *H. incongruens* such as lifespan and lifetime egg production, which would be affected when exposed to zinc. Consequently, the results showed the life span and lifetime egg production of ostracod increased under the sublethal concentration of zinc. The chronic effect of zinc to ostracod was reported, but this test was conducted without sediment. Another trial was conducted with sediment for the 28-day test under different feeding frequency and photo-periods. The endpoint for the test was the number of eggs and individuals; however, high mortality of ostracod was observed at the end of the 28-day test even under control condition. The problem to count the eggs under the presence of sediment was also reported (Tsukahara, 2014). Although numerous studies attempted to establish the chronic ostracod toxicity test, but there was a limitation in term of the test method and high variable of ostracod reproduction characteristic. The further improvements of the test procedures and considering the suitability and sensitivity of chronic endpoint are still needed to allow the quantitative measurement on reproduction and to evaluate the chronic toxicity of test sediment.

The main objective of this chapter is to propose a new chronic sediment toxicity test using the freshwater benthic ostracod *H. incongruens*. The intensive literature surveys were done to determine the general conditions and procedure of the proposed chronic test. The selection of test organisms, test phase, test conditions (temperature, feeding, photoperiod) and endpoints for this study were shown. The details procedures and measurement endpoint used in this study were described in details.

4.2. Selection of test organism, test procedure, test conditions and endpoint of chronic ostracod toxicity test

The general conditions and procedure of the proposed chronic test using the benthic ostracod *H. incongruens* were selected based on the following descriptions from literature surveys.

4.2.1. Test organism

The selection of test organisms is one of the most crucial steps in the toxicity test. It is a major influence on the relevance, success, and interpretation of a test (US EPA, 2000). Previous studies proposed the diversity of test organisms in their development of sediment toxicity tests, especially benthic organisms due to their habitat in the sediment (Burton et al., 1992; Havel and Talbott, 1995; Turesson et al., 2007; Reinier et al., 2009). The direct contact of benthic species with sediments, interstitial or overlying water for all their life cycle increases the likelihood for more accurate assessment of contaminated sediments (Burton et al., 1992).

The proposed benthic organism which is focusing in this study is ostracod species *H. incongruens*. The benthic ostracod in the aquatic system represents as the detritus feeder and primary and secondary consumer, thus ostracod is important in ecosystems as a food source for many aquatic organisms and their geographical cosmopolitan distribution (Külköylüog O., 2005; Bebon R., 2013). The brief descriptions for morphology, ecology, lifecycle and reproduction of this ostracod species showed in the previous section (section 1.1). Additionally, the great advantages of selection *H. incongruens* in toxicity testing are that easily reared in the laboratory as a cyst-based test, their comparable sensitivity with amphipod *H. aztecea* and midge larvae *C. riparius* (Chial and Persoone, 2002 c, 2003; Cooman et al., 2015). Many studies have investigated the effects and sensitivity of the ostracod to heavy metals, PAHs and organic compounds (Kudlak et al., 2011; Torokne and Toro 2010) and various solid-phase samples (Torokne and Toro, 2010; Watanabe et al., 2011; Santorufo et al., 2012; Khanal et al., 2014). The test with *H. incongruens* was standardized as ISO test (ISO,

2012) confirming the suitability of *H. in*congruens as a test organism for sediment toxicity testing.

4.2.2. Test phases

The first trial for the development of chronic ostracod toxicity test had been proposed previously by our research group (Tsukahara, 2014). The endpoints were limited to 28-day surviving- and hatched-ostracod individuals, due to the difficulty to count the eggs under the presence of sediment. Thus, the further technical development is necessary to separate the sediment and egg produced. The study of Havel and Talbott (1995) reported the first day of ostracod laying eggs at the average on the 19^{th} day ranged from 13^{th} - 25^{th} day. The decision was made to remove sediment phase before ostracod starts laying the first brood of the eggs. As a result, in the present study, the sediment exposure phase and reproduction phase were separated. The test phases were considered to separate into three consecutive parts as (I) a 14-day sediment exposure phase, (II) a reproduction phase and (III) a hatching test, which allow quantifying measurement on ostracod reproduction. The reproductions phase is decided to conduct without sediment which referred to the methodology of *H. azteca* 42-day test in US EPA (2000). The test design with amphipods is isolated from the sediment exposure phase on day 28^{th} and place into water-only chambers where the reproduction is measured on day 35^{th} and 42^{nd}. Though the test organism might recover from the effects of sediment or acclimate in reproduction phase, they are exposed to toxic sediment during their critical developmental stages, the reproduction impairment occurs before the release of the first brood in clean water (US EPA, 2000).

4.2.3. Test conditions

Many abiotic factors such as temperature, pH, water hardness, feeding and light conditions should be investigated in toxicity test. Due to the healthy, normally behave, well feeding and low mortality of test organisms need to be maintained during the long holding period and in the controls. For benthic ostracod, many factors such as temperature, photoperiod, and feeding are known to affect growth and reproduction including the hatching success (Chial and Persoone, 2002a; Rossi et al., 2011;

Tsukahara, 2014; Aguilar-Alberola et al., 2014). The following sections have described the selection of the test conditions in the proposed chronic test.

Temperature: Development of *H. incongruens* includes eight instars; egg production begins after the final molt (ninth instar) at temperatures of 10–30^{o}C (Havel and Talbott, 1995). In addition, following the study by Chial and Persoone (2002a), 25°C was selected for suitable rapid growth of the ostracod neonates. The temperature at 25±1^{o}C is used in conducting the ISO 14371 method (ISO, 2012). Thus, the current study was decided to perform at temperatures 25°C.

Photoperiod: 24h-darkness is proposed to be used in the proposed chronic test. Tsukahara (2014) reported that the light increased the algae fed as food. The algal growth affects the level of dissolved oxygen concentrations due to photosynthesis. Previous studied of Tsukahara (2014) observed the growth of algae during the test period of those well-plates without adding the algae except in the well-plate incubated for 24-h dark condition. The algae growth believed to be an excessive amount of spirulina given to ostracod neonate prior to the test. Although algae are not fed after 7 days of sediment exposure phase, the well-plate should keep under the dark condition to avoid the algae growth. Therefore, 24h-darkness was selected as photoperiod condition in this test development

Feeding: In this study, algae are proposed to be fed in the first week of the test and TetraMin in the following weeks. The purpose of feeding during the toxicity test is to maintain the health and quality of test organisms. An overestimation of sediment toxicity due to starvation as well as underestimation in the toxicity test at high food levels (Haas et al., 2004). The food of ostracod was also considered based on Sevilla et al. (2013) which reported the effect of different food on the ostracod growth and sensitivity to the toxicants. The results showed TetraMin could support ostracod growth at least equally with the algal cells and met the criteria in ISO 14371. Tsukahara (2014) proposed the usage of TetraMin fed as food to ostracod after 10-day exposure. Soucek et al., 2016 conducted the water only of a 42-day test of *Hyalella azteca* treatments in different food or combination of foods. The results showed that

the treatment with TetraMin had high survival, weight, and reproduction of amphipod *H. azteca* as well as low variability (coefficient of variation) among replicates.

Figure 4.1 shows ground TetraMin after pass through 250 µm mesh screen (taken by Image J analysis). The mean particle size of TetraMin was 103±46 µm ranged from 15-219 µm. It appeared that TetraMin is larger than algae *S acutus* (less than <10 µm in size) (NIES, 2004). From this fact, it can be inferred that the ingestion of TetraMin was difficulty when feeding to the ostracod neonates ranged from 169 to 223 µm. Thus, algae were fed in the first week of the sediment exposure phase. After 1 week of the exposure, the ostracod is larger in size (ranged from 500-700 µm). It presumed that ostracod can ingest TetraMin at this life stage. Ostracod graze for the food by carapace (shell) is open the appendages can protrude between the valves for feeding, reproduction, and locomotion. For this reason, TetraMin is selected as the feeding during the second week of sediment exposure phase and reproduction phase. The easiness in preparation and frequency of feeding also accounts for this decision.

Food amount and feeding frequency are proposed in this study following the results of two previous studies by Sevilla et al., (2013) and Tsukahara, (2014). The number of ostracod individuals and the number of eggs were relatively high under the 24-hr dark condition when fed with algae at $3.0X10^7$ cell/well/week (Tsukahara, 2014). Sevilla et al., (2013) studied the effect of different food on the growth of ostracod in 6-day exposure. The results showed all three different doses of TetraMin could support ostracod growth at least equally with the algal feeding and met the test validation criteria in the ISO protocol. Algae (*S. acutus*) at concentration $3.0X10^7$ cell/well during the first week and 2 mg/well of TetraMin in the second week were fed as a food for ostracod in 14-day sediment exposure phase.

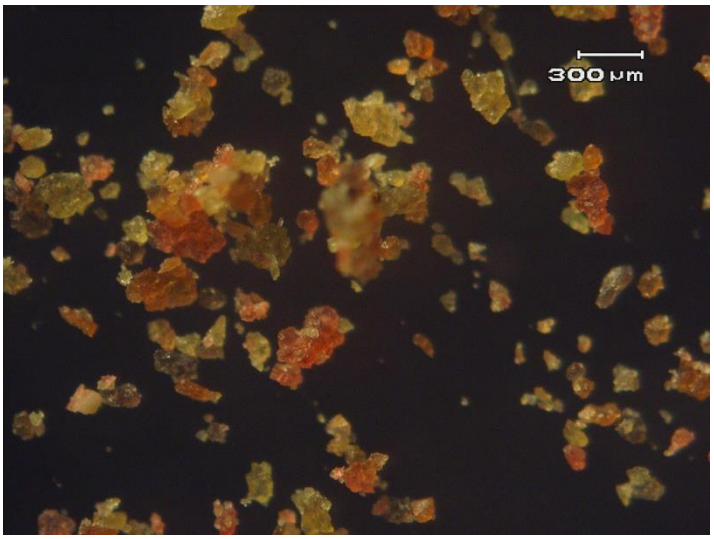


Figure 4.1. TetraMin after sieving by 250 µm mesh screen

4.2.4. Endpoint of a chronic sediment toxicity test

Previous sections 1.1 of chapter 1 mentioned the important of conducting chronic toxicity test in the evaluation of sediment contamination. Given that acute tests typically assess mortality over short-term exposures and usually indicate a high level of toxicity in sediment. To investigate the long-term effect of contaminated sediment, chronic test with reproductive endpoints is needed, which can be used to evaluate the moderate and low toxicants over long-term exposure to test organisms.

Previous researches studied the reproduction characteristics of ostracod. The ostracod *H. incongruens* reproduce by parthenogenesis. They produce both resting and non-resting eggs that have no recognizable morphological difference. Egg of ostracod is spherical eggs (about 150-200 µm in diameter) in the water or in aggregation with several layers of hundreds of eggs attached to any suitable substrate (Rossi et al., 1996, 2004, 2011, 2013, 2015). In addition, life history characteristics of an ostracod *H. incongruens* had studied by Angell and Hancock, 1989; Havel and Talbott, 1995). They showed that ostracod *H. incongruens* had a life span of 14-37 days with an average of 25 days (developed through eight instars) and reproduction start after the final molt (ninth instar). The egg production ranged 13-25th days with 1-4 days between individual broods. Lifetime egg production ranged from 1-36 eggs (means

36.5 eggs/individual). The time between hatching and laying the first brood took a mean of 19.0 days (Havel and Talbott, 1995). These life history characteristic of the ostracod can be used as reproductive endpoints in the present study. In addition, Havel and Talbott (1995) and Angell and Hancock (1989) reported that ostracod produced two types of the eggs: non-resting and resting. Resting eggs were resistant to desiccation and had a highly variable hatching time. Havel and Talbott (1995) reported that the 13% of the egg hatched within the first 10 d of observations. Angell and Hancock (1989) found that 32% of the ostracod eggs hatched within 125 d and half of them hatched within 10 d. From these two studies, it was concluded that 14 d was sufficient as the hatching test observation period. Nevertheless, hatching test in this study is required to do in parallel with the reproduction phase; thus it is necessary to develop a more feasible procedure to determine the hatching rate in order to reduce these kinds of burden. Previous studies showed that the eggs of ostracod could withstand drying and freezing at various temperatures and could be hatched afterwards (Angel and Hancock, 1989; Vandekerkhove et al., 2013). Therefore, the possible further development of the hatching test for the first attempt is pre-incubation the ostracod eggs to preserve them and performs the subsequent hatching test in the later time (the preliminary investigations is shown in Appendix D).

4.2.5. Test acceptability criteria

The acceptability criteria for the toxicity test include the acceptable growth of test organisms in the controls and variation between the replicates. In the guideline for the standard toxicity test method of US EPA stated that it is strongly recommended to conduct the test with control sediment before the test with potentially contaminated sediment. The data for control conditions can be used to determine if the use of the control sediment and other test conditions are resulting in the acceptable performance of the tests including correctly interpretation for the toxicity test results (US EPA, 2002). The following chapter will perform the proposed chronic test with control sediment (reference sediment provide from the ostracodtoxkit) to evaluate the test acceptability under control conditions and to quantify the natural variation of test endpoint. The data of the test repeatability can be used as the test acceptability criteria for control sediment in the proposed chronic test.

4.3. Proposal of the method and detailed procedure of chronic ostracod toxicity test

4.3.1. Experimental design

The following section is the summary of experimental design which composed of three consecutive phases: (I) a 14-day sediment exposure phase (II) a reproduction phase (III) a hatching test. The procedures and timeline for the chronic toxicity test were described in details in Table 4.1.

(I) 14-day sediment exposure phase

14-day sediment exposure phase was designed following the procedures of the 6-day ostracod toxicity test in Table 4.2 (ISO14371), but for a longer period of time (14 days). The hatching of the ostracod cysts was initiated 52 h prior to the toxicity test as described earlier (section 3.5). Toxicity test was started by adding 2 ml of algae suspensions (*S. acutus*) and ten ostracod neonates into each well of the 6-well microplate containing test sediment and SFW, then the microplates were incubated at 25^{o}C under 24h darkness for 14 days. Particularly on the day 7 of the sediment exposure phase, ostracod was fed with 2 ml of 1 mg/ml of TetraMin (a commercial fish food, www.tetra-jp.com) after discarding the 2 ml of overlying water in each well. Special care was taken not to disturb ostracod while changing the overlying water on day 7. Flakes of TetraMin were ground in a mortar and pestle and sieved through 250 µm screen. The pH, conductivity, and salinity of the overlying water were measured at the start of the test, on day 7 and day 14 of the sediment exposure phase (Figure 4.2).

(II) Reproduction phase

Reproduction phase follows 14-day sediment exposure phase and continues until all the ostracods die (Figure 4.2). The reproduction phase was started by transferring each surviving ostracod individually to a well of 12-well microplates (one individual per one well) containing 2 ml of SFW and 1 ml of 1 mg/ml of TetraMin suspension without sediment. Then, the well plates were incubated at 25^{o}C under 24h darkness

until the end of the reproduction phase. Every other day of reproduction phase, mortality and reproduction of ostracod are recorded by counting e number of surviving ostracods and of eggs produced in each well. Then, each surviving ostracod was again transferred into a new 12-well microplate in the same procedure on the 14^{th} day. The pH, conductivity, and salinity in the overlying water are also measured before and after transferring the ostracod. The eggs produced in each well is counted manually under a stereoscopic microscope and collected for the hatching test.

(III) Hatching test

The hatching test was simultaneously conducted in parallel with reproduction phase after first egg production. The produced eggs were collected every other day and further observed their hatching after transferred to new 6-well microplates containing used SFW (ostracod eggs were transferred with used SFW from the reproduction well to hatching well microplate and kept them submerged). Then, the microplates were kept at 25^{o}C under 24h-darkness and checked hatching every other day for 14 days (Figure 4.2).

4.3.2. Endpoints

Table 4.3 shows the endpoints both of 6 d ostracod toxicity test and chronic ostracod toxicity test. The endpoint measurements of chronic ostracod toxicity test in 14-day sediment exposure phase are 14 d mortality and 14 d growth inhibition. Endpoints in the reproduction phase are lifespan, a total number of eggs produced, the first day of brooding, mean day of egg production, lifetime egg production, and egg-laying ratio. The hatching test endpoints are hatching ratio and F2 generation rate. Lifespan is defined as the average day when its death is confirmed in the test although the hatching procedure is started 52 prior to day 0 of the test. In addition to the underestimation, the defined life span includes overestimation due to the observation frequency (every other day) in reproduction phase. First day of brooding is defined as the day on which the eggs were observed first for the individual ostracod. This index also has the same under-/overestimation with life span evaluation.

Table 4.1 Timeline of ostracod chronic toxicity test

Procedure	Time	Activity	Number of ostracod per well	Sediment	Water	Food	Photoperiod	Temperature
Hatching (52 hours)	- 52 hour	Immerse the cysts in SFW to hatch the ostracod	1 tube of ostracod cysts* (A: per petri dish)	Without sediment	standard freshwater (SFW)	-	24L	25°C
	- 4 hour	Pre-feed with Spirulina powder				Spirulina		
Sediment exposure phase (14 days)	0	Prepare the 6-well microplate test (B) with sediment, SFW and food algae Measure pH, salinity and conductivity of the overlying water Transfer the ostracod neonate from petri dish (A) to the 6-well microplate test (B) to start chronic toxicity test	10 (B: 6- well microplate)	sediment to be tested		green algae 3.0×10^7 cells/well	24D	
	Day 7	Measure pH, salinity, and conductivity Feed with TetraMin				TetraMin 2mg/well		
	Day 14	Measure pH, salinity, and conductivity						
Reproduction phase		Record the number of surviving ostracods and measure body length Prepare the 12-well microplate test (C) with SFW and TetraMin (no sediment) Transfer the live ostracods from the 6-well microplate test (B) to new 12-well microplates (C) (one individual per well) to observe the egg production Measure pH, salinity, and conductivity before and after transferring the ostracod	1 (C:12-well microplate)	without sediment		TetraMin 1 mg/well		

	Every other day until all the ostracods die. (Day 16, 18, 20, ...)	Repeat the procedure of transferring the live ostracods and to change the water and food Record the number of surviving ostracods Count the number of eggs in each well and collect them for the hatching test						
Hatching test	Day of egg collection (Day 16, 18, 20, ...until reproduction phase ends)	Conducted the hatching test in parallel with reproduction phase (start on the first day of brooding)	as produced (1 to 347 eggs in this study) (D: 6-well microplate)		used SFW	without food		
		Prepare the 6-well microplate test (D) with used SFW (the overlying water where the eggs were produced) (no sediment and food) Transfer the collected eggs from the 12-well microplate (C) produced on the same day to a well of 6-well microplates to observe hatching						
	Every other day for 14 days (+2, 4, 6, ..., 14 days).	Record the number of hatched ostracod and remove them from the well.						

*Freshwater benthic ostracod *H. incongruens* was purchased as dormant eggs (cysts) from MicroBioTests Inc., Belgium (www.microbiotests.be) ** At least each of six replicates for the test and control sediment is recommended to perform in ISO method (Chial and Persoone, 2002b, ISO, 2012).In total: seven replicates (70 ostracod individuals) are prepared. One replicate are for measurement of body length of day 14 and remaining 6 replicates are used for reproduction test.

Table 4.2 Comparison of test conditions between 6-day ostracod toxicity test and chronic ostracod toxicity test

Test Type	6-day ostracod toxicity test[1]	Chronic ostracod toxicity test
Temperature	25±1 ^{o}C	25±1 ^{o}C
Photoperiod	24h-Darkness	24h-Darkness
Renewal of overlying water	NO	Every other day after day 14 for reproduction phase
Age of organism	0 to 2d at the start of test	0 to 2d at the start of test
Food	Algae (*S. acutus*) at 3.0×10^7 cells/well	Algae (*S. acutus*) at 3.0×10^7 cells/well during the first week, and feeding TetraMin suspension for the remainder of the test.
Test duration	6 day	Total 26-48 day: [2] (plus 14 days for hatching observation periods)

1. ISO14371 (ISO, 2012), 2. Total length of test is 50-62 day including the hatching test

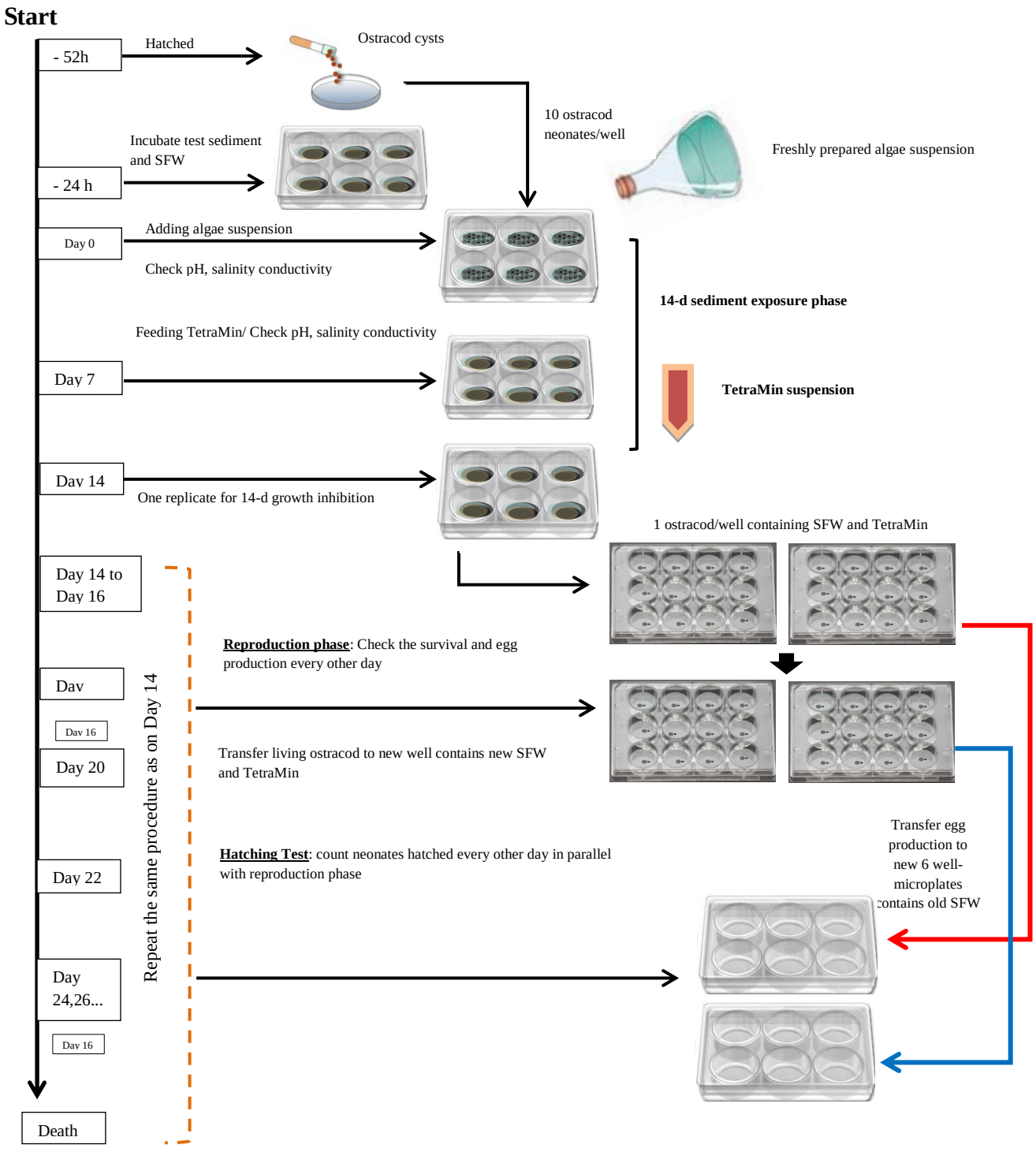


Figure 4.2 The hatching of ostracod cysts and procedure of 14-day sediment exposure phase, the procedure of reproduction phase and hatching test

Table 4.3 Endpoints of the ostracod toxicity test

Toxicity test	Endpoints	Definition/ References
6-day ostracod toxicity test	1) 6-d mortality 2) 6-d growth inhibition	ISO 14371 (ISO, 2012)
Chronic ostracod toxicity test	1)**14-day sediment exposure Phase**: 1.1 14-d mortality 1.2 14-d growth inhibition	Same definition with 6-day ostracod toxicity test but for longer exposure time (14d)
	2)**Reproduction phase**:	
	2.1 Life span	**Life span** (LS_i) is defined as the day of ostracod death observation (for individual *i*).
	2.2 Egg-laying ratio	**The egg-laying ratio** is expressed as $(n_e/n) \times 100$, where n_e is the number of ostracods which produces eggs during the test and *n* is the total number of ostracod tested.
	2.3 First day of brooding	**First day of brooding** is the day on which the eggs were observed first for each individual ostracod.
	2.4 Mean day of egg production	**Mean day of egg production** ($\Sigma_t tN_{i,t} / \Sigma_t N_{i,t}$) is the average day of egg production from an ostracod (*i*) which has several broods in a lifetime. **Total egg produced for each individual ostracod** ($\Sigma_t N_{i,t}$) is the number of egg produced from the individual *i*

	2.5 Lifetime egg production	**Lifetime egg production** is the average number of egg production from an ostracod (*i*) which calculate from the total number of eggs produced **(total number of eggs produced** is expressed as $\Sigma_i\Sigma_t N_{i,t}$, where $N_{i,t}$ is defined as egg numbers produced by the ostracod individual *i* on day *t*.)
	3) **Hatching test:** 3.1 Hatching ratio	**Hatching ratio** (%) is the proportion of the hatched eggs within the test period to all the eggs tested; defined as $(n_{F2}/\Sigma_i\Sigma_t N_{i,t})\times 100$, where n_{F2} is the number of hatched eggs (neonates).
	3.2 F2 generation rate	**F2 generation rate** is an index of the species continuation defined as n_{F2}/n; where *n* is the total number of ostracod tested.

4.4. Summary

In this chapter, the methodology of a new chronic sediment toxicity test using *H. incongruens* is given and the test procedure is proposed from the intensive literature surveys.

- The selection of the benthic ostracod *H. incongruens* in sediment toxicity test based on their features such as small size, asexual reproduction, and physical contact with sediment. The great advantage is that the test with *H. incongruens* was developed as a cyst-based test to reduce the burden of maintaining and culturing the live stocks. Their life history and reproduction characteristics of ostracod such as life span, first day of brooding, lifetime egg production, and hatching ratio e.g. can be used as reproductive endpoints for chronic sediment bioassays.

- The test is separated into three consecutive phases (1) a 14-day sediment exposure phase (with sediment) (2) a reproduction phase (without sediment), (3) a hatching test (the ostracod eggs produced and laid in the reproduction phase were transferred and observed in the hatching test for 14 d). This design was proposed which allows a quantitative measurement of ostracod reproduction.

- Incubation of the multi-well plates in 24h-darkness at 25°C and feeding the green algae (*S.acutus*) at 3.0×10^7 cells/well in the first week and TetraMin suspensions in the following weeks of test experiments were decided as the test parameters in this study.

- Determination of the mortality and growth inhibition compared with the controls as 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 endpoint in the reproduction phase. Hatching rate and F2 generation rate of ostracod are the endpoint of the hatching test. The test repeatability as the measurement of the variability (coefficient variation) in the test endpoints and the acceptability criteria for control sediment were determined in the following chapter.

CHAPTER 5

EVALUATION OF TEST REPEATABILITY FOR A CHRONIC SEDIMENT TOXICITY TEST USING THE BENTHIC OSTRACOD *HETEROCYPRIS INCONGRUENS*

5.1. Introduction

To demonstrate the acceptable laboratory performance, intra-laboratory precision (repeatability) expressed as a coefficient of variation (CV) need to be determined (US EPA, 2000). Before conducting the sediment toxicity test with potentially contaminated sediment, the test with control sediment(s) should be performed. It helps to identify the acceptable responses of test organisms and good performance of the test method. In the previous chapter (chapter 4), the chronic sediment toxicity test method was proposed. To establish a new method, the test repeatability and test acceptability criteria for control sediments as test validation are needed to perform. Many factors can have considerable effects on the test result and strongly influence by intra-laboratory variability (e.g., changes in test conditions, test duration, organism health and condition and choice of reference toxicant and feeding) (Moore et al., 2000). The repeatability of the test can measure by the intra-laboratory precision. The intra-laboratory precision was expressed to quantify the variability of an endpoint for a specific toxicity test in order to interpret toxicity test results correctly and be able to apply it on sediments risk assessment (Bebon R., 2013). Additionally, the data of test repeatability can be used as test acceptability criteria.

This chapter aimed to evaluate the repeatability of the test method based on the coefficient of variation in each endpoint of proposed test. The life history and reproduction characteristics of ostracod *H. incongruen*s in reference sediment (supplied from the ostracodtoxkit) were investigated. The seven batches of the control tests (total 500 ostracods) were conducted and the results were used to determine the test acceptability criteria for control sediment which will be necessary for future users of chronic ostracod toxicity test.

5.2. Materials and methods

5.2.1. Test medium

The composition of the test medium showed in chapter 3. Standard freshwater (SFW) was commonly used for ostracod culturing. Green algae *Scenedesmus acutus* N-94 used as food for ostracod in the first week of sediment exposure phase was cultured in C medium. TetraMin suspension was fed to ostracod in the second week of sediment exposure phase and reproduction phase. The details of hatching ostracod cysts prior to the test were also described in chapter 3 (section 3.3).

5.2.2. Test organisms

Freshwater benthic ostracod *H. incongruens* was purchased as dormant eggs (cysts) from MicroBioTests Inc., Belgium. The cysts were hatched before starting the toxicity test in standard freshwater (SFW). The detail procedures are already written in chapter 3.

5.2.3. Test sediment and test preparation

The clean reference sediment (RF) was obtained as a part of the Ostracodtoxkit F (MicroBioTests Inc., Belgium) and used as the control sediment in this study. The clean reference sediment (RF) was mixed with standard fresh water (SFW) at the ratio of 1:2 V/V (RF: SFW) in each well of the six-well microplates (IWAKI) and held in the incubator for 24 h before inoculating the test organisms (ostracod). The 24 h holding time was considered as equilibration time with SFW by storing at 25^{o}C in the 24 h dark conditions prior to the test.

5.2.4. Repeatability of chronic ostracod toxicity test

Seven different batches of the control tests were performed in order to determine test repeatability (intra-laboratory variability) and were expressed as the CV. The chronic ostracod toxicity test method was performed as described above under control conditions. The seven batches of the control tests (total 500 ostracods) were conducted

using clean RF sediment as a part of the Ostracodtoxkit F (MicroBioTests Inc., Belgium). The sample was prepared by mixing the RF with SFW in the ratio of 1:2 v/v (RF: SFW) in each well of the 6-well microplates and held in an incubator for 24 h before inoculating the test organisms. The 24 h holding time was considered as the equilibration time with SFW by storing at 25°C in 24 h darkness prior to the test. The test was started by inoculating 10 freshly hatched ostracod neonates into incubated well plates and the toxicity test was terminated after all of the ostracods used died following the procedure of the chronic ostracod toxicity test.

5.2.5. Statistical analysis

All data and results were reported with standard deviations. The Grubb's test was applied to test outliers of the control data using Minitab 17 (State College, PA: Minitab Inc.). The CV was calculated as CV = standard deviation/mean × 100% for each endpoint to determine test repeatability.

5.3. Results and discussion

5.3.1. Repeatability of the chronic ostracod toxicity test

Seven batches of the control tests were used to determine the CVs for test repeatability. Table 5.1 shows the overlying water quality, 14 d mortality and 14 d body lengths of ostracods in the RF sediment for each batch of the control tests. At the end of the 14 d sediment exposure phase, the reproduction phase was continually performed; the life history and reproduction characteristics of ostracods in RF sediments are shown in Table 5.2. Grubbs' test was then applied to identify the outliers for each endpoint of the control tables. The results showed good repeatability; only the first day of brooding in control_3 was detected as an outlier and was removed from the control dataset. The mean values after identifying outliers are shown in Table 5.3.

Table 5.3 shows the summary of 14 d mortality, 14 d body length, life history, and reproduction characteristics of *H. incongruens* in RF sediments. The CVs for the endpoints were determined to demonstrate and quantify the repeatability of the test. Most of the CV results for each endpoint showed good test repeatability, with CVs below 15%, whereas variability was higher for lifetime egg production, hatching ratio, and F2 generation rate endpoints with CVs ranging from 29.5% to 51.9% (Table 5.3). For the mortality endpoint, the average for 14 d mortality was 6.1%, with a CV of 42.6%. In this case, this should be interpreted with caution as the CV value may be high simply because of low mean; thus CV can be misleading while ascertaining the variability of such a dataset.

Table 5.1. The overlying water quality, 14 d mortality and 14 d body lengths of ostracods in the RF sediment for each batch of the control tests.

Sample	Control_1 (nw=6)	Control_2 (nw=8)	Control_3 (nw=8)	Control_4 (nw=10)	Control_5 (nw=6)	Control_6 (nw=6)	Control_7 (nw=6)
Date	**May, 2014**	**Sep, 2014**	**Dec, 2014**	**May, 2015**	**July, 2015**	**Dec, 2015**	**Mar, 2016**
pH	**8.1±0.0**	**8.3±0.1**	**8.2±0.00**	**8.2±0.3**	**7.6±0.30**	**8.3±0.20**	**8.2±0.10**
Conductivity (mS/cm)	**1.04±0.05**	**0.78±0.06**	**1.16±0.11**	**1±0.04**	**1.16±0.06**	**1.12±0.20**	**1.30±0.13**
Salinity (%)	**0.05±0.00**	**0.03±0.01**	**0.05±0.00**	**0.04±0.01**	**0.05±0.01**	**0.06±0.01**	**0.07±0.01**
14 d mortality (%)	**6.7±5.2**	**8.8±9.9**	**1.3±3.5**	**4±0.5**	**6.7±8.2**	**6.7±8.2**	**8.3±4.1**
14 d body length (µm)	**1032±111**	**980±32**	**1017±27**	**996±103**	**1171±42.3**	**1230±93.7**	**1247±36.3**

nw= number of wells

Table 5.2 The life history and reproduction characteristics of ostracods in RF sediments.

Parameters	Control_1		Control_2		Control_3		Control_4		Control_5		Control_6		Control_7	
	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals
n **(ostracod tested)**	60	38	80	55	80	48	100	69	60	41	60	38	60	45
Egg-laying ratio (%)	63.3±12.1		68.8±23.0		60.0±11.2		69.0±21.3		68.3±13.3		63.3±15.1		75.0±18.7	
Life span (day) mean±SD [range]	27.4±1.8 [25-30] (nw=6)	30.2±1.8 [28-32] (nw=6)	25.9±2.8 [20-30] (nw=8)	30.2±3.2 [25-35] (nw=8)	27.4±2.0 [24-30] (nw=8	32.3±3.1 [26-36] (nw=8)	24.1±2.0 [19-27] (nw=10)	25.0±1.2 [23-27] (nw=10)	21.2±1.0 [20-23] (nw=6)	22.3±0.9 [21-24] (nw=6)	22.8±1.8 [21-26] (nw=6)	24.2±2.4 [21-27] (nw=6)	23.8±2.7 [21-28] (nw=6)	25.8±2.1 [22-28] (nw=6)
First day of brooding (day) mean±SD [range]	-	21.0±1.4 [19-23] (nw=6)	-	22.1±0.9 [21-23] (nw=8)	-	25.5±1.1 [24-27] (nw=8)	-	21.2±0.6 [20-22] (nw=10)	-	20.0±0.9 [19-22] (nw=6)	-	21.4±0.9 [20-23] (nw=6)	-	19.5±0.5 [19-20] (nw=6)
Mean day of egg production mean±SD [range]	-	23.7±1.4 [22-26] (nw=6)	-	24.6±1.8 [23-28] (nw=8)	-	27.8±1.4 [26-30] (nw=8)	-	22.3±0.5 [21-23] (nw=10)	-	20.4±0.8 [20-22] (nw=6)	-	22.8±1.9 [21-25] (nw=6)	-	21.7±1.1 [20-23] (nw=6)
Total no. of eggs produced (egg)	1221		1714		1340		1295		668		645		1796	
Lifetime egg production (egg/ind.) mean±SD [range]	20.4±8.2 [12-33] (nw=6)	32.1±21.9 [2-78] (n=38)	21.4±10.8 [10-43] (nw=8)	31.2±29.9 [1-109] (n=55)	16.8±7.5 [8-28] (nw=8)	27.9±19.8 [1-82] (n=48)	13.0±7.9 [3-27] (nw=10)	18.8±15.6 [1-68] (n=69)	11.1±3.6 [7-16] (nw=6)	16.3±10.7 [1-39] (n=41)	10.8±8.4 [1-23] (nw=6)	17.0±24.7 [1-109] (n=39)	29.9±15.8 [13-53] (nw=6)	32.5±30.0 [1-109] (n=45)
Hatching ratio (%)	Not determined		12.8		7.4		18.8		28.0		14.1		17.0	
F2 generation rate	Not determined		2.74		1.24		0.62		3.12		1.52		5.08	

nw = number of wells

5.3.2. Test acceptability criteria

The test acceptability criteria for the chronic ostracod toxicity test are proposed in Table 5.3. These values are presented as the mean and upper and lower control limits (±2 standard deviations) of each endpoint for *H. incongruens* reproduction except for mortality and body length. Control limits of ±2 standard deviation were proposed to demonstrate acceptable test performance and establish the control toxicity test charts in the United States Environmental Protection Agency (US EPA) method (US EPA, 2000). However, due to the fact that a high CV of ≥30% was observed at the endpoints for lifetime egg production, hatching ratio, and F2 generation rate, acceptability criteria were not defined for these parameters.

In terms of setting acceptability criteria for mortality, mortality and growth as a second "sublethal" effect criteria was published in 2002 (Chial B and Persoone G., 2002b). These criteria effects were based on the 6 d ostracod toxicity test. In this study, the responses of ostracods in control sediments after 14 d of exposure were used to set the validity criteria for ostracod mortality and body length. The mean 14 d mortality in the control tests was 6.1% (95% confidence interval 3.7–8.5%); the threshold for mortality criterion could be set at 10%. However, the ISO 14371 method (ISO, 2012) proposed a 20% mortality as a valid threshold for 6 d ostracod mortality and most of the sediment tests were conducted with freshwater invertebrates in the US EPA method (US EPA, 2000) and the minimum mean control survival was set at 80%. Thus, 20% mortality was decided as the acceptability threshold for 14 d ostracod mortality in this new proposed chronic ostracod toxicity test.

For setting ostracod body length, the mean final ostracod length after 14 d of exposure of all seven control test batches was 1096 µm with a lower confidence interval of 989 µm (Table 5.3). According to the Chial and Persoone study (Chial B and Persoone G., 2002b); a lower 95% confidence interval of body length was used to set minimum ostracod growth in control sediments. Following that previously mentioned, we decided to impose a 14 d ostracod body length of at least 900 µm as the acceptability criterion in control sediments. In addition, the further development of ostracod body length criteria is possible, and a growth factor criterion

of 1.5 has been proposed in the ISO 14371 method instructions (ISO, 2012) based on conclusions from inter-laboratory comparisons. The toxicity test was considered valid when the mean ostracod body length in control sediments was at least 1.5 times greater than the mean body length at the start of the test. This criterion was laid instead of a strict mathematical increase of 400 µm body length as proposed by Chial and Persoone (2002b). The proposed test acceptability criteria for control sediment will be used to determine the validity of the control test and to help interpret the toxicity results correctly in the following chapters.

Table 5.3. The summary of 14 d mortality, 14 d body length, life history, and reproduction characteristics of *H. incongruens* in RF sediments and the test acceptability criteria for the chronic ostracod toxicity test.

Parameters	Control_ALL(N=7)		Proposed test acceptability criteria for control sediment	
	All individuals	Egg-laying individuals		
14 d mortality (%)	**6.1±2.6 (CV = 42.6%)**		**≤20% mortality**	
14 d body length (µm)	**1096±116 (CV = 10.6%)**		**≥ 900 µm**	
Egg-laying ratio (%)	**66.8±5.0 (CV = 7.5%)**		**56.8 – 76.8**	
Life span (day) mean±SD [range]	**24.7±2.3 [21.2-27.4] (CV = 9.3%)**	**27.1±3.7 [22.3-32.3] (CV = 13.7%)**	**20.1 - 29.3**	**19.7 - 34.5**
First day of brooding (day) mean±SD [range]	**-**	**20.9±1.0 [19.5-22.1]** (N=6*) **(CV = 4.8%)**	**-**	**18.9 – 22.9**
Mean day of egg production mean±SD [range]	**-**	**23.3±2.4 [20.4-27.8] (CV = 10.3%)**	**-**	**18.5 – 28.1**
Lifetime egg production (egg/ind.) mean±SD [range]	**17.6±6.9 [10.8-29.9] (CV = 39.2%)**	**25.1±7.4 [16.3-32.5] (CV = 29.5%)**	**n.d. (not determined since the high coefficient of variations)**	
Hatching ratio (%)	**16.4±6.9 (CV = 42.1%)** (N=6**)			
F2 generation rate	**2.7±1.4 (CV = 51.9%)** (N=6**)			

Calculation based on 7 batched of experiments in control conditions, #Control_3 was identified as outlier using Grubbs' test and excluded from calculation, CV= coefficient of variation, **Hatching rate, and F_2 generation rate was calculated from all batch of the experiments except for Control_1, 95% confident interval (C.I.) for 14 d body length of ostracod is 989-1203 µm.

5.3.3. Effect of food on *H. incongruens* reproduction

Havel and Talbott (1995) showed that the average life span of ostracods was 25 d (14–37 d) and that reproduction started from day 19 (day 13–25). These values were not considerably different from this study of a 24.7 d mean life span and the average first day of brooding as day 20.9. The lifetime egg production of ostracods in the Havel and Talbott study (1995) was 36.5 eggs/individual (0–64 eggs), which was considerably different from this study of 17.6 eggs/ individual. The possible explanations could be different conditions of these two studies that included the amount, type, and frequency of feeding. Algae *Selenastrum* sp. at a final cell density of 8 x 10^6 cells/ml were fed to each individual three times a week that was about 2.7 times higher than that of this study. In terms of reproduction characteristics, ostracods and daphnia reproduce by parthenogenesis (Rossi et al., 1996, 2013). Pavlaki et al., (2014) reported that the reproduction of *Daphnia magna* increased as the food level increasing after a 21 d exposure test to different food levels. *D. magna* was fed every day with *Raphidocelis subcapitata* at a rate of 0, 1, 2, 3, 4, 5, and 6 x 10^6 cells/ml of medium and the number of offspring gradually increased with increasing food amounts. Future research is needed to quantify ostracod reproduction and the effects of different foods (e.g., different algal species and nonliving food) to optimize the feeding conditions of the proposed test method. These would be useful to identify the variability and possibility that food contained in the control sediment might affect their reproduction rate.

5.3.4. Endpoint variability in the chronic ostracod toxicity test

In this study, a high CV (≥30%) was found in lifetime egg production, hatching ratio, and F2 generation rate compare with other endpoints. Havel and Talbott (1995) showed that lifetime fecundity and egg development time of ostracods were variable (CV of 73% and 116%, respectively) under control conditions in contrast to a low variation in body length (CV of 21%). Based on the variability of the endpoints, the CV in control sediment has also been documented in *Chironomus tentans* life-cycle test, survival (<20%), growth weight (<15%), emergence (<30%), reproduction as mean eggs produced/female (<20%), percent hatch

(<10%) (Sibley et al., 1996; Benoit et al., 1997). Ingersoll et al., (1998) showed the reproduction of *Hyalella azteca* more variable than growth. The CVs were typically <10% for growth and >20% for reproduction. These studies indicated that more replicates might be needed for reproductive endpoint in order to determine the statistical differences among treatments (Ingersoll et al., 1998; US EPA, 2000). In general, the variations were dependent on either environmental factors in the test conditions such as overlying water quality or incubation temperature or biological factors such as the age of the test organisms, food quality and quantity, and the experience of those conducting the tests. Given that a high CV may affect the statistical power of the comparisons, increasing the number of test replicates should be considered to minimize test variability; the CV for mortality decreased from around 38% to 8% when the test replicates increased from 3 to 4 to 8 replicates in a 6 d exposure test of *H. incongruens* with a toxic sediment (Chial B and Persoone G., 2002b).

Likewise, the hatching ratio as well as the F2 generation rate was highly variable among the control tests. The hatching characteristics of benthic ostracods may account for the large variation in hatching time. Ostracod species are known to lay both rapidly- and delayed-hatched eggs (Havel and Talbott, 1995; Angell and Hancock, 1989; Rossi et al., 1996; Retrum et al., 2011); hence, the eggs that remain after an observation period may be still viable and may be expected to hatch after some time. Angell and Hancock (1989) showed that *H. incongruens* eggs could be hatched under drying and freezing at different temperatures. Havel and Talbott (1995) reported a highly variable hatching time that ranged from 1 to 157 d for 90 eggs. Further studies are necessary to investigate the hatching characteristics of *H. incongruens* in order to reduce test variations.

5.4. Summary

A new chronic sediment toxicity test using benthic ostracod *H. incongruens* was previously proposed. This chapter showed the repeatability of the test by determining the variability of test endpoints (measured as coefficient of variations) and test acceptability criteria for control sediment. The seven batches (500 in a total of individual ostracods used) of the control tests using reference sediment were conducted. The results are summarized as follows.

- Almost all endpoints in the proposed chronic method showed good test repeatability with CVs below 15%, whereas variability was higher in lifetime egg production, hatching ratio, and F2 generation rate endpoints with CVs ranging from 29.5% to 51.9%.

- 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*, and

- 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.

- As a results of a high CV of ≥30% was observed in lifetime egg production both of all individual and egg-laying individual, hatching ratio, and F2 generation rate. Thus, the acceptability criteria of these endpoints were not defined. The results showed the natural variability of the endpoint in the control sediment. The next chapters will conduct the test with reference toxicants and environmental sediment sample to observe the sensitivity and suitability of proposed chronic test. The proposed test acceptability criteria for control sediment will be used to determine the validity of the control test and to help interpret the toxicity results correctly in the following chapters.

CHAPTER 6

TOXICITY ASSESSMENTS OF NICKEL ON REPRODUCTION OF THE BENTHIC OSTRACOD *HETEROCYPRIS INCONGRUENS*

6.1. Introduction

Heavy metals are abundant, persistent and potentially toxic contaminants in aquatic ecosystems (Tang et al., 2014). Heavy metal contamination in the environment can be naturally produced, but mostly by anthropogenic activities such as agriculture, mining, combustion of fuels and vehicles and industrial or manufacturing (Luoma et al., 2008). Throughout releasing into the aquatic environment, heavy metals have persisted in water and accumulated in sediment. Therefore, they cause an adverse effect on aquatic organisms particularly on benthic organisms via direct absorption through water or sediment ingestion (Iwasaki et al., 2009; Stankovic et al., 2014; Magalhães et al., 2015).

Nickel cause disorders of the respiratory system, Mg^{2+} antagonism and kidney lesions in fish and other aquatic animals (Shuhaimi-Othman et al., 2012). Pane et al., (2004) studied the toxic effects of chronic waterborne nickel exposure which caused significantly decreases in survival, growth and reproduction of *Daphnia magna*. Additionally, nickel is defined as one of the priority pollutants in EPA's Aquatic Life Criteria Table. As of that sensitivity and chronic toxicity of nickel has been investigated for a number of invertebrates' species such as larvae of *Chironomus riparius* (Powlesland and George, 1986), cladoceran *Ceriodaphnia Dubia* (Kszos et al., 1992; Keithly et al., 2004) and amphipod *Hyalella azteca* (Borgmann et al., 2001; Keithly et al., 2004). Since the availability of ostracod toxicity test using *H. incongruens* which were developed and standardized (Chial and Persoone, 2002a, b, c; ISO, 2012), it has been extensive used to test on various metals toxicity studies (Kudlak et al., 2011; Sevilla et al., 2013, 2014). Kudlak et al., (2011) showed toxicity for a variety of metals (Cd, Hg, Cu, Cr, Ni, Mn, Zn, Pb, Li, Fe) spiked in reference sediment by determining median effective concentration (EC50) and the median lethal concentration (LC50) using *H. incongruens*. Sevilla et al., (2013) reported the sensitivity of *H. incongruens* to copper (Cu) and zinc (Zn) under different foods and photoperiods. Sevilla et al., (2014) determined the effects of metals on

H. incongruens through aquatic and dietary exposure using cadmium (Cd), copper (Cu) and zinc (Zn). Relative to other metals such as copper (Cu) and zinc (Zn), sensitivity and toxicity of nickel to ostracod has been showed less attention. To date, there is only a study of nickel toxicity to ostracod mortality by Kudlak et al., (2011). Additionally, the toxicity study of nickel was mainly focused based on 6-d mortality and growth inhibition of ostracod. As far as we known, the toxic effects of nickel on the reproduction of ostracod *H. incongruens* have never been reported.

In this chapter, the aim was to assess the toxicity of nickel on the reproduction of benthic ostracod *H. incongruens*. Previous chapters showed the detail methodology and the results of test repeatability for the proposed chronic test. The test was conducted to determine the chronic toxicity of nickel where ostracod was exposed with serial dilutions of nickel by diluting with standard freshwater. The acute to chronic ratio (ACR) of nickel was calculated to compare the sensitivity between the other test species.

(Toxicity assessments using zinc, chromium III, and VI on the ostracod reproduction were also performed. The results are shown in Appendix A. The chronic toxicity of chromium III, and VI were not detected in the tested range concentrations. Zinc showed high 14 d mortality, thus toxicity of zinc on ostracod reproduction was not obtained by this method.)

6.2. Materials and methods

6.2.1. Test chemicals

The preparation of nickel solution was described in chapter 3. The stock solutions of nickel were measured before diluting with standard freshwater (SFW) and test series were prepared in 10 times interval. The exposure concentrations were estimated as half as measured concertation from the stock solutions because of the subsequent dilution by algal food suspension. Exposure concentrations of C0 = SFW, C1= 0.012, C2 = 0.12, C3 = 1.2, C4 = 12, C5 = 120 and C6 = 1200 µg/l of Ni were used for the ostracod toxicity test.

6.2.2. Test organisms

Freshwater benthic ostracod *H. incongruens* was purchased as dormant eggs (cysts) from MicroBioTests Inc., Belgium. The cysts were hatched before starting the toxicity test in standard freshwater (SFW). The detail procedures are already written in chapter 3.

6.2.3. Chronic ostracod toxicity test

A chronic ostracod toxicity test was only performed in this study. The procedures and timeline of each phase in the chronic toxicity test were shown in chapter 4. Ostracod neonates were exposed to different concentrations of the nickel which were spiked in reference sediment prior starting the toxicity test for 24h darkness in 25^{o}C incubator. The experiments were carried out in six replicates in each concentration of nickel and one replicate for 14 d body length measurement of ostracod.

6.2.4. Statistical analysis

The experimental data were analyzed following the description of statistical analysis in chapter 3. The 50% and 20% lethal concentrations (LC50 and LC20) were calculated using Probit analysis while the non- observed effect concentrations (NOE(L)C) and lowest observed effect concentrations (LOEC) were determined using Dunnett's test or Chi-square test. The acute to chronic ratios (ACRs) were calculated as the ratio of the LC50/EC50 to the chronic MATC, NOEC or EC20.

6.3. Results and discussions

Table 6.1 shows the results of 14 d mortality, 14 d body length, the life history and reproduction characteristics of ostracod *H. incongruens* under control condition. All the data set in control tests were evaluated based on the proposed test acceptability criteria for control sediments in chapter 5 (acceptability criteria of lifetime egg production, hatching ratio and F2 generation rate were not defined). The results met all the acceptability criteria for the chronic ostracod toxicity test to be acceptable.

Table 6.1 Test validity: 14 d mortality, 14 d body length and life history reproduction characteristics of ostracod *H. incongruens* under control condition

Parameters	**Control**	
	All individuals	**Egg-laying individuals**
14 d mortality (%)	6.7±8.2	
14 d body length (µm)	1171±42.3	
Egg-laying ratio (%)	68.3±13.3	
Life span (day) mean±SD [range]	21.2±1.0 [20-23]	22.3±0.9 [21-24]
First day of brooding (day) mean±SD [range]	-	20.0±0.9 [19-22]
Mean day of egg production mean±SD [range]	-	20.4±0.8 [20-22]
Lifetime egg production (egg/ind.) mean±SD [range]	11.1±3.6 [7-16]	16.3±10.7 [1-39]
Hatching ratio (%)	28	
F2 generation rate	3.12	

6.3.1. Toxicity of nickel on reproduction of a benthic ostracod *H. incongruens*

Figures 6.1a shows the dose response relationship between 14 d mortality and different concentrations of nickel. Under the highest nickel exposure concentrations (C6 = 1200 µgNi/l), the mortality of ostracod after 14-day exposure was 41.7% (detailed data are shown in Appendix D). Thus, the estimated 14-d LC50 was $2.95x10^3$ µgNi/l with an NOLC of 12 µgNi/l for 14 d mortality. The 14-d LC20 was calculated as 5.03 µgNi/l. There was a statistically significant difference ($p<0.05$) in C5=120 µgNi/l and C6=1200 µgNi/l for 14 d mortality. The only study of nickel toxicity to ostracod *H. incongruens* was reported by Kudlak et al. (2011) showing 6d-LC50 of 2.41 mgNi/l. The differences in exposure time showed slight difference in LC50. Another possibility is the difference in cationic concentrations of the test water, because distilled water was used instead of standard freshwater (SFW) in Kudlak et al. (2011) study.

Figure 6.1b shows the dose response relationship between14 d growth inhibition of ostracod with nickel concentrations. Ostracod growth decreased with increased nickel concentrations. Statistically significant difference was found between the control case (C0) and all diluted nickel concentrations (C1-C6). The growth of ostracod was more sensitive than mortality with LOEC ≤ 0.012 µgNi/l for 14-d growth inhibition. In contrast to amphipod *H. azteca*, Keithly et al., (2004) showed survival was more sensitive than growth in the 14-day nickel test with *H. azteca*. The basis for the differences between the two studies may be due to variability of the test species or slight differences in the test methodology (e.g., organism age, or feeding conditions). The ages of amphipod used were 7- to 8-d old at the start of the test (Keithly et al., 2004). In addition, they used a different feeding, a standard mixture of yeast, chlorophyll, and trout chow (YCT) were fed in the amphipod 14-d test. In the present study, the ostracod neonates ≤52 h old and the fresh algal suspensions *S. acutus* were used.

It has to be kept in mind that the results are presented in exposure concentrations with test series was prepared in 10 times interval. This would have an effect on the accuracy for log scale-wide range of nickel concentrations and the actual concentrations of non-observed effect (lethal) concentration (NOECs or NOLCs). The results of the ostracod reproduction exposure to different concentrations of nickel are shown in Figure 6.2a, b, c, d. and Table

6.2. Figure 6.2a shows the relationship of mean life span by all individual and the egg-laying ratio of ostracod with nickel concentrations. The mean life span of all individual was considerably decreased in a high concentration of nickel (C5 and C6). There was a statistically significant difference in these two nickel exposure cases ($p<0.05$). Egg-laying ratios were varied by nickel concentrations and two highest concentrations of nickel (C5 and C6) caused a statistically significant decreased egg-laying ratio.

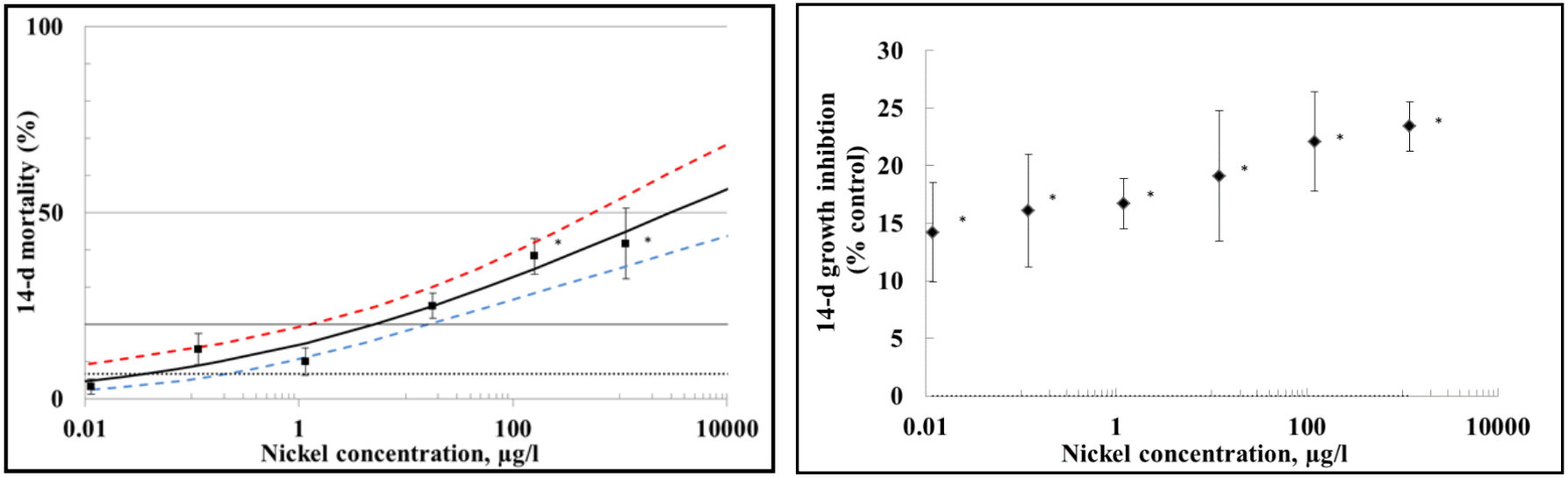
The mean life span of egg-laying individuals, first day of brooding and mean day of egg production under nickel exposure cases are shown in Figure 6.2b. The mean life span of egg-laying individuals slightly longer in nickel exposure cases than in control condition. There was a statistically significant difference in C4 and C6. First day of brooding and mean day of egg productions were slightly changed with nickel concentrations, but the only significant difference was observed in C4 in mean day of egg production. The total number of eggs produced in the control case and under different nickel concentrations as shown in Table 6.2. Mean lifetime egg production by all individuals and by egg-laying individuals are shown in Figure 6.2c. Mean lifetime egg production both of all individuals and egg-laying individuals varied with nickel concentrations, but no significant difference was observed. The results showed the mean lifespan of egg-laying individuals increased with increased nickel concentrations. Thus, lifetime egg production of ostracod egg-laying individuals increased with increase of nickel concentrations though at the highest concentration of nickel (C6) was slightly decreased. Based on the results, mean lifetime egg production was not a reliable endpoint in the current chronic ostracod toxicity test.

Although the two highest concentration of nickel (C5 and C6) was not kill more than 50% of ostracod in 14d-sediment exposure phase; under these concentrations of nickel, it has an impact on ostracod reproduction. Based on this study, the average first day of brooding in the control and by nickel exposure cases is about 20 day whereas the mean life span of all individual less than 20 days in two high concentration of nickel (C5 and C6) (Table 6.2). The results showed that some ostracods died before producing their first brood. As a result, the egg-laying ratio was statistically significantly low in C5 and C6, which implied the toxic effect of nickel on the ostracod reproduction at these two concentrations.

Figure 6.2d and Table 6.2 shows the results of hatching test with nickel concentrations. Under different nickel concentrations, the lowest hatching ratio was in the highest concentrations of nickel (C6) in contrast to higher hatching ratio than control in C1. Statistically significant difference in hatching ratio ($p<0.05$, Chi-square test) was obtained in C1, C3-C6. F2 generation rate became lowest in the highest concentration of nickel (C6). F2 generation rate is an index of the species continuation, and the value ≤ 1 in C4-C6 indicates the possibility of extinction of the ostracod population.

The 14d-LC20 shows the concentrations of the nickel that kill 20% of ostracod during a 14-day exposure to sediment phase, but it was not evaluated the effect on reproduction or the next generations of ostracod. The hatching ratio and F2 generation rate were highly variable which were also demonstrated in chapter 5. Considering the aspects of the accuracy and repeatability of test endpoints, egg-laying ratio and lifespan for all individual are selected as the reliable and sensitive chronic endpoint. The results of the proposed chronic test demonstrated non observed effect concentration (NOEC) of 12 μg Ni/l in egg-laying ratio and lifespan for all individual.

The results of chronic nickel effect as waterborne toxicity showed statistically significant difference in term of ostracod life span and egg-laying ratio. However, ostracod is epi-benthic species which ostracod contact to toxicants in various pathways such as ingests the contaminated sediment or food-contaminated toxicants. The different of toxicity pathway may affect in different sensitivity to ostracod which showing in the different sensitive endpoint. Previous study of Pane et al. (2004) showed the 21 d chronic Ni waterborne exposure caused significant decrease in survival, reproduction and growth of *Daphnia magna*. In contrast, there is no decisive evidence that dietary pathway of nickel exposure has affected on growth or total reproductive output of *Daphnia magna* (Evens et al., 2009). Further research such as Ni-spiking in sediment, dietary Ni exposure as food-contaminated toxicants is required to test if this could also be the case for ostracod.



a. **b.**

Figure 6.1 Dose-response relationships between (a) 14 d mortality of ostracod and (b) 14 d growth inhibition with nickel concentrations. The broken lines show 95% upper and lower confidence limits (95%UCL and LCL). Error bar represents standard error.

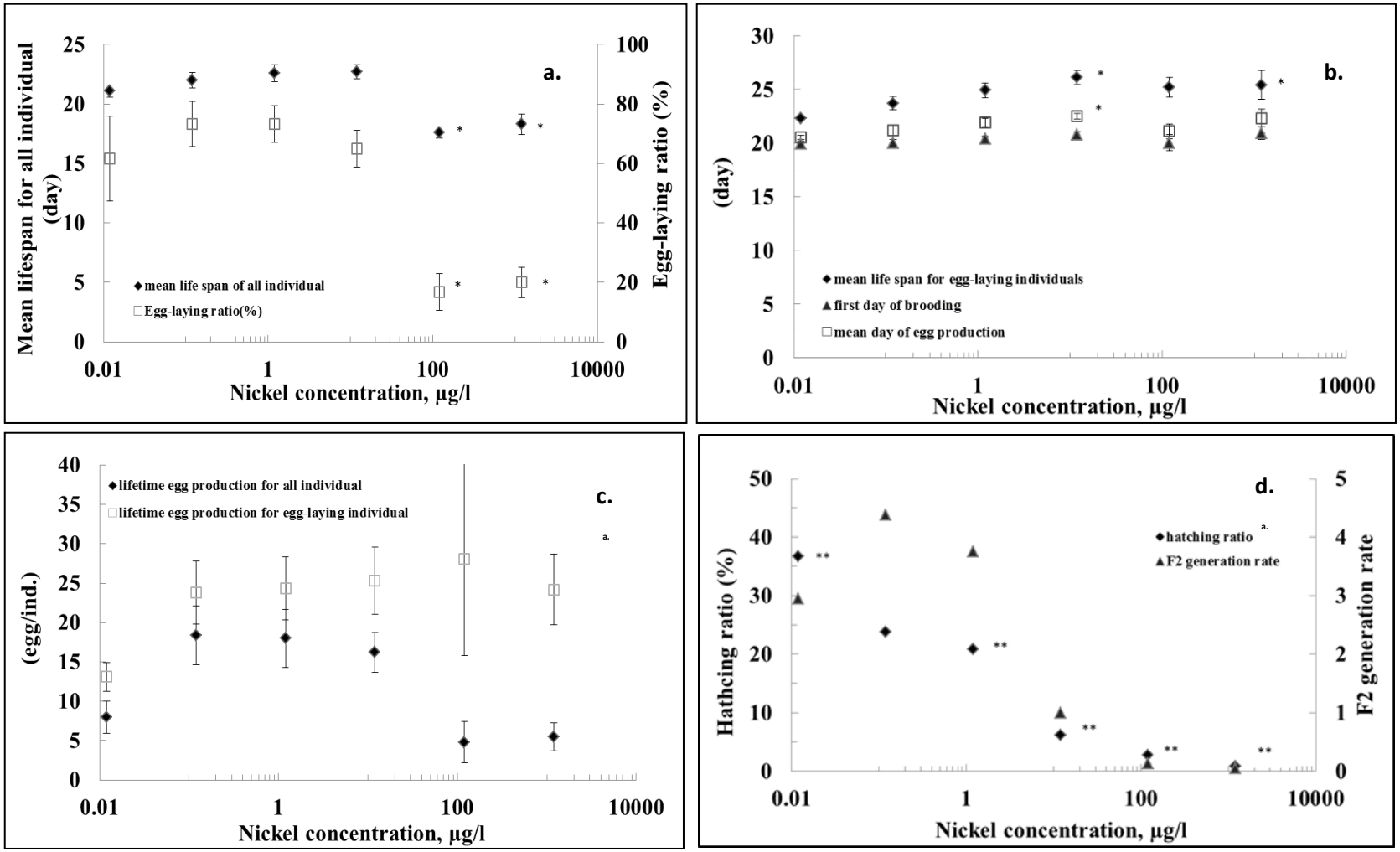


Figure 6.2 Relationships between reproduction of ostracod and nickel concentrations (a) mean lifespan for all individual and egg-laying ratio, (b) mean lifespan for egg-laying individual, first day of brooding and mean day of egg production, (c) lifetime egg production for all individual and egg-laying individual, (d) hatching ratio and F2 generation rate. Error bar represents standard error.

Table 6.2 Life history characteristics of ostracod under control and after exposure to different concentrations of nickel

Parameters	Control (C0)		Exposure concentrations (µgNi/l)											
			C1= 0.012		C2 = 0.12		C3 = 1.2		C4 = 12		C5 = 120		C6 = 1200	
	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals
n **(ostracod tested)**	60	41	60	37	60	44	60	44	60	39	60	10	60	12
Egg-laying ratio (%)	68.3±13.3		61.7±34.9		73.3±18.6		73.3±15.1		65±15.2		**16.7±15.1***		**20.0±12.6***	
Life span (day) mean±SD [range]	21.2±1.0 [20-23] (nw=6)	22.3±0.9 [21-24] (nw=6)	21.1±1.2 [19-22] (nw=6)	22.3±0.4 [22-23] (nw=5)	22.0±1.6 [20-24] (nw=6)	23.7±1.6 [22-26] (nw=6)	22.6±1.7 [20-25] (nw=6)	24.9±1.6 [22-26] (nw=6)	22.7±1.4 [21-25] (nw=6)	**26.1±1.6* [24-29] (nw=6)**	**17.6±1.2* [16-19] (nw=6)**	25.2±2.3 [22-28] (nw=5)	**18.3±2.2* [16-21] (nw=6)**	**25.4±3.3* [20-29] (nw=5)**
First day of brooding (day) mean±SD [range]	-	20.0±0.9 [19-22] (nw=6)	-	19.9±0.7 [19-21] (nw=5)	-	20.0±0.7 [19-21] (nw=6)	-	20.4±0.7 [20-22] (nw=6)	-	20.8±0.6 [20-22] (nw=6)	-	20.0±1.9 [18-22] (nw=5)	-	20.9±1.5 [19-23] (nw=5)
Mean day of egg production mean±SD [range]	-	20.4±0.8 [20-22] (nw=6)	-	20.5±0.5 [20-21] (nw=5)	-	21.2±1.2 [20-23] (nw=6)	-	21.9±0.9 [20-23] (nw=6)	-	**22.5±0.7* [22-24] (nw=6)**	-	21.1±1.7 [18-22] (nw=5)	-	22.3±2.0 [20-25] (nw=5)
Total eggs produced (egg)	668		482		1103		1081		973		289		328	
Lifetime egg production (egg/ind.) mean±SD [range]	11.1±3.6 [7-16] (nw=6)	16.3±10.7 [1-39] (n=41)	8.0±5.0 [0-13] [nw=6)	13.0±9.3 [1-37] (n=37)	18.4±9.2 [4-29] (nw=6)	25.1±16.9 [3-62] (n=44)	18.0±9.0 [8-31] (nw=6)	24.6±19.4 [2-83] (n=44)	16.2±6.2 [8-24] (nw=6)	24.9±15.0 [4-74] (n=39)	4.8±6.4 [0-17] (nw=6)	28.9±25.2 [2-75] (n=10)	5.5±4.4 [0-10] (nw=6)	27.3±15.8 [6-62] (n=12)
Hatching ratio (%)	28		**36.7****		23.8		**20.8****		**6.2****		**2.8****		**0.9****	
F2 generation rate	3.12		2.95		4.38		3.75		1.00		0.13		0.05	

* $p<0.05$ in Dunnett' test. ** $p<0.05$ in Chi-square test. nw= number of wells

6.3.2. Acute to chronic ratio (ACR) of nickel and sensitivity comparison with other test species

Table 6.3 summarizes the toxicity data of nickel to ostracod *H. incongruens* and other invertebrate species. To the best of our knowledge, this is the first report on nickel chronic toxicity to ostracod *H. incongruens*. The chronic nickel toxicity to ostracod *H. incongruens* was compared to the previously reports of chronic Ni exposure with other test species. Chronic Ni toxicity in the amphipod *Hyalella azteca* was reported as EC25 for growth of 17 µgNi/l (Borgmann et al., 2001) and 14d-EC20 of 61µg/L (Keithly et al., 2004) at a hardness of 98 mg/L (as $CaCO_3$) which was comparable with this study. Powlesland and George (1986) reported the chronic toxicity of nickel to larvae of *Chironomus riparius* (Meigen) as MATC of 1.1 mgNi/l for 30 d-growth. The effect of nickel to cladoceran *Ceriodaphnia Dubia* was reported as NOEC (7d-reproduction) of 5 µgNi/l by Kszos et al., (1992) and 7d-EC20 of 4.7µgNi/l by Keithly et al., (2004). Another study showed the nickel NOEC (21d-reproduction) for *Daphnia magna* as 15 µgNi/l (Biesinger and Christensen, 1972), 13 µgNi/l (Kszos et al., 1992) and 42 µgNi/l (Pane et al., 2004). The results show different sensitivity in different endpoint investigated as well as test species.

The mechanism of nickel toxicity to aquatic organisms has been awaited for fully understanding because the difference in physiology among organisms may affect mechanisms of toxicity. Nickel toxicity appears to act more like an ionoregulatory toxicant in aquatic invertebrates. In contrast, nickel has been found to be impaired the respiratory function to fish (Niyogi et al., 2014). Pane et al. (2003, 2004) reported the mechanisms of chronic waterborne nickel toxicity in the freshwater cladoceran, *D. magna*. Toxicity of nickel in both acute and chronic exposures causes disruption of Mg^{2+} uptake and reduction of whole body Mg^{2+} concentrations to *D. magna*. Additionally, chronic nickel exposure impaired respiratory function, as both oxygen consumption and whole-body hemoglobin content of *D. magna* were significantly decreased by 31 and 68%, respectively (Pane et al., 2003).

Table 6.3 Freshwater toxicity data of nickel and acute to chronic ratio (ACR)

Species	Acute (mg/l)	Chronic (µg/l)	ACR	Median ACR	References
Heterocypris incongruens	6d-LC50: 2.41[a]	NOEC (egg-laying ratio, lifespan for all individual): 12[b]	201	201	[a]Kudlak et al., 2011 and [b]This study
Hyalella azteca	96 h-LC50: 3.05[d]	IC25 for growth (4weeks): 17[c]	179	115	[c] Borgmann et al., 2001
		14d-EC20 (growth): 61[d]	50		[d]Keithly et al., 2004
Chironomus riparius	48 h-LC50: 79.5[e]	MATC (30d- growth of larvae):1100[e]	72	72	[e]Powlesland and George 1986
Chironomus tentans	24h- EC50 (immobilization): 78.1[f] 48hr- EC50 (immobilization): 69.5[f]	-	-	-	[f]Khangarot and Ray, 1989
Ceriodaphnia dubia	48 hr-LC50: 0.15[d]	NOEC (7d-reproduction): 5[g]	30	31	[g]Kszos et al. 1992
		7d- EC20 (reproduction): 4.7[d]	32		[d] Keithly et al., 2004
Daphnia magna	48 hr-LC50 (immobilization): 0.51[h]	NOEC (21 d-reproduction): 15[h]	34	34	[h]Biesinger and Christensen 1972
		NOEC (21 d-reproduction): 13[i]	39		[i]Kszos et al. 1992
		NOEC (21 d-reproduction, and growth of maternal (F_0 generation)): 42[j]	12		[j]Pane et al., 2004

NOEC = no observed effect concentration; EC20, = 20% effect concentrations; IC25= 25% Inhibition concentrations; MATC= maximum acceptable toxic concentration

To facilitate in sensitivity comparison, acute to chronic ratios (ACR, unitless) were calculated based on the available chronic and acute toxicity data for freshwater invertebrates of nickel from Table 6.3. In the United States, ACRs are commonly calculated as an acute LC50/MATC and used for deriving water quality criteria for aquatic life (Raimondo et al. 2007; Mebane et al., 2008). ACRs have also been reported as LC50/EC20s. US EPA (2007) used chronic EC20 values as a

replacement of the MATC in ACRs to derive water quality criteria for copper. In the European Union, Australia and New Zealand, ACRs are usually calculated as LC50/NOEC when used to derive water quality guidelines or to register industrial substances for use. Canada has a national goal for environmental quality of no observable adverse effects on aquatic and terrestrial ecosystems over the long-term, and uses chronic NOECs or EC10 (Mebane et al., 2008). Additionally, ACRs can be calculated as an acute LC50 divided by long-term LC50 in the study of Grosell et al., (2006). Mebane et al., (2008) stated that these calculated ACRs by using the same endpoint over different exposure times is not used to estimate protective concentrations for untested species, but only reflect the influence of duration or acclimation on lethality. Thus, the ACRs in this study were calculated from acute toxicity data (LC50) by the chronic MATC, NOEC, and EC20s on growth and reproduction. The results of ACRs indicated the difference in sensitivity of the species. Median nickel ACR of *H. incongruens* was 201 followed by median ACRs of *H. azteca, C. riparius*, *D. magna* and *C. dubia*. Based on this study, 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 also indicates the importance of conducting the chronic toxicity test proposed in this study. However, it has to be considered that nickel concentrations were prepared in 10 time interval as the initial range-finding test of this study. This would have a great effect on accuracy of ACRs and discussing the ostracod's sensitivity compared to other species, as well as the evaluation of the risk that environmental concentrations pose to them.

The variability of ACRs among species in Table 6.3 may be partly due to the difference in the endpoint of the chronic toxicity tests. The endpoint for *H. azteca* and *C. riparius* was growth inhibition while that for other species was reproduction. The mode of action is naturally different in different endpoints, and the difference will results in the variability of ACRs. Table 6.3 also shows a great difference in acute toxicity data among test species. In general, the planktonic crustaceans showed high sensitivity in acute toxicity compared with the benthic organisms in Table 6.3.

Although the mechanism of low sensitivity in the benthic organisms is not well understood, the different sensitivity in acute toxicity tests caused the variability of ACRs. Raimondo et al., (2007) reported that ACR variability related to chemical MOA and class, taxa (invertebrates and fish), and ambient media (freshwater and saltwater) and should be considered when applying ACRs in risk assessment.

Shuhaimi-Othman et al., (2012) showed the application of using an acute-to-chronic ratio (ACR) to derive freshwater quality criteria for iron, lead, nickel, and zinc for the protection of aquatic life. The results of this study would be useful for further derive the ACR and criterion continuous concentration of nickel for the protection of benthic community. Further studies are required for test sensitivity of ostracod in different metals, a group of chemicals or their mixture to represent environmental contaminated sediments (usually mixtures of different chemicals). This chapter showed the first report of the chronic nickel toxicity to ostracod *H. incongruens*. The next chapter reported an application example of the proposed chronic test using urban road dust as an environmental sediment sample.

6.4. Summary

In this chapter, the aim was to assess the toxicity of nickel on the reproduction of benthic ostracod *H. incongruens* using the proposed chronic ostracod toxicity test and to compare the sensitivity of other test species using acute to chronic ratios (ACRs).

- The mortality of ostracod after a 14-day exposure to a highest concentration of nickel (C6=1200 µgNi/l) was 41.7%. Thus, the estimated 14-d LC50 was $2.95x10^3$ µgNi/l with an NOLC (14-d mortality) of 12 µgNi/l and the 14-d LC20 was calculated as 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. The resulted showed mean life span of all individual was considerably decreased and statistically significant difference (p<0.05) found in 120 and 1200 µgNi/l of nickel exposure cases. The egg-laying ratio was statistically significantly low in these two concentrations with NOEC of 12 µgNi/l for egg-ratio and life span for all individual.

- 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. The high variability of these two endpoints was also shown in chapter 5.

- 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 also indicates the importance of conducting the chronic toxicity test proposed in this study. However, it has to be considered about the accuracy of an ACR for nickel concentrations were in 10 time interval as the initial range-finding test of this study.

CHAPTER 7

TOXICITY ASSESSMENTS OF URBAN ROAD DUST ON REPRODUCTION OF THE BENTHIC OSTRACOD *HETEROCYPRIS INCONGRUENS*

7.1. Introduction

Urban road dust (URD) is one of the potential sources of sediment pollution as solid particles in urban road runoffs. They can potentially be transferred into water bodies by rainfall. Urban road dust in an aquatic ecosystem can deteriorate water quality and show the adverse effect to aquatic organisms particularly on benthic organisms, due to their habitat in sediment. Previous studies reported that urban road dust contained high concentrations of many potentially toxic substances such as hydrophobic organic compounds, heavy metals, polycyclic aromatic hydrocarbons and perfluorinated surfactants (Fang et al., 2004; Murakami and Takada, 2008; Atiemo et al., 2011).

Since the availability of sediment toxicity test using benthic ostracod *H. incongruens* was standardized as ISO14371 (ISO, 2012), the test has been applied to test the freshwater sediment for various solid samples such as sediment (Torokne and Toro, 2010), soils (Chial et al., 2003; Santorufo et al., 2012) and road dust (Watanabe et al., 2011, 2013; Khanal et al., 2013, 2014). The toxicity assessments of URD to *H. incongruens* were done in term of simulating the runoff process in the aquatic systems such as the amount of rainfall and holding time (Watanabe et al., 2011) and evaluating size-fractioned of URD (Khanal et al., 2014). Watanabe et al. (2013) and Khanal et al. (2015) applied the whole sediment toxicity identification evaluation to characterize the potential toxicants in URD. Previous studies assessed the toxicity of URD to benthic ostracod *H. incongruens* based on the two criteria in ISO as 6 d mortality and 6 d growth inhibition. However, as far as we know, there is no studies report the effect of URD dust on the reproduction of ostracod. From the ecological

point of view, chronic toxicity should be evaluated to represent a long-term exposure of toxic sediments to organisms which affect reproduction.

The objective of this chapter aimed to assess the toxic effect of urban road dust (solid phase of urban road runoff) on the reproduction of ostracod and to provide an application example of the proposed chronic method using URD. 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*.

7.2. Materials and methods

7.2.1. Test organism

H. incongruens were purchased as dormant eggs (cysts) from MicroBioTests Inc., Belgium. The procedure for hatching ostracod cysts is as described in section 3.3 of chapter 3.

7.2.2. Standard ostracod toxicity test (ISO14371)

The method and test materials followed ISO 14371; the method is hereafter referred to as the 6 d ostracod toxicity test in this study. The detailed procedure is shown in chapter 3.

7.2.3. Chronic ostracod toxicity test

The proposed chronic ostracod toxicity test consisted of three consecutive parts: (I) a 14 d sediment exposure phase, (II) a reproduction phase, and (III) a hatching test. The procedures and timelines for the chronic ostracod toxicity test are shown in chapter 4.

7.2.4. Toxicity assessment of URD

After determining test repeatability in the previous chapter, the chronic ostracod toxicity test was applied to assess the toxicity of URD in parallel with the 6 d ostracod toxicity test. An URD (<2000 µm) sample that was previously collected by Khanal et al., (2014) from a roadside in Tokyo in 2012, was used in this study, and the clean RF sediment was used as the control sediment. The dose-response relationship for ostracod mortality was determined by diluting (v/v) URD with the RF sediment to obtain concentrations of 3.125%, 6.25%, 12.5%, 25%, and 50% (v/v). The sediment sample of both the RF sediment and URD was prepared following the procedure described in the previous section. The chronic and 6 d ostracod toxicity test of control sediment and URD samples were conducted in two

batches. The first RF sediment batch (RF_1) was conducted with 3.125%, 6.25%, and 50% of the URD sample. The second RF sediment batch (RF_2) was conducted with 12.5% and 25% of the URD sample. Nine test replicates were conducted for each concentration in the chronic ostracod toxicity test; one replicate was used for body length measurements on day 14 and the rest were used for reproduction. The 6 d ostracod toxicity tests were performed following the same method of the chronic toxicity test and each concentration of the control and URD samples were conducted in sextuplicate.

7.2.5. Statistical analysis

All data and results were reported with standard deviations. The Grubb's test was applied to test outliers of the control data using Minitab 17 (State College, PA: Minitab Inc.). Dunnett's test was used to determine the significant difference ($p < 0.05$) between URD samples and control sediment using Minitab 17. Dunnett's test was used in ANOVA to create confidence intervals for differences between the means of control and treatment groups. A chi-square analysis was applied to the hatching ratio. The LC50 of URD was calculated using a Probit analysis.

7.3. Results and discussions

7.3.1. Toxicity assessment of URD on *H. incongruens*

(I). 6-day ostracod toxicity test on the URD sample

Table 7.1 shows the results of the overlying water quality, 6 d mortality, and 6 d growth inhibition. The pH of the overlying water was considered not lethally toxic to ostracods. Previous studies reported that average pH ranges from 5.5 to 8.7 during the 6 d ostracod toxicity test did not have lethal effects on ostracods (Watanabe et al., 2011). The 6 d mortality was in the range of 1.7–72% and increased with higher concentrations of URD. There was a statistically significant difference ($p < 0.05$) in 6 d mortality between the control and 25% URD and 50% URD sample. The 6 d growth inhibition also increased with increasing concentrations of URD. We also found a statistically significant difference ($p < 0.05$) in 6 d growth inhibition between the control and 12.5% RD sample. Non-observed effect concentrations in 6 d mortality and growth inhibition were 12.5% and 6.25% URD, respectively. Figure 7.1 shows the dose-response relationships between 6 d mortality and different concentrations of URD (v/v) in RF sediments. The calculated LC50 with 95% upper and lower confidence limits (95% UCL and LCL) of URD samples was 30% (26–37%). The LC20 was the mortality threshold in a 6 d toxicity test and was calculated as 14% (11–17%). The LC50 was furthered represented by a toxic unit (TU50 =100/LC50) to help in toxic comparisons. The TU50 for URD was 3.3.

The results of 6 d ostracod toxicity were only determined by a high concentration of URD samples of 12.5% RD, 25% RD, and 50% RD. The 6 d toxicity test was too short to evaluate low to moderate levels of contamination and long-term exposure that may affect ostracod reproduction. Therefore, one of our concerns was to determine whether 3.125% and 6.25% URD samples had any detectable effect in the chronic toxicity test.

Table 7.1. The overlying water quality, 6 d mortality, and 6 d growth inhibition in 6 d ostracod toxicity test with different URD concentrations.

Parameters	RF[a]	3.125% URD	6.25% URD	12.5% URD	25% URD	50% URD
pH	8.2 ± 0.0	8.3 ± 0.1	8.5 ± 0.0	8.4 ± 0.1	8.5 ± 0.1	8.1 ± 0.1
Conductivity (mS/cm)	1.30 ± 0.32	0.93 ± 0.04	0.91 ± 0.07	1.09 ± 0.03	1.21 ± 0.06	1.16 ± 0.09
6 d mortality (%)	1.7±3.9	1.7 ± 4.1	5.0 ± 8.4	10 ± 16	45 ± 23*	72 ± 10*
6 d growth inhibition (%)	0.0±9.0	0.9 ± 4.1	1.2 ± 7.3	15.1 ± 10.7*	N/a**	N/a**

Values indicate mean ± standard deviation.* $p < 0.05$ in Dunnett's test.** N/a: Not available due to the high mortality (>30%, the criteria in ISO 14371) [a] mean value from RF_1 and RF_2, control conditions with the 1st batch of RF_1 was conducted with 3.125%, 6.25%, and 50%Rsample and RF_2 was conducted with 12.5% and 25%RD sample.

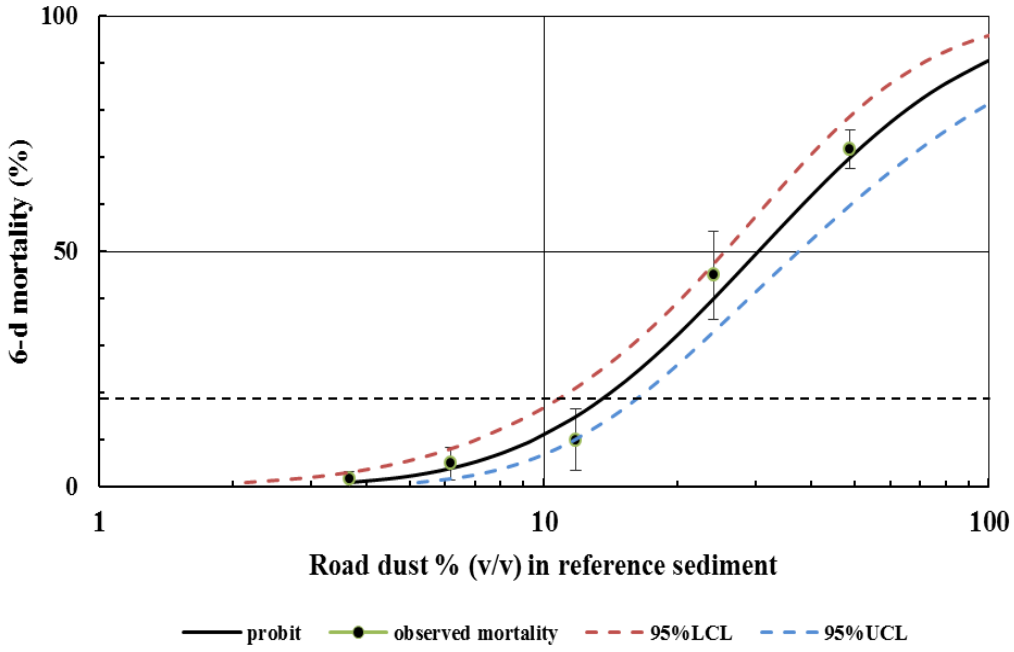


Figure 7.1. Dose-response relationships between 6 d *H. incongruens* mortality with different concentrations of urban road dust (v/v) in reference sediments. The broken lines express 95% upper and lower confidence limits (95% UCL and LCL). Error bars represent standard errors.

(II). Chronic ostracod toxicity test on URD samples

Figure 7.2 shows the dose-response relationships between 14 d mortality and different concentrations of URD (v/v) in RF sediments. The calculated LC50 with 95% UCL and LCL of URD samples was 15% (3–82%) with a TU50 of 6.67. The overlying water quality, 14 d mortality, and 14 d growth inhibition in the chronic toxicity test with different URD concentrations are presented in Table 7.2. The observed 14 d mortality increased with increasing concentrations of URD mixed with RF sediment (v/v), except for 3.125% URD that showed a lower mortality than the control. 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. As shown in Table 7.2, a statistically significant difference ($p < 0.05$) in 14 d growth inhibition was found for all concentration of URD.

After 14 d in the sediment exposure phase, *H. incongruens* reproductions were observed under exposure to different URD concentrations. Due to the high 14 d mortality in 25% URD and 50% URD samples where *H. incongruens* died before producing eggs, these two samples were not accounted for reproduction characteristics. Then, data set in each

endpoint of control sediments were also evaluated based on the proposed acceptability criteria in chapter 5. The values for the first day of brooding in the control RF_2 test fell outside of the expected range. Thus, this control dataset with 12.5% URD and 25% URD results were excluded.

Table 7.3 shows the life history and reproduction characteristics of *H. incongruens* under control conditions and after exposure to different URD concentrations. The mean life span of all individuals was slightly shorter but was longer for egg-laying individuals when exposed to URD than in the control condition. The first day of brooding changed slightly with different URD concentrations. There was a statistically significant difference between RF_1 and the 6.25% URD sample ($p < 0.05$) on the first day of brooding. These results showed that a low URD concentration retarded *H. incongruens* reproduction. The mean day of egg production was slightly delayed in URD samples compared with the control but no statistically significant difference was found.

The number of eggs produced was recorded for each individual at each URD concentration until all *H. incongruens* died in order to calculate the lifetime egg production and the egg-laying ratio (Table 7.3). The egg-laying ratio in both of 3.125% URD and 6.25% URD and lifetime egg production in 6.25% URD were lower than that in control sediments although the results were not significantly different. For the hatching test, a statistically significant difference was found in 6.25% URD that had the lowest hatching ratio of 4.4% and an F2 generation rate <1 indicating the possibility of *H. incongruens* extinction.

We found that at 6.25%URD in the chronic test impacted *H. incongruens* reproduction as showing in delayed first day of brooding. The application of chronic sediment toxicity test using *H. incongruens* would help to predict and assess the long term effect of URD on the benthic water environment. Thus, identification of principal toxicants in urban road dust is matter for further investigation. Toxicity Identification and Evaluation (TIE) approach initially developed to identify the cause of toxicity in various sediment samples including URDs. The sediment TIE process involves a series of processes and consisted of three phases: phase I (characterization), phase II (identification), and phase III (confirmation) (US EPA, 2007). Previously, the TIE approach has been applied to identify the major toxicants in URD with the application of benthic ostracod *H. incongruens* (Watanabe et

al., 2013; Khanal et al., 2015). Hydrophobic organic compounds (Watanabe et al., 2013) and heavy metals (Khanal et al., 2015) were estimated as the major toxicants in URD. In addition, Hiki et al. (2016) investigated dissolved nicotine as a causative toxicant in URD by performing sediment TIE with an estuarine amphipod, *Grandidierella japonica*. These previous studies indicated responsible acute-toxicant in urban road dust using TIE. Further collaboration of TIE procedure with the chronic ostracod toxicity test is necessary to identify the causative toxicants in the samples exhibiting chronic toxicity.

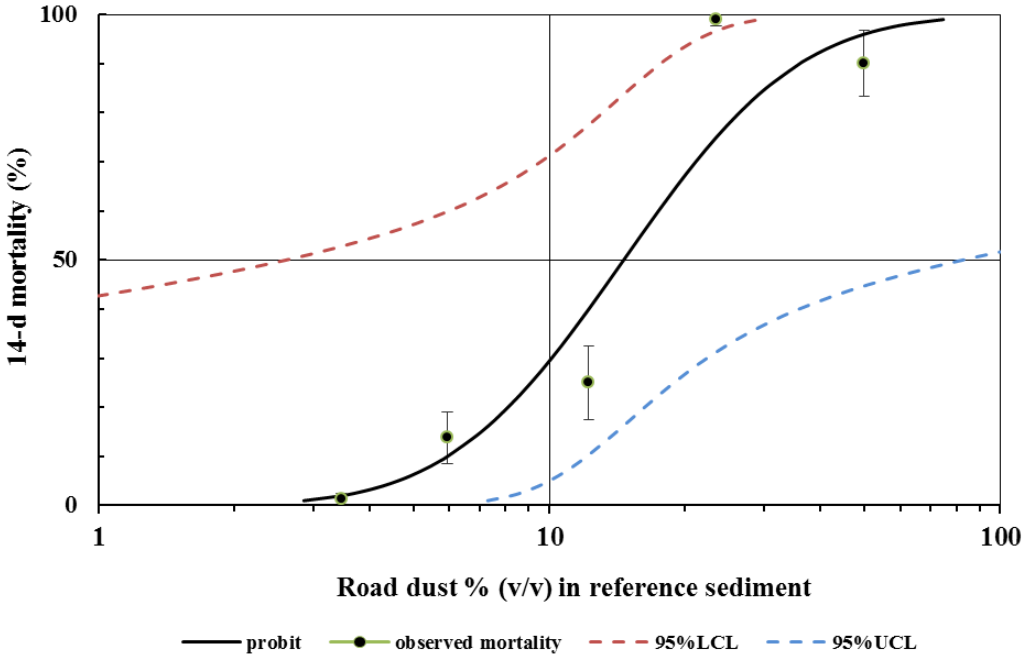


Figure 7.2. Dose-response relationships between 14 d *H. incongruens* mortality with different concentrations of urban road dust (v/v) in reference sediments. The broken lines express 95% upper and lower confidence limits (95% UCL and LCL). Error bars represent standard errors.

Table 7.2. The overlying water quality, 14 d mortality, and 14 d growth inhibition in the chronic toxicity test with different URD concentrations.

Parameters	RF[a]	3.125%URD	6.25%URD	12.5%URD	25%URD	50%URD
pH	8.2 ± 0.1	8.4 ± 0.1	8.4 ± 0.1	8.3 ± 0.2	8.4 ± 0.1	8.6 ± 0.2
Conductivity (mS/cm)	1.06 ± 0.20	0.87 ± 0.06	0.94 ± 0.12	1.13 ± 0.11	1.21 ± 0.09	1.08 ± 0.01
14 d mortality (%)	5.0 ± 8.2	1.25 ± 3.5	13.8 ± 15	25 ± 21*	99 ± 3.5*	90 ± 19*
14 d growth inhibition (%)	0.0 ± 3.5	5.5 ± 4.3*	15.9 ± 6.0*	7.2 ± 2.9*	N/a**	N/a**

[a] mean value from RF_1 and RF_2, control conditions with the 1st batch of RF_1 was conducted with 3.125%, 6.25%, and 50%Rsample and RF_2 was conducted with 12.5% and 25%RD sample.

Table 7.3. The life history and reproduction characteristics of *H. incongruens* under control conditions and after exposure to different URD concentrations**.**

Parameters	RF_1		3.125%URD		6.25%URD		50%URD
	All individuals	**Egg-laying individuals**	**All individuals**	**Egg-laying individuals**	**All individuals**	**Egg-laying individuals**	**All individuals**
n (number of ostracod)	80	55	80	43	80	36	80
Egg-laying ratio (%)	68.8±23.0		54.0±22.6		45.0±25.6		0
Life span (day) **[range]**	25.9±2.8 [20-30]	30.2±3.2 [25-35]	25.9 ± 3.9 [21-31]	31.6 ± 3.3 [27-38]	24.2±4.1 [19-28]	31.0±2.5 [29-36]	14.5±1.5
First day of brooding (day) **[range]**	-	22.1±0.9 [21-23]	-	22.5±1.8 [20-26]	-	**24.2±2.1*** [23-28]	-
Mean day of egg production (day) **[range]**	-	24.6±1.8 [23-28]	-	26.0±2.5 [24-31]	-	26.6±1.4 [25-28]	-
Total number of eggs produced (egg)	1714		1793		1264		-
Lifetime egg production (egg/ind.) [range]	21.4±10.8 [10-43]	31.2±29.9 [1-109]	22.4±13.4 [4-48]	41.7±37.5 [1-154]	15.8±10.8 [1-31]	35.1±29.9 [1-122]	0
Hatching ratio (%)	12.8		12.8		**4.4****		-
F2 generation rate	2.7		2.9		0.69		0

mean ± standard deviation; values in parenthesis indicate the observed range in min-max. * p<0.05 in Dunnett's test, ** p<0.05 in Chi-square test
3.125%, 6.25%, and 50%URD sample was conducted with control sediment as the batch of RF_1

7.3.2. Toxicity variability in URD

In this study, an URD sample that was previously collected in a study by Khanal et al., (2014) from a roadside in Tokyo in 2012, was analyzed by a 6 d toxicity test after 2 years of storage, and the difference between the toxicity results was compared. In the previous toxicity test by Khanal et al., (2014), ostracod mortalities of less than 50% were observed for all concentrations of the diluted URD. In contrast, 6 d toxicity result from the current study showed highest of 72% ostracod mortality at 50% diluted URD concentration. There are a number of possible reasons for toxicity differences between their and our study. The first possibility might be the different storage times of the sediment sample before toxicity tests were conducted. Dillon et al., (1994) showed that toxicity increased over time for sediment stored at -22^{o}C and 4^{o}C. Another study indicated that sediment storage time could substantially influence toxicity as determined by the results of three toxicity tests that varied significantly by storage time (Becker et al., 1995). Another possible reason is the heterogeneity of URD samples. The bioavailability and leaching characteristics of toxicants in sediment samples are also influenced by the grain size distribution (Simpson et al., 2005). In general, fine fractions have been found to accumulate a higher concentration of toxicants than sandy or coarse grain particles. Thus, fine particles were expected to be more toxic to benthic organisms (Simpson et al., 2005, Murakami et al., 2005, 2008). During the two toxicity tests that were conducted separately, the heterogeneity of sediment samples and the use of sediment stored over some period of time may have caused the variability and difference in the URD toxicity results between these two studies.

Generally, the process of sediment collection, handling, and storage may alter bioavailability and concentration of toxicants. These changes in the physical, chemical, or biological characteristics of the sediment cause the variability of toxicity test results. Therefore, the pre-caution regarding the information about sample transport, collection, storage and handling before conducting the sediment test can refer to US EPA guideline (US EPA, 2001b).

The 6 d and 14 d sediment exposure phase in this study represented the difference in exposure time of *H. incongruens* to URD. The mortality rate under a series of

concentrations of URD after 6 d and 14 d of exposure are shown in Tables 7.1 and 7.2. The toxicity (mortality and growth inhibition) after 14 d (Table 7.2) was greater compared to 6 d (Table 7.1), except for mortality by 3.125% URD and growth inhibition by 12.5% URD. The TU50 was greater for 14 d mortality than that for 6 d mortality. The possible reasons need to explain how toxicity increased with increasing exposure time. Khanal et al., (2013) proposed the hypothesis on changes in toxicity of wet road dust. At the initial exposure time, the toxicity of URD to *H. incongruens* was due to the presence of a labile toxicant. As the exposure time increased, more of the bound toxicants in URD were released and exposed to *H. incongruens*. This led to increasing or changes in toxicity of URD with a prolonged exposure time. In addition, biochemical processes are believed to occur at the latter stage of exposure, including exchangeability of toxicants, change in toxicant speciation, and the formation of new toxicants. The further explanation was proposed in the study of Watanabe et al., (2011) as the toxicity of road dust in a 7 d holding time showed a higher ostracod mortality than at 1 h and 24 h holding times. This phenomenon was due to the more non-labile toxic compounds at the initial stage of exposure that started to dissolve and form new toxic compounds such as hydrogen sulfide by microbial reactions. In addition, the formation of unionized ammonia was also suspected to be one toxicant in URD that enhanced ostracod mortality over long periods of time (Watanabe et al., 2013). The previous studies showed the effect of long holding time on the mortality of the ostracod. This phenomenon should be further tested for the chronic toxicity to ostracod as reproductive output. The proposed future plan can be conducted to observe the effect of various holding time or incubation time of sediment before inoculation of the ostracod.

Toxicity of sediment may vary due to the bioavailability of toxicants in the sediment. The bioavailability would depend on many factors such as pH, organic carbon content, and particle size distribution. And those parameters may change with time as mentioned in the last paragraph. Measuring only pH, conductivity and salinity in the overlying water may not be sufficient to investigate the bioavailability of toxicants. Further study may be proposed to measure the dissolve organic carbon content in the overlying water (UV absorbance is a possible index of organic content in the limited amount of sample). This measurement would help to determine the effect of dissolve organic carbon content during the 14 d sediment exposure phase on the chronic toxicity to ostracod.

7.3.3. Acute to chronic ratio (ACR) of URD

ACR expresses the acute toxicity of an effluent or a toxicant to its chronic toxicity (Raimondo et al., 2007). In this study, the results of URD toxicity were used to predict the threshold concentration at which no negative effect would occur during a chronic exposure in the receiving water. ACRs were calculated as the ratio of the lethal concentration (LC50) and non-observed effect concentrations (NOEC) or maximum acceptable toxicant concentration (MATC) resulting from the most sensitive chronic endpoint. Based on this study, the estimated ACR for URD and *H. incongruens* was 6.8, which was calculated from the LC50 of 6 d mortality (%) and hatching ratio MATC or the first day of brooding in URD.

ACR can be applied to estimate the chronic toxicity or dilution ratio required for a clean RF sediment of URD to become non-toxic. Thus, chronic TUs (TUc = 100/NOEC, MATC, or NOEC) can be calculated to facilitate comparisons with other samples and can be defined as the percentage of URD in a sediment sample. Considering TUs as a safety threshold, this URD could become non-toxic if mixed with 23 times clean sediment (MATC = 4.42) volume of the road dust. This sample was indicated as a non-significant toxicity in the 6 d toxicity tests by Khanal et al., (2014). Using ACR, we can calculate TUs to compare chronic toxicity with those of URD samples. The TUs of URD in Khanal et al., (2014) ranged from a low of 10.1 (LC50 = 67%) to a high of 179 (LC50 = 3.8%), or the dilution required for URD to become non-toxic. To the best of our knowledge, the ACR for URD using *H. incongruens* has never been reported. 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.

7.4. Summary

This chapter shows an application example of the proposed chronic test using URD. The toxic effect of URD on ostracod reproduction was carried out in comparison with ISO 14371. The results are summarized as follows.

- The 6d-LC50 and LC20 (with 95% UCL and LCL) of URD sample was 30% (26 - 37%) and 14% (11-17%), respectively. The TU50 was 3.3. Non-observed effect concentrations were 12.5% and 6.25% URD in 6 d mortality and growth inhibition, respectively.

- The 14d-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.

- In term of the URD toxicity on ostracod reproduction, 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 showed a toxic effect on *H. incongruens* reproduction, which was not identified as toxic in standard 6 d toxicity tests (ISO 14371).

- ACR of URD in this study can be applied and 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. Considering chronic TUs as a safety threshold, this URD could become non-toxic if mixed with a 23 times clean sediment volume of the road dust.

CHAPTER 8

CONCLUSIONS AND RECOMMENDATIONS

8.1. Conclusions

The main objective of this study was to develop a new chronic sediment toxicity test using the freshwater benthic ostracod *H. incongruens.* The following conclusions were drawn.

A chronic sediment toxicity test using a benthic ostracod was proposed based on a literature survey to identify the suitability of the freshwater benthic ostracod *H. incongruens* as a cosmopolitan and representative test species including the advantage as cyst-based toxicity test.

The detailed procedure and test conditions are outlined in Chapter 4. The proposed chronic test is composed of three consecutive phases of a 14-day sediment exposure phase, a reproductive phase, and a hatching test. The 14-day sediment exposure phase was designed to expose the ostracod in sediment until a few days before release of the first brood. Then, reproduction and hatching were examined and evaluated. The hatching test was conducted simultaneously in parallel with the reproductive phase after the ostracod laid their first brood of eggs. 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 3.0×10^7 cells/well during the first week, and feeding a TetraMin suspension for the remainder of the test.

Mortality and growth inhibition as the endpoints used in the ISO 14371 method. Reproductive parameters of ostracods, such as egg production, the first day of brooding, egg-laying ratio, and hatching ratio, were used as new endpoints for the chronic test proposed in this study. The test was validated by determining repeatability of the method after seven control tests. The results showed good repeatability of most endpoints with coefficients of variation (CVs) < 15%. However, lifetime egg production, hatching ratio, and F2 generation rate were highly variable with CVs of 29.5%–51.9%. Thus, the results of the life history and reproductive characteristics of the ostracod *H. incongruens* under

the reference (control) sediment and the variability in the test endpoints were used to set the test acceptability criteria. 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 test acceptability criteria were used to test the validity of the control sediment.

After validating the test, nickel was used as a reference toxicant to assess the reproductive toxic effect on *H. incongruens* and to compare sensitivity to nickel among the other test species used in the sediment toxicity test. Ostracod mortality after a 14-day exposure to the highest concentration of nickel (1,200 μgNi/l) was 41.7%. Thus, the estimated 14-d LC_{50} was 2.95×10^3 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. *H. incongruens* reproduction varied with nickel concentration. 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. Based on this study, 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 also indicates the importance of conducting the chronic toxicity test proposed in this study. However, it has to be mentioned for the wide range of exposure nickel concentrations in 10 times interval used in this study; this would effect on accuracy of ACRs and sensitivity comparison of the ostracod among other species. Further research should consider investigating the sensitivity of ostracod to nickel as definitive toxicity test. Moreover, further studies are proposed to determine the sensitivity of the chronic test to other reference chemicals and specific groups of contaminants, such as metals, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and pesticides, including their mixtures, which would be more relevant as sediment contaminants.

Urban road dust (URD) was used as an example application of the proposed chronic test. A series of dilutions of URD and reference sediment were tested in a 6-d ostracod toxicity test. The results of 6-d ostracod toxicity test demonstrated a NOEC of 12.5% and

6.25%URD in mortality and growth inhibition, respectively. The proposed chronic toxicity method showed significant differences on the first day of brooding and a hatching ratio of 6.25% URD. These results suggest that low concentrations of URD were toxic to *H. incongruens* reproduction, which was not previously identified as toxic by a standard 6 d toxicity test (ISO 14371).

In this study, the chronic toxicity test results of a URD were used to predict the threshold concentration at which no negative effect would occur during chronic exposure in receiving water. 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 (MATC = 4.42). The ACR for URD using *H. incongruens* has never been reported. This approach could be used to effectively manage URD in urban runoff and to estimate chronic thresholds of URD to protect the benthic community.

Most of the endpoints as CV below 15% reflect good repeatability of test method and can be established for routine use in the chronic ostracod toxicity test. The test acceptability criteria can be used to validate the control and correctly interpret the chronic toxicity test results. Although the sensitivity of the ostracods to certain chemicals was limited and more toxicants and studies are required, 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, only performing acute test may not be sufficient and underestimate the toxicity of nickel to ostracod. This also indicates the importance of conducting the chronic toxicity test proposed in this study. The results of the URD environmental sediment sample demonstrated that low concentrations of URD were toxic to *H. incongruens* reproduction, which was not determined by a standard 6 d toxicity test (ISO 14371). However, the calculated ACR of URD can help to estimate the chronic thresholds that would be protective of the aquatic community and help effectively manage URD in urban runoff pollution.

8.2. Recommendations

Further studies are needed based on the results of this research. The following points are recommended to consider in future studies.

- Hatching ratio and F2 generation rate were highly variable in the control tests as reported in Chapter 5. As the hatching ratio and F2 generation rate are critical indices to evaluate the continuation of a species, a theoretical investigation of hatching characteristics of the benthic ostracod *H. incongruens* is necessary to minimize test variations. Further research is required to determine the effects of abiotic conditions on life history traits of *H. incongruens*, such as the effects of different food or combination of foods.

- *H. incongruens* was the most sensitive to chronic nickel, which showed the highest ACR, compared with the other test species. Further research should identify additional chemicals that ostracods are sensitive to as well as specific groups of pollutants in relevant to sediment contamination. The test can also be used for sediment spiked with certain chemicals to evaluate the sensitivity of benthic ostracods. These further experiments may help to determine the future risks of environmental contaminants before they are released into the environment.

- Future studies should consider conducting the chronic ostracod toxicity test with standard mixtures of metals and/or other inorganic/organics compounds, as these kinds of tests may provide a more accurate assessment and appropriate response by the benthic ostracod to measure interactive toxic effects of complex contaminant mixtures. Mahar and Watzin (2005) observed the effects of mixtures of metals and organophosphates on *Ceriodaphnia dubia* survival and reproduction. The results showed differences in the toxic response, which varied with sensitivity to the chemicals and the specific endpoints evaluated.

- In this study, the chronic toxic effect of metal on the ostracod was first considered using dissolved toxicants. Dietary toxicity from contaminated food should be incorporated when assessing the biological effect of contaminated sediment as diet is a potential exposure routes of benthic species. Different toxicity pathway may change the effect and

sensitive on endpoint investigated. De Schamphelaere et al., (2004) demonstrated a decrease in reproduction in response to dietary Zn (accumulated in green algae) in the cladoceran *Daphnia magna*, but no effects on survival, growth, or feeding rate. Further research such as Ni-spiking in sediment, dietary Ni exposure as food-contaminated toxicants is required.

- Low concentrations of URD showed a toxic effect on *H. incongruens* reproduction, which demonstrated a chronic effect of URD. URD contributes to the toxicity of urban runoff and pollution. Thus, ACR of URD was calculated to estimate chronic thresholds of URD, which could be used to protect the benthic community. This finding would be beneficial for effectively managing URD in urban runoff pollution; however, testing with more types of URD samples is needed to define more reliable ACR criteria.

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APPENDIX A

Analytical grade of chromium chloride hexahydrate ($CrCl_3.6H_2O$), potassium dichromate ($K_2Cr_2O_7$) and zinc chloride ($ZnCl_2$) were used as the reference toxicants. The 100 mg/l stock solution was prepared by dissolving 10 mg $CrCl_3.6H_2O$ and $K_2Cr_2O_7$ in 100 ml Milli-Q water, separately. For zinc solution, 21 mg of zinc chloride ($ZnCl_2$) were dissolved in 100 ml Milli-Q water. The stock solutions of each metal were measured, and the serial dilutions from each measured stock solutions were prepared by diluting with standard freshwater (SFW) in 10 times interval. Exposure concentrations to 0.048, 0.48, 4.8, 48 and 480 µg/l of Cr (III), 0.11, 1.1, 11, 105 and 1050 µg/l of Cr (VI) and 0.26, 2.6, 26, 255, 2550 µg/l of Zn were used for the ostracod toxicity test. The exposure concentrations were half as much as measured values because of the subsequent dilution by algal food suspension.

Table A1. Overlying water quality, 14-d mortality and 14-d growth inhibition after the sediment exposure phase to different concentrations of Cr (III) solution.

Cr (III) (µg/l)	Control	0.048	0.48	4.8	48	480
pH	8.3±0.20	8.3±0.05	8.5±0.08	8.2±0.12	8.3±0.13	8.3±0.05
Conductivity (mS/cm)	1.12±0.20	0.96±0.08	1.11±0.19	1.00±0.09	0.96±0.03	0.95±0.03
Salinity (%)	0.06±0.01	0.05±0.01	0.05±0.01	0.04±0.00	0.04±0.00	0.04±0.00
14-d mortality%	6.7±8.2	21.7±14.7	20.0±6.32	25.0±15.2*	25.0±5.5*	20.0±0.00
14-d body length (µm)	1230±93.7	1191±100	1107±146	1176±72	1214±47	1202±131
Growth Inhibition	0.0±9.0	3.7±9.6	11.8±13.9	5.2±6.9	1.5±4.5	2.7±12.6

Values indicate the mean ± standard deviation.***** $p<0.05$ in Dunnett's test

Table A2. Overlying water quality, 14-d mortality and 14-d growth inhibition after the sediment exposure phase to different concentrations of Cr (VI) solution.

Cr(VI) (µg/l)	Control	0.11	1.1	11	105	1050
pH	8.3±0.20	8.5±0.00	8.4±0.12	8.4±0.11	8.4±0.00	8.3±0.07
Conductivity (mS/cm)	1.12±0.20	1.05±0.01	1.05±0.07	1.04±0.06	1.02±0.01	0.98±0.08
Salinity (%)	0.06±0.01	0.05±0.01	0.05±0.00	0.05±0.00	0.05±0.01	0.05±0.00
14-d mortality%	6.7±8.2	18.3±13.3	30.0±15.5*	25.0±12.2*	21.7±9.8	26.7±8.2*
14-d body length (µm)	1230±93.7	1221±32	1153±75	1174±67	1134±89	1047±107*
Growth Inhibition	0.0±9.0	0.9±3.1	7.4±7.2	5.4±6.4	9.2±8.5	17.5±10.2*

Values indicate the mean ± standard deviation.***** $p<0.05$ in Dunnett's test

Table A3. Overlying water quality, 14-d mortality and 14-d growth inhibition after the sediment exposure phase to different concentrations of Zn solution.

Zn (µg/l)	Control	0.26	2.6	26	255	2550
pH	8.2±0.10	8.6±0.07	8.5±0.07	8.3±0.00	8.6±0.07	8.5±0.00
Conductivity (mS/cm)	1.30±0.13	1.26±0.13	1.29±0.13	1.30±0.09	1.53±0.11	1.38±0.03
Salinity (%)	0.07±0.01	0.06±0.00	0.07±0.01	0.06±0.00	0.08±0.01	0.06±0.01
14-d mortality%	8.3±4.1	66.7±16.3*	86.7±5.2*	86.7±12.1*	81.7±18.3*	63.3±26.6*
14-d body length (µm)	1247±36.3	1157±86	1161±66	1164±77	1107±153*	1129±126*
Growth Inhibition	0.0±3.4	8.4±8.1	8.1±6.2	7.8±7.2	13.1±14.4*	11.1±11.8*

Table A4. Life history and reproduction characteristics of ostracod under control condition and after the exposure to the different concentrations of chromium (III)

Parameters	Control		Exposure concentrations (µg Cr(III)/l)									
			0.048		0.48		4.8		48		480	
	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals
N (ostracod tested)	60	38	60	6	60	25	60	12	60	18	60	27
Egg-laying ratio (%) (n_w=6)	63.3±15.1		10±12.7*		41.7±27.1		20±11*		30±21*		45±19.7	
Life span (day) mean±SD [range]	22.8±1.8 [21-26] (nw=6)	24.2±2.4 [21-27] (nw=6)	19.0±1.8* [17-21] (nw=6)	27.9±4.2 [25-33] (nw=3)	20.5±2.7 [16-24] (nw=6)	24.8.6±0.8 [24-26] (nw=5)	19.5±1.6* [17-22] (nw=6)	24.3±1.5 [22-26] (nw=5)	20.3±2.1 [17-23] (nw=6)	26.3±1.8 [23-28] (nw=6)	21.8±1.3 [20-23] (nw=6)	26.9±2.4 [24-31] (nw=6)
	22.8±5.2 [14-38] (n=60)	24.4±5.3 [14-38] (n=38)	19.0±5.1* [14-38] (n=60)	29.0±6.3 [24-38] (n=6)	20.5±4.9 [14-32] (n=60)	24.9±2.6 [20-32] (n=25)	19.5±4.4* [14-28] (n=60)	24.3±2.5 [20-28] (n=12)	20.3±5.4* [14-38] (n=60)	26.2±3.7 [20-38] (n=18)	21.8±6.0 [14-40] (n=60)	26.7±4.3 [22-40] (n=27)
First day of brooding (day) mean±SD [range]	-	21.4±0.9 [20-23] (nw=6)	-	23.3±1.2* [22-24] (nw=3)	-	22.4±0.8 [22-24] (nw=5)	-	21.8±0.8 [21-23] (nw=5)	-	22.4±1.3 [21-24] (nw=6)	-	22.1±0.8 [21-23] (nw=6)
		21.4±2.2 [18-26] (n=38)		23.0±1.1 [22-24] (n=6)		22.6±2.1 [20-28] (n=25)		21.8±1.3 [20-24] (n=12)		22.0±1.5 [20-24] (n=18)		21.9±1.7 [24-26] (n=27)
Mean day of egg production mean±SD [range]	-	22.8±1.9 [21-25] (nw=6)	-	25.2±1.8 [24-28] (nw=3)	-	22.9±0.9 [22-24] (nw=5)	-	22.4±1.3 [21-24] (nw=5)	-	23.8±1.4 [22-26] (nw=6)	-	23.3±1.1 [22-25] (nw=6)
		23.0±3.3 [18-31] (n=38)		25.7±3.4 [22-31] (n=6)		23.1±2.2 [20-29] (n=25)		22.4±1.7 [20-26] (n=12)		23.5±2.6 [20-31] (n=18)		23.1±2.6 [20-31] (n=27)
Total eggs produced (egg)	645		216		265		133		249		341	
Lifetime egg production (egg/ind.) mean±SD [range]	10.8±8.4 [1-23] (nw=6)	15.9±10.8 [3-32] (nw=6)	3.6±8.2 [0-20] (nw=6)	24.9±36.8 [1-67] (nw=3)	4.4±4.2 [0-12] (nw=6)	9.5±4.3 [5-16] (nw=5)	2.2±2.4 [0-6] (nw=6)	10.9±8.8 [2-21] (nw=5)	4.2±3.9 [0-11] (nw=6)	13.0±5.5 [6-19] (nw=6)	5.7±3.3 [2-10] (nw=6)	12.6±6.2 [8-24] (nw=6)
	10.8±21.2 [0-109] (n=60)	17.0±24.7 [1-109] (n=38)	3.6±17.8* [0-126] (n=60)	36.0±48.4 [1-126] (n=6)	4.4±9.4* [0-50] (n=60)	10.6±12.2 [1-50] (n=25)	2.2±6.6* [0-37] (n=60)	11.1±11.2 [1-37] (n=12)	4.2±9.7* [0-63] (n=60)	13.8±13.5 [4-63] (n=18)	5.7±11.1 [0-71] (n=60)	12.6±13.7 [1-71] (n=27)
Hatching ratio (%)	14.1		15.7		5.3**		12.8		8.4**		4.7**	
F2 generation rate	1.52		0.57		0.23		0.28		0.35		0.27	

Values indicate the mean ± standard deviation.* p<0.05 in Dunnett' test, ** p<0.05 in Chi-square' test, n= calculated based on individual basis, nw=number of well (10 individual each) those endpoint were evaluated based on well replicates, not individual basis.

Table A5. Life history and reproduction characteristics of ostracod under control condition and after the exposure to the different concentrations of chromium (VI)

Parameters	Control		Exposure concentrations (µg Cr(VI)/l)									
			0.11		1.1		11		105		1050	
	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals
N (ostracod tested)	60	38	60	9	60	16	60	14	60	24	60	24
Egg-laying ratio (%) (nw=6)	63.3±15.1		15±16.4*		26.7±26.6*		23.3±17.5*		40±25.3		40±14.1	
Life span (day) mean±SD [range]	22.8±1.8 [21-26] (nw=6)	24.2±2.4 [21-27] (nw=6)	18.5±2.8 [16-24] (nw=6)	22.6±4.3 [18-27] (nw=4)	19.4±4.0 [16-26] (nw=6)	26.3±4.2 [20-29] (nw=4)	18.9±2.1 [17-22] (nw=6)	25.5±4.9[18-32] (nw=6)	21.4±3.8 [17-28] (nw=6)	27.5±3.3 [22-31] (nw=6)	20.4±1.6 [18-23] (nw=6)	27.2±2.3 [24-30] (nw=6)
	22.8±5.2 [14-38] (n=60)	24.4±5.3 [14-38] (n=38)	18.5±4.2* [14-36] (n=60)	24.7±5.7 [18-36] (n=9)	19.4±6.1* [14-42] (n=60)	27.4±5.8 [18-42] (n=16)	18.9±4.9* [14-32] (n=60)	26.0±3.6 [18-32] (n=14)	21.4±7.3 [14-38] (n=60)	29.0±5.0* [22-38] (n=24)	20.4±6.5 [14-42] (n=60)	26.8±5.3 [18-42] (n=24)
First day of brooding (day) mean±SD [range]	-	21.4±0.9 [20-23] (nw=6)	-	20.4±2.1 [18-23] (nw=4)	-	21.6±1.3 [20-23] (nw=4)	-	21.7±1.9 [18-23] (nw=6)	-	21.9±1.0 [21-24] (nw=6)	-	21.7±0.5 [21-23] (nw=6)
		21.4±2.2 [18-26] (n=38)		21.3±2.2 [18-26] (n=9)		21.9±1.7 [18-26] (n=16)		22.1±1.8 [18-24] (n=14)		21.8±1.6 [20-26] (n=24)		21.8±1.6 [18-26] (n=24)
Mean day of egg production mean±SD [range]	-	22.8±1.9 [21-25] (nw=6)	-	21.2±2.7 [18-24] (nw=4)	-	23.2±2.2 [20-25] (nw=4)	-	23.1±2.7 [18-25] (nw=6)	-	24.2±1.9 [22-27] (nw=6)	-	23.6±1.2 [22-25] (nw=6)
		23.0±3.3 [18-31] (n=38)		22.5±3.1 [18-27] (n=9)		23.9±2.5 [18-29] (n=16)		23.9±2.4 [18-27] (n=14)		24.8±2.4 [22-30] (n=24)		23.4±2.7 [18-30] (n=24)
Total eggs produced (egg)	645		189		548		268		860		734	
Lifetime egg production (egg/ind.) mean±SD [range]	10.8±8.4 [1-23] (nw=6)	15.9±10.8 [3-32] (nw=6)	3.2±5.5 [0-14] (nw=6)	15.8±19.9 [1-44] (nw=4)	9.1±11.3 [0-29] (nw=6)	30.0±16.5 [7-42] (nw=4)	4.5±4.0 [0-9] (nw=6)	17.2±12.9 [2-38] (nw=6)	14.3±15.6 [1-43] (nw=6)	27.2±17.4 [7-54] (nw=6)	12.2±5.8 [6-23] (nw=6)	32.1±13.1 [16-53] (nw=6)
	10.8±21.2 [0-109] (n=60)	17.0±24.7 [1-109] (n=38)	3.2±15.6 [0-116] (n=60)	21.0±37.0 [1-116] (n=9)	9.1±22.2 [0-101] (n=60)	34.3±32.1 [1-101] (n=16)	4.5±10.4 [0-40] (n=60)	19.1±13.7 [2-40] (n=14)	14.3±28.0 [0-155] (n=60)	35.8±34.7 [5-155] (n=24)	12.2±22.0 [0-112] (n=60)	30.6±25.7 [2-112] (n=24)
Hatching ratio (%)	14.1		3.7**		14.8		23.1**		16.6**		12.5**	
F2 generation rate	1.52		0.12		1.35		1.03		2.38		1.53	

Values indicate the mean ± standard deviation.* $p<0.05$ in Dunnett' test, ** $p<0.05$ in Chi-square' test, n= calculated based on individual basis, nw=number of well (10 individual each) those endpoint were evaluated based on well replicates, not individual basis.

Table A6. Life history characteristics of ostracod under control condition and after the exposure to the different concentrations of zinc

Parameters	Control		Exposure concentrations (µg Zn/l)									
			0.26		2.6		26		255		2550	
	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals
n **(ostracod tested)**	60	45	60	11	60	6	60	8	60	9	60	13
Egg-laying ratio (%) (nw=6)	75±18.7		18.3±18.3*		10.0±6.3*		13.3±12.1*		15.0±18.7*		21.7±19.4*	
Life span (day) mean±SD [range]	23.8±2.7 [21-28] (nw=6)	25.8±2.1 [22-28] (nw=6)	17.3±2.7* [15-22] (nw=6)	27.1±3.4 [24-32] (nw=4)	15.3±0.6 * [14-16] (nw=6)	26.2±3.5 [23-32] (nw=5)	15.2±1.2* [14-17] (nw=6)	23.0±1.2 [22-24] (nw=4)	15.7±1.7* [14-19] (nw=6)	23.6±2.4 [22-27] (nw=4)	16.3±1.8* [15-20] (nw=6)	20.9±2.6* [18-24] (nw=6)
First day of brooding (day) mean±SD [range]	—	19.5±0.5 [19-20] (nw=6)	—	19.3±1.6 [18-22] (nw=4)	—	21.0±1.0 [20-22] (nw=5)	—	19.3±1.0 [18-20] (nw=4)	—	18.5±1.0 [18-20] (nw=4)	—	18.4±0.5 [18-19] (nw=6)
Mean day of egg production mean±SD [range]	—	21.7±1.1 [20-23] (nw=6)	—	22.4±2.2 [20-25] (nw=4)	—	22.3±1.5 [21-25] (nw=5)	—	20.4±1.0 [19-22] (nw=4)	—	20.4±1.1 [19-22] (nw=4)	—	19.0±1.0* [18-20] (nw=5)
Total eggs produced (egg)	1796		597		146		234		327		170	
Lifetime egg production (egg/ind.) mean±SD [range]	29.9±15.8 [13-53] (nw=6)	36.9±12.4 [26-53] (nw=6)	11.9±11.5* [1-26] (nw=5)	38.5±24.1 [6-65] (nw=5)	2.9±2.3* [1-7] (nw=5)	27.8±24.6 [7-66] (nw=5)	5.9±4.8* [2-13] (nw=4)	26.8±12.1 [14-42] (nw=4)	8.2±8.2* [1-18] (nw=4)	29.5±22.1 [6-57] (nw=4)	2.8±3.2* [1-9] (nw=6)	11.0±5.2 [6-20] (nw=6)
Hatching ratio (%)	17		23.6**		8.2**		26.9**		16.2		35.9**	
F2 generation rate	5.08		2.35		0.20		1.05		0.88		1.02	

Values indicate the mean ± standard deviation.* $p<0.05$ in Dunnett' test, ** $p<0.05$ in Chi-square' test, n= calculated based on individual basis, nw=number of well (10 individual each) those endpoint were evaluated based on well replicates, not individual basis.

APPENDIX B

SAMPLING AND SAMPLE DETAILS OF URBAN ROAD DUST

Road dust (<2000 μm) used in this study was collected from road side in Tokyo in 2012 (described in Khanal et al, 2014 as sample#St.6). Samples were sieved through 2000 μm nylon mesh. Then, first air dried at room temperature before freeze drying and stored in refrigerator till analysis. The dried samples were then sieved from respective cut of size (250 μm and 63 μm). The <2000 μm fraction defined as the total fraction, which represents the portion carried away by road runoff (Watanabe et al., 2011; Khanal et al., 2014)**,** 250 to 63 μm cut off size represents the dominant fraction in road runoff (Aryal et al.,2005) and 63 μm is the fraction separating the sand (>63 μm) and fine fraction (silt and clay, <63 μm) (Tsakovski et al., 2011) represents the dominant fraction contained the pollutants carried from runoff suspension in sediment (De-Groot et al., 1982). Table B1shows the sampling details and Table B2 shows the sample details.

Table. B1 Sampling details of the urban road dust (Khanal et al., 2014).

Sample Collector	Highway sweeping vehicles
Road Type	Urban highway
Location	Tama river to Chidori cho
Coordinates	N35° 31’01”44, E 139° 47’ 30’’11 N35° 40’23”00, E 139° 55’ 38’’22
Sampling Length	30 km
Sampling Date	September 6, 2012
Antecedent dry weather period[a]	64 hour/(0.5mm)
Vehicles frequency [b]	64 - 171 × 10^3 vehicles/day

[a] Japan Weather Association, 2013.
[b] Ministry of Land, Infrastructure, Transport and Tourism, Japan, 2013.
The figure in parentheses indicates rainfall (mm) in the last 24 h

Table B2 Sample details of the urban road dust (Khanal et al., 2014).

Particle size distribution (by weight)	**Organic matter content (as ignition loss)**	**Heavy metal concentration (mg/kg dry wt.)**	**PAH concentration (mg/kg dry wt.)**
250–2000 µm:98.8% 63–250µm:0.8% <63 µm:0.4%	4.1%	Cr:51 Ni:28 Cu:74 Zn:264 Cd:0.33 Pb:11	PHE:0.14 ANT:0.03 FLT:0.07 PYR:0.11 BaA:0.02 CHR:0.05 BbF:0.17 BkF:0.04 BaP:0.05 IPY:0.10 DBA:0.02 BPE:0.15 **∑12-PAHs:1.0**

PHE: phenanthrene, ANT:anthracene, FLT: Fluoranthene, PYR: pyrene, BaA:Benzo(a)anthracene, CHR:chrysene, BbF:Benzo(b)fluoranthene, BkF:Benzo(k)fluoranthene, BaP: Benzo(a)pyrene, IPY:Indeno(1,2,3-c,d)pyrene, DBA: Dibenzene(a,h) antracene, BPE: Benzo(g,h,i)perylene.

APPENDIX C

Table C1. Life history characteristics of ostracod under control condition and after the exposure to the different concentrations of road dust.

Parameters	RF_1		RF_2		3.125%RD		6.25%RD		12.5%RD		25% RD	50% RD
	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	Egg-laying individuals	All individuals	All individuals
n (number of ostracod)	80	55	80	48	80	43	80	36	80	36	80	80
Egg-laying ratio (%)	68.8±23.0		60.0±11.2		54.0±22.6		45.0±25.6		45.0±29.8		0	0
Life span (day) [range]	25.9±2.8 [20-30]	30.2±3.2 [25-35]	27.4±2.0 [24-30]	32.3±3.1 [26-36]	25.9 ± 3.9 [21-31]	31.6 ± 3.3 [27-38]	24.2±4.1 [19-28]	31.0±2.5 [29-36]	24.5±4.0 [19-30]	32.5±1.8 [30-35]	14.1±0.1	14.5±1.5
First day of brooding (day) [range]	-	22.1±0.9 [21-23]	-	25.5±1.1 [24-27]	-	22.5±1.8 [20-26]	-	**24.2±2.1**[a*] [23-28]	-	25.2±1.6 [23-28]	-	-
Mean day of egg production (day) [range]	-	24.6±1.8 [23-28]	-	27.8±1.4 [26-30]	-	26.0±2.5 [24-31]	-	26.6±1.4 [25-28]	-	27.6±1.2 [26-30]	-	-
Total number of eggs produced (egg)	1714		1340		1793		1264		974		-	-
Mean lifetime egg production (egg/ind.) [range]	21.4±10.8 [10-43]	31.2±29.9 [1-109]	16.8±7.5 [8-28]	27.9±19.8 [1-82]	22.4±13.4 [4-48]	41.7±37.5 [1-154]	15.8±10.8 [1-31]	35.1±29.9 [1-122]	12.2±9.80 [3-29]	27.1±21.4 [3-82]	0	0
Hatching ratio (%)	12.8		7.4		12.8		**4.4**[a**]		**16.8**[b**]		-	-
F2 generation rate	2.7		1.24		2.9		0.69		2.1		0	0

mean ± standard deviation; values in parenthesis indicate the observed range in min-max. * $p<0.05$ in Dunnett's test, ** $p<0.05$ in Chi-square test

[a] The 1st batch of RF_1 was conducted with 3.125%, 6.25%, and 50%RD sample. The 2nd batch of the test (RF_2) was conducted with 12.5% and 25%RD sample.

APPENDIX D

Effect of pre-incubation conditions on hatching success of ostracod *H. incongruens*

1. Introductions

Hatching ratio is one of the key parameters to evaluate species continuation. *H. incongruens* is known to lay both rapidly- and delayed-hatching eggs (Havel and Talbott, 1995; Rossi et al., 1996; Retrum et al., 2011; Rossi et al., 2011, 2013); hence, the remaining eggs after the hatching observation may be still viable as resting eggs which are expected to be hatched after some time. Rossi et al. (2004) reported that the hatching time of the ostracod was related to water chemistry, in particular to oxygen concentration and pH. It has also been proposed that variability in hatching might be linked to its genetic variability or to the so-called "mother effect" (i.e., age, feeding history and environmental condition of the mother) (Rossi et al., 1996). Temperature is also an important abiotic factor influencing survival, reproduction and hatching success of organisms. The various combinations effects of freezing, drying, high temperatures on the eggs of ostracod, *H. incongruens* were studied. The experiments showed that some ostracod eggs were capable of hatch despite being wet, or dried under different temperature (Angel and Hancock, 1989).

Virtually all the chronic toxicity test required the long time to conduct in addition to the hatching test. Hatching test in this study is required to do in parallel with the reproduction phase. In order to reduce these kinds of burden, it is necessary to develop a feasible procedure to determine hatching rate of the eggs produced. We presumed that it may be possible either to keep the produced eggs dry, wet or frozen (-20^{o}C) for a period of time and subsequently to hatch them in an appropriate situation. The initial idea of this study is pre-incubation the ostracod egg to preserve them and perform the hatching test in the later time.

The objectives of this chapter is to determine the hatching success of ostracod egg *H. incongruens* under different pre-incubation conditions and temperature in order to further develop feasibility procedures on hatching test in the chronic ostracod toxicity test.

2. Materials and Methods

2.1 Reagents and Test organisms

Reagents used in this chapter were reported in details in chapter 3. Standard freshwater (SFW) was commonly used for ostracod culturing and preparation for TetraMin suspension. Culture medium: green algae *Scenedesmus acutus* N-94, used as food for ostracod, were cultured by culture medium. Freshwater benthic ostracod *Heterocypris incongruens* was purchased as dormant eggs (cysts) from MicroBioTests Inc. (www.microbiotests.be). A detail of hatching of ostracod cysts prior to the toxicity test was also described in chapter 3 (section 3.3).

2.2 Effect of pre-incubation conditions on hatching response of ostracod, *H. incongruens*

To determine the hatching success of ostracod egg under different pre-incubation conditions and temperature, the ostracod neonates were exposed to reference sediment provide in the Ostracodtoxkit following the procedure in the 14d-sediment exposure phase. After the reproduction phase was started, continually counted the ostracod egg produced and separately transferred the ostracod eggs in the following four diffrent treatments (Table G1) until all ostracod used death. The produced eggs were collected and separated in different treatments, then further observed the hatching ratio for 14 days after all ostracod tested death (Table G2). The hatching ratio for each concentrations was defined as the number of hatched eggs (neonates) within 14 days divided by the total number of egg produced.

2.3 Statistical analysis

All data and results were reported with standard deviation. Chi-square analysis was applied to determine the significant difference ($p<0.05$) between samples and control using Minitab 17 software.

Table D4 Pre-incubation conditions of egg produced from ostracod

Treatment	Pre-incubation conditions	Medium	Tempt.	Photo period	Well plate
1	Control condition*	old SFW	25°C	24D	6-well microplate
2	Wet Condition	old SFW	4°C		
3	Dry Condition**	No SFW	4°C		
4	Frozen wet	old SFW	-20°C		

* hatching test procedures as described in chapter 3
** remove SFW from the eggs and kept the well plate in the desiccator for at least 1 h to confirm the dryness

Table D5 Determination the hatching success of ostracod eggs under different treatments

Treatment	Medium	Tempt.	photoperiod	Hatching determination
1	Old SFW	25°C	24D	Conducting in parallel with reproduction phase Every other day for 14 days (+2, 4, 6,..., 14 days).
2-4*	Immerse in 4 ml of new SFW			Start after all ostracod death, Every day for first three days and after that conducting every other day (total is 14 days) (+1,2,3,5,7,9, ..., 14 days)

*for frozen wet, thaw at 4°C for 24h-Dark before immerse in SFW

3. Results and discussion

3.1 Overlying water quality, culturing conditions and reproduction characteristics of ostracod

Overlying water quality, 14 d mortality, 14 d body length and reproduction characteristics of ostracod were reported in Table G3 and Table G4.

Table D6. Overlying water quality, mortality and body length of ostracod in sediment exposure test phase for 14 days (reference sediment).

Parameters	
pH	8.2±0.3
Conductivity	1±0.04
Salinity	0.04±0.01
14-d mortality (%)	4±0.5
14-d body length (µm)	996±103

Table D7. Life history and reproduction characteristics of ostracod *H. incongruens* under control conditions**.**

Parameters	Control	
	All individuals	**Egg-laying individuals**
N (ostracod tested)	100	69
Egg-laying ratio (%)	69	
Life span (day) mean±SD [range]	24.1±2.0 [19-27] (n_w=10)	25.0±1.2 [23-27] (n_w=10)
First day of brooding (day) mean±SD [range]	-	21.2±0.6 [20-22] (n_w=10)
Mean day of egg production mean±SD [range]	-	22.3±0.5 [21-23] (n_w=10)
Total eggs produced (egg)	1295	
Lifetime egg production (egg/ind.) mean±SD [range]	13.0±7.9 [3-27] (n_w=10)	18.8±15.6 [1-68] (n=69)

nw = Calculation based on well replicates

Total egg produced of ostracod was 1295 eggs and distributed into following four treatments as shown in Table G5. After that the hatching test was done to determine the hatching ratio in each treatment.

Table D8. Distribution number of eggs in each treatment

Treatment	Pre-incubation	No. of egg distributed in each day of egg production							
		Day 18	**Day 20**	**Day 22**	**Day 24**	**Day 26**	**Day 28**	**Day 30**	**Total eggs**
1	Control condition 25^{o}C	6	56	118	109	29	7	5	330
2	Wet Condition 4^{o}C	6	56	118	109	29	7	-	325
3	Dry Condition 4^{o}C	5	55	117	108	29	7	-	321
4	Frozen wet -20^{o}C	5	55	116	108	28	7	-	319

3.2 Effect of pre-incubation conditions on hatching success of ostracod *H. incongruens*

The hatching determination in the treatments 2 - 4 was performed after all ostracod death. For the treatment 4, the well plates were thaw at 4^{o}c overnight before conducting the hatching test. Observation in hatching response was done every day for first three days and after that checking in every other day. Table G6 showed the hatching ratio of ostracod under different pre-incubation conditions. Statistically significance was found in the wet 4^{o}C condition (p<0.05). Egg produced in dry 4^{o}C and WET -20 ^{o}C were not hatched during observation periods.

Table D**9** Hatching ratio of ostracod under pre-incubation conditions

Treatment	1 (Control 25^{o}C)	2 (WET 4^{o}C)	3 (DRY 4^{o}C)	4 (Frozen wet -20^{o}C)
No.of egg	330	325	321	319
Individual Hatched	62	108	0	0
Hatching rate (%)	18.8	33.2**	0	0

** p<0.05 in Chi-square test