Ecological Risk Assessment of a River Water on Agricultural Area in West Java Province, Indonesia and Comparison with Whole Effluent Toxicity Test

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Summary

This thesis comprises the ecological risk assessment in Cipeles River, West Java Province, Indonesia and its comparison to the Whole Effluent Toxicity (WET) test. The thesis was divided into 8 chapters as followed:

Chapter 1 Introduction

This chapter described the background, the objective, the study area, and the systematic of this thesis. The study area was Cipeles River, a tributary of Cimanuk River located in the agricultural area of Sumedang District, West Java Province, Indonesia.

Chapter 2 Literature review

This chapter reviewed the ecological risk assessment, Fish Embryo Toxicity (FET) test, the test that conducted in determining the toxicity level in this study, and also physicochemical toxicity such as ammonia and zinc to the aquatic environment. It also reviews the organophosphorus (ORP) pesticide, its occurrence in aquatic environment, and the toxicity level in previous studies.

Chapter 3 Determination ORP Pesticides in River Water Sample using Solid Phase Extraction and Gas Chromatographic-Mass Spectrometer

This chapter discussed the problem formulation of organic compounds that might emerged at the study area. The objective was to determine the ORP concentration in water samples from several sampling locations simultaneously using the SPE coupled with GC-MS. The recovery using multiple ORP standards was within the acceptable range and the coefficient of determination R^2 of each 13 compounds >0.98-0.99. The chlorpyrifos was detected in the water sample from upstream (St.1), city area (St.4), and downstream (St.10), at 1.19, 0.49, and 0.28$\mu g.L^{-1}$, respectively. While terbufos and thiometon were detected on the St.1 sample at 0.93$\mu g.L^{-1}$ and 0.07$\mu g.L^{-1}$, respectively.

Chapter 4 Distribution, Source Identification, and Assessment of Heavy Metal Pollution in the Surface and Pore Waters of Cipeles River, West Java, Indonesia

This chapter focused to determine the heavy metal in surface and pore water by ICP-MS for a basic database on pollution assessment using heavy metal pollution indices (HMPI). A spatial analysis using GIS also conducted here. The dominant heavy metals, Fe and Mn, had detected as the results of runoff

from the laterite and alluvial soils in the study area. The presence of other micro concentrations of heavy metals in the city area showed the anthropogenic source of non-mining activities, such as fertilizer and pesticide usage in agriculture. As a result, the heavy metal occurrence in the pore water was Mn>Fe>Ba>Co>Zn>Cu>Pb>Cr in the order of abundance, while in the surface water, i.e. Fe>Mn>Zn>Ba>Cu>Pb>Co>Cr. The high concentration of zinc in St.4 and St.8 samples contributed to their high HMPI.

Chapter 5 Lethal and Sublethal Effect on Early-life Stage of Zebrafish by Organophosphorus Phorate Exposure

This chapter highlighted the determination of acute lethal and sublethal toxicity of ORP phorate exposure using zebrafish early-life stage toxicity test on a prolonged period. This bioassay procedure would reduce the requirement of the sample volume, while the prolonged period 120-hpf, than usual 96-hpf, was to accommodate the zebrafish larvae ability to swim-up as an important stage to survive. This research revealed that increasing of phorate concentration and the exposure time (t_{exp}) statistically significant differs from the control of lethal rate and hatching rate. The probit analysis on lethal LC_{50} resulted 4.54 $mg.L^{-1}$, while the estimation of no effect concentration was found 7.67 $\mu g.L^{-1}$ at 30 days. The EC_{50} on hatching rate and swim-up failure was 9.75 $mg.L^{-1}$ and 2.14 $\mu g.L^{-1}$, respectively. By observation, the phorate concentration treatment affected the swim-up failure rate, even statistically not significant. Only t_{exp} gave a significant difference to the swim-up failure rate. So, the prolonged period 120-hpf was significant to be monitored as proposed. Rely on the result of a lethal effect, even LC_{50} endpoint was higher than the fish acute toxicity, this bioassay could be used as the previous screening to fish acute toxicity to support the 3Rs principle

Chapter 6 Whole Effluent Toxicity (WET) Test of River Water Sample using Early-life stage Zebrafish

WET test to determine the lethal and sublethal effect on salinity variation and field water sample, which implemented the previous zebrafish early-life stage bioassay, was discussed in this chapter. Salinity treatment only had a significant effect on the lethal rate when above 0.17psu. Meanwhile, salinity treatment had no significant difference to the hatching rate and swim-up failure rate. This study also revealed that zebrafish embryo could be survived on salinity as low as 0.04 psu. From the WET test, significant difference analysis showed on lethal effect from sample St.1, St.4, and St.7, whereas the hatching rate from sample St.4, St.5, and St.8. The swim-up failure rate was significantly different at the sample St.1, St.5, St.7.

Chapter 7 Ecological Risk Assessment (ERA) estimation

ERA calculation especially on detected ORP in the surface water sample was discussed in this chapter. Regarding the risk quotient (RQ) on three trophic levels of aquatic organisms, the ORPs detected in water samples put concern not only the acute lethal-sublethal effect to the local aquatic organisms that have sensitivity as early-life stage *Lepomis m.* but also the chronic effect on the lower trophic level, such as daphnids. At St.1, the RQs resulted by chlorpyrifos, i.e. on daphnid (1.45) and on adult *Lepomis m.* (0.699); by terbufos, i.e. on daphnid (2.33), on adult *Lepomis m.*(0.518), and on juvenile *Lepomis m.* (1.211). At St.4, the RQ by chlorpyrifos on daphnid is 0.595. The concern also addressed to St.7 because of ammonia (RQ =1.69), meanwhile to St. 4 (RQ = 2.04) and St.8 (RQ = 0.76) because of zinc.

Chapter 8 General Conclusion and Future Recommendation

This chapter summarized the conclusion of the various findings and their implications. The analysis result of the physicochemical parameter and ERA concerning ORP pesticide had been reflected in WET test result. The recommendations for further research also discussed in this chapter.

Table of Content

List of Figures

List of Tables

List of Abbreviation

BOD	Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
dpf	day post fertilization
EC_{50}	Effect Concentration 50
ECOTOX	ECOTOXicology knowledgebase
FET	Fish Embryo Toxicity
GC-MS	Gas chromatography-mass spectrometry
GDP	Gross Domestic Product
HMPI	Heavy metal pollution index
hpf	hour post fertilization
LC_{50}	Lethal Concentration 50
LOEL	Lowest Observed Effect Level
$\mu g.L^{-1}$	microgram per liter
$mg.L^{-1}$	milligram per liter
NOEC	No Observed Effect Concentration
NOEL	No Observed Effect Level
ORC	Organochlorine
ORP	Organophosphorus
RQ	Risk Quotient
USEPA	United State Environmental Protection Agency
WET	Whole Effluent Toxicity

Chapter 1

Introduction

1.1 Background

Agriculture contributed 13% for Indonesian GDP (The World Bank, 2017) and the government concerns to increase the agriculture production to support national domestic consumption. Paddy fields covers 70% of total harvested area among others major agricultural food product, for example maize, cassava, and sweet potatoes (Ministry of Agriculture, 2016).

To maintain the productivity, any improvement of intensification is being taken. The disturbances such as long drought, flood, pests are diminished. However, the distribution and application of pesticide is managed by several regulations.

Indonesia has stated and updated some regulations about the circulation, storage and usage of pesticide, the pesticide registration and inspection of pesticide. Use of pesticides is prohibited if the pesticide is not registered. The application of pesticide in agriculture not limited on paddy field but also other agriculture and forestry commodities. Various registered pesticides, as shown in Fig. 1.1, are used depend on the specific purpose.

On the other side, the application of pesticide has negative effect for human health or environment. Many pathways took direct or indirectly for pesticide entering the water bodies, such like spills during mixing, spraying, and/or loading of the pesticides (Isworo, Purwanto, & Sabdono, 2015), unmanned disposal, spraying against

the wind, or contaminated by run-off. Indonesia has minimum precipitation 460 – 905 mm/year and maximum 3,323 – 5,435 mm/year (2010-2017) (BPS-Statistics Indonesia, 2015),(BPS-Statistics Indonesia, 2016),(BPS-Statistics Indonesia, 2017),(BPS-Statistics Indonesia, 2019). This annual rainfall is quite high compare to other countries, which allow run-off of chemicals in the ground into water bodies easily.

In environment, pesticides may be degraded or transformed by chemical processes such reactions as photolysis (photochemical degradation), hydrolysis (reaction with water), oxidation-reduction, and also biological process, which those later contribute to river water quality by washing out. Since Indonesia have many main rivers in agricultural area, the pesticide pollution in the river potentially occurs.

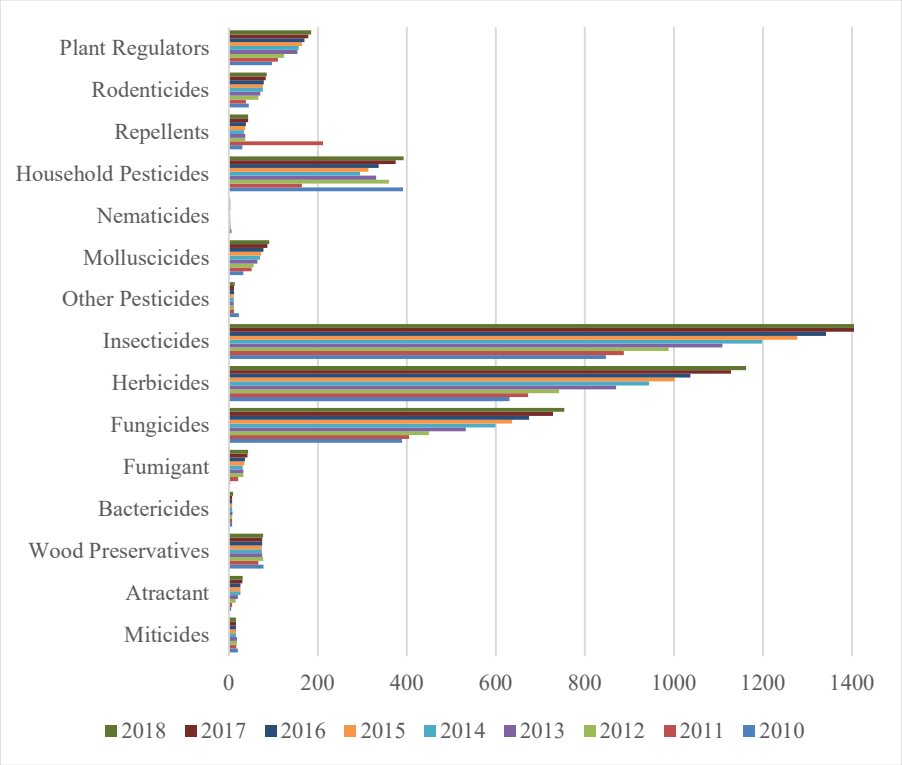

Fig. 1.1 Registered Pesticide in Indonesia (2010-2018)(Ministry of Agriculture, 2018)

In West Java Province, many main rivers flow around agricultural area, such as Cimanuk River, which its quality as reported previously was exceeded the standard (Indonesia Ministry of Public Work, 2010), especially on the organic loading (as COD and BOD), phosphate and chloride. However, there was limited study to confirm about the specific source that affects those water source qualities.

As we know, river water quality is influenced by non-point source not only from agriculture, but also domestic and industrial activities nearby. The chemical emerged from those activities later will enter the river and impact to aquatic biota as first receptor. Regarding those risks into aquatic biota, river quality should be controlled and monitored base on the fact that the downstream of the river sometime used for irrigation, fish farming, or raw water for water treatment. Further, to understand the risk on aquatic biota, the comprehensive assessment would better to be conducted than single exposure, due to accommodate various sources of pollution.

The toxicity assessment methods of aggregate effluent have been developed in previous studies (Heys, Shore, Pereira, Jones, & Martin, 2016), (Stenström, 2013). Whole Effluent Toxicity (WET) test (USEPA, 2000), or also known as Whole Effluent Assessment in European country, represent toxicity assessment of total effluent or receiving water, not only from single compound source. This study would like to apply the whole effluent toxicity test to assess the water sample from study area.

1.2 Topic Development

The WET test usually conducts the acute (USEPA, 2002a) or chronic (USEPA, 2002b) toxicity from effluent or receiving water using aquatic plant, e.g algae, aquatic

invertebrate (daphnids), or aquatic vertebrates, e.g. fish. Considering the risk assessment that commonly use the most sensitive organism, other approach has been developed, such kind of zebrafish (*Danio rerio*) embryo. This also appropriate for the study area that has a local-fish farming (Hermawan, 2010), thus put the embryo as vulnerable receptor in aquatic ecosystem of study area. The Fish Embryo Toxicity (FET) test no.236 (OECD, 2013) was conducted in WET test, to determine the toxicity level of field sample.

The agricultural product, in this study was pesticide residue toxicity that might washing out into surface water also determined through FET test. Regarding the preferences of insecticide which less persistent, more degradable in environment (Stoytcheva & Zlatev, 2011) and have shorter half-life, organophosphate (ORP) become one of the focus in this study. Other physicochemical, such as heavy metal and ammonia which usually occur in agricultural area, also become another focus in this study. The lethal and sublethal effect were determined which later calculated on ecological risk assessment for certain compound, and compared to WET test result.

1.3 Research Objective

Regarding ecological risk assessment which include the problem formulation and analysis, this research was aim (i) to determine the occurrence of ORP concentration in the water sample and (ii) to determine the heavy metal concentration in surface water and pore water. For certain ORP, this research also (iii) determine acute toxicity of phorate by FET test. The bioassay using zebrafish embryo also carried out (iv) to obtain the toxicity level of water sample using WET test. Finally, (v) the ecological risk

assessment using risk quotient (RQ) from certain compound found in the water sample was assessed and compared with WET test result.

1.4 Study Area

West Java Province, Indonesia is the second paddy rice producer in Indonesia. The paddy harvested area, 15% of total area for national scope. Study area located at Cipeles segment, the largest subsystem of Cimanuk watershed, where Cipeles River watershed covers 445,70 km^2 area (Sulaksana, Sukiyah, Sjafrudin, & Haryanto, 2013) on eight sub-districts with 108 villages and population 1.7 million people (Kementerian Pekerjaan Umum, 2010) at Sumedang and Tomo District, which geographically situated between $107^0$45'34"- $108^0$01'57" E and $06^0$45'57"- $06^0$57'59" S (Salim & Kusuma, 2006), as shown in Fig. 1.2. Along Cipeles River, land usage are divided into paddy field, residence, farm non-paddy, land, and forest (Salim & Kusuma, 2006), which 12-86% area for agriculture, whereas other land usage 45-88% as forest, residence, trade area and industry (BPS-Statistics of Sumedang Regency, 2018). Land usage of Cimanuk Cisanggarung Watershed, where the Cipeles River situated (Fig. 1.3).

Sample was collected from eleven locations, represent the upstream, city center, residential, farm, food industry and downstream, as shown in Fig. 1.4.

Fig. 1.2 Study Area, Cipeles River at Sumedang District

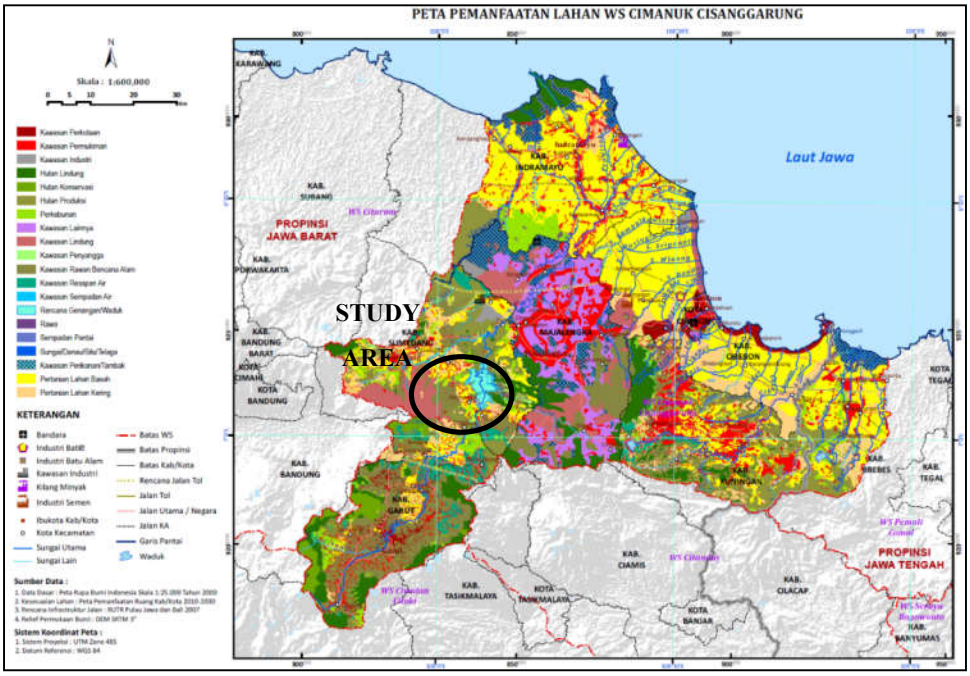

Fig. 1.3 Land Usage Cimanuk Cisanggarung Watershed (Kementerian Pekerjaan Umum, 2016)

Fig. 1.4 Situation at Sampling Location

1.4.1 Cimanuk River

Cimanuk River start the upstream from Papandayan Mountain, Garut district along ±180 km and ended in Jawa Sea, Indramayu district. Cipeles River as the tributary has 60-61 km length, located in middle area of Cimanuk catchment area (Kementerian Pekerjaan Umum, 2010), (Salim & Kusuma, 2006). Soil type in Cipeles River mostly consist of laterite/latosol (55%), especially in highland, whereas alluvial mostly found in near riverbank (Kementerian Pekerjaan Umum, 2016).

Average flowrate of Cimanuk River at downstream Sumedang-Tomo Q_{max} 429 $m^3.s^{-1}$ and Q_{min} 24.5 $m^3.s^{-1}$ (Statistical Yearbook 2010-2016).

1.4.2 Physicochemical Characteristic

In order to obtain the representative data, some physicochemical parameter is directly measured at sampling sites. Those insitu parameter of temperature, pH, and conductivity were measured using Water Quality Checker Horiba (Japan) and N-NH_3 was analyzed based on EPA 350.1 method. Sample was collected on last wet season of April 2018 (67% rainy days in a month). The measurement and analysis result were shown in Table 1.1.

Another chemical parameter, Chemical Oxygen Demand (COD), was analyzed in accredited Indonesian laboratory based on APHA5220-D (2012). The field sample was acidified by nitric acid until pH below 2 and immediately analyzed within 48 hours. Based on analysis result, COD value was varied from 5 $mg.L^{-1}$ until 46 $mg.L^{-1}$. Spatial plotted of COD was described in Fig. 1.5.

Table 1.1 Insitu parameter and ammonia concentration

	pH	Temperature, ^oC	Conductivity, $mS.cm^{-1}$	Ammonia, $mg.L^{-1}$
Upstream				
St.1, UPS	7.09	27.4	0.084	0.26
St.2, UPS	7.73	27.2	0.094	0.03
St.3, SUB	7.43	28.1	0.095	0.04
City area				
St.4, RIV	8.62	28.1	0.127	0.22
St.5, CITY	9.01	25.4	0.123	0.15
St.6, FOOD	8.14	26.2	0.088	0.09
St. 7, FARM	7.85	30.5	0.261	1.49
St.8, DOM	7.81	25.2	0.089	0.09
Downstream				
St.9, JUNCT	7.62	31.5	0.177	0.09
St.10, DOS	6.99	29.5	0.131	0.05
St.11, DOS	7.49	30.5	0.142	0.07

UPS, upstream; SUB, suburban; RIV, river; FOOD, food industry; FARM, farming; DOM, domestic; JUNCT, junction; DOS, downstream).

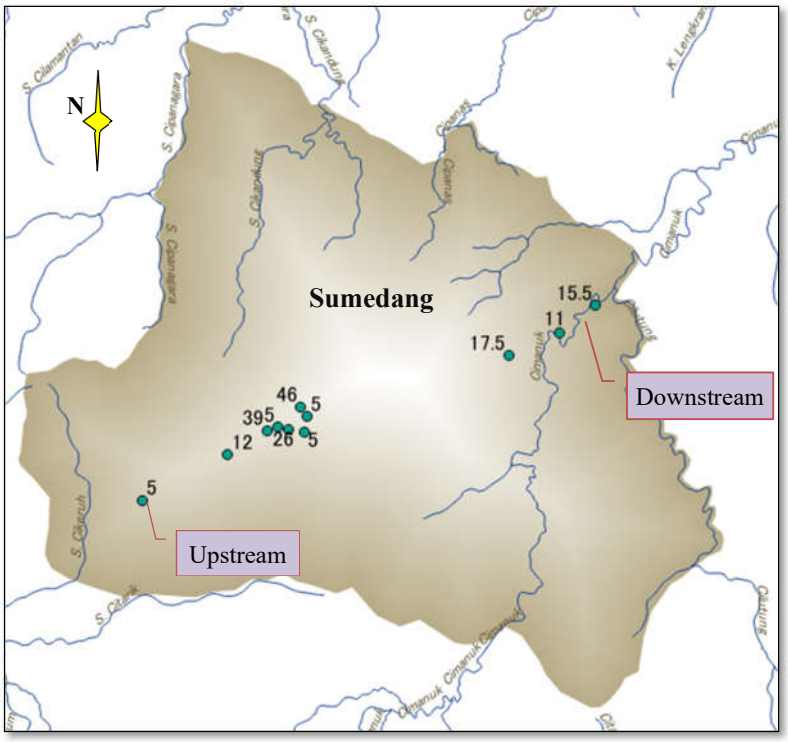

Fig. 1.5 Spatial Plot of COD at Sampling Location

1.5 Outline of Thesis

This thesis is written as the partial requirement for degree Doctor of Engineering. The dissertation consists of eight chapters.

Chapter one described the background, the objective, the study area, and the systematic of this thesis. The study area was Cipeles River, tributary of Cimanuk River that located on agricultural area of Sumedang District, West Java Province, Indonesia.

Chapter two consisted of review the ecological risk assessment, Fish Embryo Toxicity (FET) test, the test that conducted in determining the toxicity level in this study. It also reviews the ORP pesticide, its occurrence and the toxicity level in previous

studies.

Chapter three described the determination of ORP pesticides in river water sample using solid phase extraction (SPE) and gas chromatograph/mass spectrometer (GC/MS), the methods that has advantages on extracting and analysing the organic compound in water matrix.

Chapter four explained about the determination of heavy metal from the surface water sample and pore water using Induce Couple Plasma/mass spectrometer (ICP/MS). This inorganic element later might be related when evaluating the WET test result.

Chapter three and chapter four described about chemical analysis of the compounds on field samples. Meanwhile, chapter five and chapter six described about bioassay study using FET test.

Chapter five described about the application of FET test using early-stage life zebrafish on specific ORP pesticide, phorate.

Chapter six mainly discussed about WET test of river water sample using early-life stage zebrafish. The salinity effect to zebrafish embryo also conducted in this chapter.

Chapter seven explained about ecological risk assessment (ERA) of ORP and the other concerned compound that found in the sample.

Chapter eight wrapped up the important results found in this study including the comparison between WET test result and ERA, and also discussed the future recommendation.

The structure of this dissertation is summarized in Fig. 1.6.

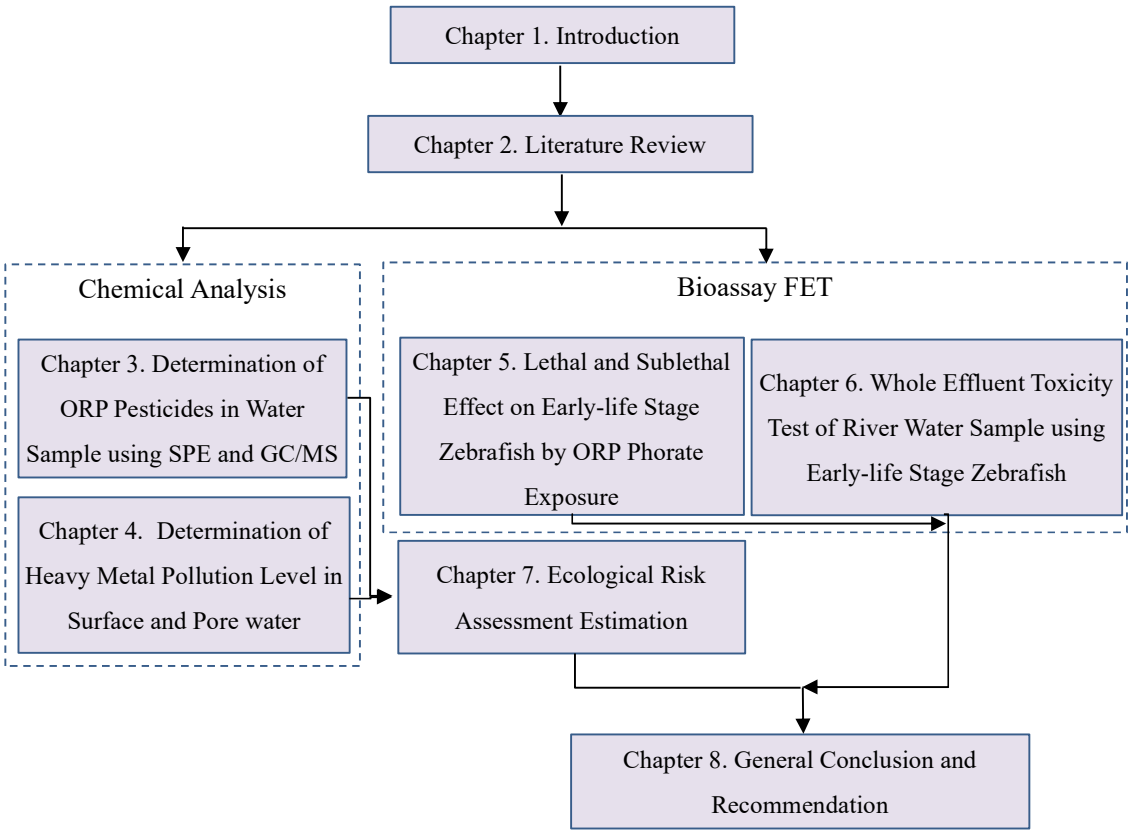

Fig. 1.6 Structure of Dissertation

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Chapter 2

Literature Review

2.1 Ecological Risk Assessment

Ecological Risk Assessment means to analyse the risk to the ecology of area in response to human activities (EPA, 2017). It evaluates the environment that receive the adverse effect as a result of exposure to one or more stressors. The framework scope includes the problem formulation or also hazard identification, analyses on characterization of exposure and characterization of ecological effects, and risk characterization (Oost, Beyer, & Vermeulen, 2003), shown in Fig. 2.1.

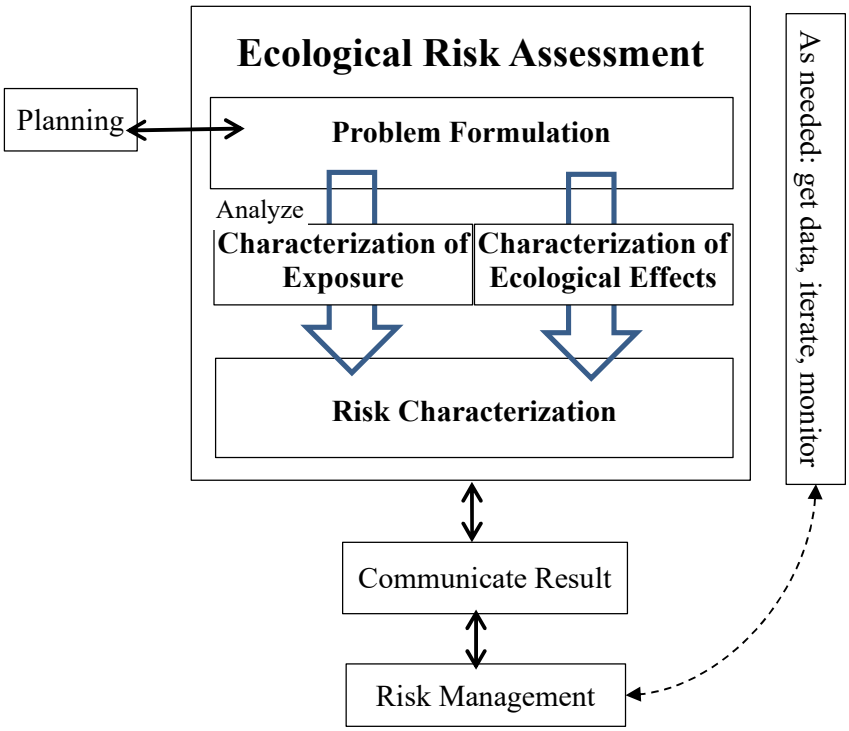

Fig. 2.1 Framework of Ecological Risk Assessment
(*www.epa.gov/risk/conducting-ecological-risk-assessment*)

The problem formulation started from good planning that includes the objective recipient, determine the compound(s) and it source, the exposure time, which endpoint should be carried out and also the route of exposure. On problem formulation it already defined the endpoint and the concerning organism that will be protected. Thus, the exposure and effect characterization are conducted on analysis before the risk characterization step. On the risk characterization, risk estimation and risk description are the common components (USEPA, 2017).

The risk estimation compares the estimated environmental concentration (EEC) which might expose to the organism with the toxicity level, which regards to the Level of Concerns (LOC). The calculation of risk quotient uses the equation Eq. **2.1**.

$$RQ = \frac{estimated\ concentration\ of\ exposure}{LC_{50} or\ EC_{50}} \qquad \text{Eq. 2.1}$$

For aquatic organism, the LOC categorized into four class, which indicates the concern at different endpoint (acute or chronic) and also by the specific character species or the character of targeted compounds, as shown in Table 2.1. The RQ result is analyzed on the risk description to interpret the concern should be made.

Table 2.1 Risk presumption for Aquatic Animals (USEPA, 2017)

Risk presumption	RQ	LOC
Acute High Risk	EEC / LC_{50} or EC_{50}	0.5
Acute Restricted Use	EEC / LC_{50} or EC_{50}	1.0
Acute Endangered Species	EEC / LC_{50} or EC_{50}	0.05
Chronic Risk	EEC / NOAEC	1.0

2.2 Pesticide Residue in Water Bodies

Indonesia, one country that has two seasons throughout the year i.e. dry season and wet season, has the annual rainfall minimum 460 – 905 mm/year and maximum 3,323 – 5,435 mm/year (2010-2017) (BPS-Statistics Indonesia, 2017),(BPS-Statistics Indonesia, 2019). On the other side, pesticide trade in Indonesia showed significant increase (Mariyono et al., 2018) despite to support the agricultural sector nationwide.

In the agricultural area, the important parameter to be considered from high annual rainfall is the runoff that allows the residue of agricultural products, including pesticide, entering the water body. Although some pesticides e.g. organochlorine has been banned by the government (Rahmawati et al., 2013), but the occurrence of pesticide residue that revealed in previous studies (Rahmianna et al., 2015) should be considered. To prevent the harvest failure, local farmers still allowed to use the registered pesticide (Ministry of Agriculture Republic of Indonesia, 2016) that easily buy at agricultural stores or cooperation. Carbofuran, a carbamate pesticide with wide spectrum to control insects, and chlorpyrifos, an organophosphate insecticide, acaricide and miticide used primarily to control foliage and soil-borne insect pests on a variety of food and feed crops are just two examples of it (EPA, n.d).

Chemical-specific properties influence the solubility of pesticides. This property such as the octanol-water partition coefficient (K_{ow}) may describe the behaviour in water environment, where high K_{ow} represent that the compound prefers to less soluble in water, or hydrophobic(Oost et al., 2003). K_{ow} or sometimes expressed in logarithmic scale, log K_{ow}, also common to estimate other properties. Some examples of K_{ow} or log K_{ow} was listed in Table 2.2. Pesticides that easy dissolve in water are

considered to be highly soluble. These chemicals tend to percolate into the soil to groundwater or washed-out as surface water by runoff from the rainfall. While pesticides with high vapor pressures are easily evaporate to the atmosphere.

Table 2.2 Octanol-water partition coefficient of selected organophosphorus pesticide

Pesticide	MW, $g.mol^{-1}$	Log K_{ow} [a)]
Phorate	260	3.925 [b)]
Dimethoate	229	0.699 [b)]
Terbufos	288	4.518 [b)]
Chlorpyrifos	350	4.699 [b)]
Fenthion (MPP)	278	4.091 [b)]
Methidathion (DMTP)	302	4.724 [b)]
Butamifos	332	4.62 [a)]
Prothiofos	345	5.67 [a)]

[a)] PubChem .https://pubchem.ncbi.nlm.nih.gov, [b)](edited by Michael A.Kamrin, 2000)

Pesticides may be adsorbed into soil particles, particularly the clays and soil organic matter. Differ from highly soluble pesticide, it tends to bound to the soil or the sediment (National Research Council, 1993).

2.3 Organophosphorus Toxicity Risk to Aquatic Organism

Various exposures potentially pollute the surface water and later affect the aquatic organisms. Agricultural product, domestic and surrounding human activities might end up in the receiving surface water. These chemicals are known to cause acute lethal or sublethal effect either in their abundance, morphology or behaviour on aquatic biota. If the exposure last in long term period, it eventually results the chronic risk.

Organophosphorus (ORP) pesticide that has been widely used in agricultural activity is known more toxic that previously organochlorine (ORC) (edited by Michael A.Kamrin, 2000). The activity as acetylcholinesterase inhibitor effectively interfere the nerve system, do the low-level exposure might be harmful. For some cases, it damages immune systems.

2.4 Physicochemical Parameter in Surface Water

In collecting the water sample from the field, some standard procedures should be complied in order to get the representative sample. Several physicochemical must be measured insitu, i.e. pH, temperature, dissolved oxygen (DO), conductivity and also salinity. Preservative method also important to notice to prevent the loss of target analyte, especially if the distance of sampling site and analysis laboratory quite far. Related to the objective of study, the selection of physicochemical properties to be analyzed is important to get the description of the sample. For surface water, national or local stream standard usually become the reference, depend on proper class.

For agricultural area, stream standard parameter is not as much as parameter for raw water of water treatment. Organic compound analysis, nitrogen and phosphorus compound analysis are common requirement.

Nitrate, nitrite and ammonium are major nitrogen parameter, as the agricultural product contained. Ammonia in urea fertilizer is oxidized by autotrophic nitrifying bacteria, thus the nitrite is oxidized by nitrifying bacteria to nitrate, following the equation Eq. 2.2 (Sawyer et al., 2003). The excess could be carried out by the rainfall and enter the groundwater by percolation or surface water by runoff. Nitrate in certain

level could be harmful for aquatic organism, but nitrite is more. Water containing nitrate which consume by human could convert into nitrite. Later, the interaction of nitrite with hemoglobin in human blood result methemoglobinemia, where blood cannot carry sufficient oxygen. Indonesian water quality standard for agricultural and fishery purposes mentioned the maximum nitrite, nitrate and ammonium concentration are 0.06, 20, and 0.02 mg.L^{-1} respectively. About acute criterion for ammonia in water, USEPA (2013) recommended 17 mg total ammonia nitrogen (TAN) per liter for 1-hour duration, meanwhile acutely toxicity of ammonia to 29 freshwater fish species was reported between range 0.88 to 4.6 mg.L^{-1} (Australian Government Initiative, 2000). High concentration of ammonia in water imposed difficulty for aquatic organisms to excrete it, thus affected the internal tissues and blood, and potentially death (USEPA, 2013).

$$2NH_3 + 3O_2 \xrightarrow{Nitrosomonas} 2NO_2^- + 2H^+ + 2H_20$$

$$2NO_2^- + 3O_2 \xrightarrow{Nitrobacter} 2NO_3^- \qquad \textbf{Eq. 2.2}$$

Inorganic heavy metal also contained in fertilizer in macro or micronutrient (Anonim, 2011). More attention is carried out when the washed-out residue enters the water body thus potentially polluting it. In their report, WHO mentioned the detected zinc in natural surface water was below 0.01 mg.L^{-1} while in groundwater 0.01-0.04 mg.L^{-1}(WHO, 1996). Especially for zinc, Japan standard stated in the environmental quality standard for preservation of living environment must below 0.03 mg.L^{-1}. Study by Li (2019) mentioned the short term hazardous concentration Zn at pH 7 was 230.6 µg.L^{-1}. While the delaying on hatching was shown when zebrafish exposed to Zn(II) at 0.47 mg.L^{-1}(Küçükoğlu et al., 2013). Zinc toxicosis which symptom such as weakness,

weight loss, anemia, diarrhea in mammal has reported as result of over consumption of zinc(WHO, 1996).

2.5 Determination of Pesticide in Water

The detection of ORP compounds in the environmental water matrix becomes the challenge in research, mainly caused by their short half-life and easy to degrade (edited by Michael A.Kamrin, 2000). Solid phase extraction (SPE) has been shown the good result on preconcentration method of ORPs from water sample (Barceló & Alpendurada, 1996), (Picó, Fernández, Ruiz, & Font, 2007), (Ruiz-Gil, Romero-González, Garrido Frenich, & Martínez Vidal, 2008). The preconcentration was using the C_{18} cartridge that suitable for many matrices (Ma et al., 2009). As the separation and detection method, gas chromatographic-mass spectrometer (GC-MS) has proved the good result (Ma et al., 2009), (Li, Gan, Peng, Huang, & Yu, 2010), (Vijaya Bhaskar Reddy et al., 2016).

SPE was introduced on extraction and clean-up the compound from water matrix on mid-1970 (Sabik, Jeannot, & Rondeau, 2000), especially to eliminate the chemical consumption in previous liquid-liquid extraction. The principle is to select the target compound to attach to the sorbent material, clean up the matrix and impurities, then elute the target compound using proper solvents. SPE sorbent selection become very important consideration to get the exact target compound, whether sample matrix more aqueous or organic, more soluble in organic solvent or in water, thus whether the target analyte neutral or charged (-, 1998). Several types of SPE, i.e. normal phase, reversed phase and ion exchange, have specific allocation. Reversed phase separates the polar or moderately polar sample matrix and a nonpolar stationary phase, which target

compound usually nonpolar(-, 1998). For pesticides, silica bond-18 type is commonly used to extract from water matrix (Picó et al., 2007). Simple five steps to extract the target compound using SPE is shown in Fig. 2.2.

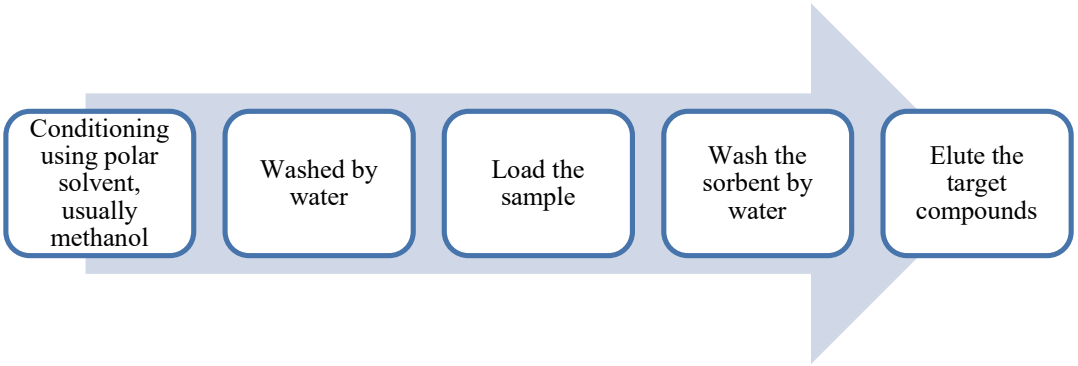

Fig. 2.2 Step of SPE extraction

Quality assurance in the compound extraction using SPE must be established to ensure the laboratory and method performance, e.g. the recovery rate between 70-130% should be made (USEPA, 2007). Some factors will influence the recovery, i.e. sample pretreatment, flowrate of loading and elution, and manual or automation of SPE. Flowrate of 4-5 $mL.min^{-1}$ is a standard value that usually take otherwise drop-by-drop. Sample pretreatment such as filtering or centrifuging the samples to reduce the interference by solid particle, or pH adjustment were crucial step prior to extraction.

2.6 Fish Embryo Toxicity

Fish Embryo Toxicity (FET) test, proposed on year 2006 (Braunbeck & Lammer, 2006) and published by OECD as the guidelines for chemical toxicity testing using zebrafish (*Danio rerio*) embryo (OECD, 2013). This test is conducted to support the principle on minimizing the pain of acute test organism, or known 3Rs principle (Replace, Reduce, Refine) which have shown good correlation with the acute fish

toxicity test on more than a hundred chemical substances (Lammer et al., 2009). This FET test was carried out using less chemical volume only 2 mL on each concentration, so less consume of budget and also minimize the chemical waste. Zebrafish embryo usage also another advantage which has shorter early-life stage compare to other organism that usually use for toxicity test. Zebrafish hatches in first 48-hour post fertilization (hpf) (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995), meanwhile nile tilapia 120-hpf and Japanese medaka in 9dpf (Iwamatsu, 2004). The clear embryo of zebrafish (*Danio rerio*) also help the observation of stage development.

2.6.1 Culturing Zebrafish

In order to produce the good embryo, zebrafish (*Danio rerio*) should be maintain under certain condition. The culture water should be chlorine-free, in temperature range between 27$\pm$1 ^{o}C and pH between 6.0-8.0 (OECD, 2013). The tap water could be used but pretreatment should be conducted, by tiered filter through 120-micron filter pad, carbon filter, biological filter, pH adjustment and UV light on recirculation system (Avdesh et al., 2012). Water conductivity maintained between 0.5$\pm$0.02 mS.cm^{-1} to keep their normal osmoregularity. The system water is static flow following by small drop-by-drop flow on 200-220 mL.min^{-1}. The photoperiod is kept in 14 hours light and 10 hours dark. The zebrafish were fed twice a day using dry flakes food and once a day using brine shrimp (*Artemia sp.)*.

2.6.2 Zebrafish Breeding

For breeding, the plastic mess wire was put before off set, in order to avoid the adult fish eat the egg produced. The fertilization between mature female and male

(ratio 1:2), Fig. 2.3 , usually occurs during first 30minutes onset light period.

Normal embryo will develop and hatched in 48-72 hours into larvae, and begin to takes the vertical position to the water surface. This 96-hpf position will inflate the swim bladder that will help the larvae to swim (Lindsey, Smith, & Croll, 2010). Thus in 120 hours, the normal larvae usually swim on the water surface.

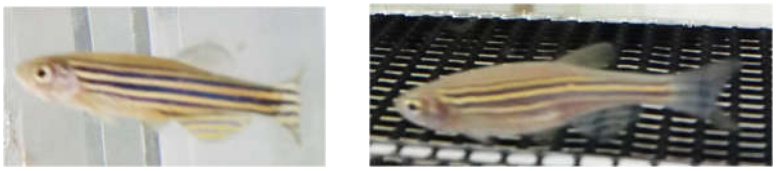

Fig. 2.3 Adult male (left) and female (right) zebrafish between 10-12months old

Zebrafish early-life stage, from embryo until larvae, was easy observed using microscope 20x. The lethal effect showed on milky appearance embryo, or dark under microscope, lack of somite, tail non-detachment from the somite, or lack of heartbeat. The normal heartbeat usually between 70-80 beats.min^{-1} on first 24-hpf, on faster until 160 beat.min^{-1} on 48-hpf. The other normal development in first 24-hpf was shown by success delivering process from cleavage, blastula until gastrula phase. On next 24-hpf the somite starts to detach, spontaneous movement, pigment and eye bud clearly appear, fin already formed and blood circulation start to flow. Between 48-hpf until 72-hpf, embryo usually begin to hatch from its chorion, depend on the temperature of incubation (Villamizar, Ribas, Piferrer, Vera, & Sánchez-Vázquez, 2012). Straight body shape was shown after dechorionated and the larvae still posed on bottom of chamber. During 72-hpf, the eye movement and fin flap were observed. Since the movement ability has developed at 96-hpf, the larvae tend to move horizontally until meet the vertical obstruct such as wall of test chamber. Further, the larvae tend to take the vertical

position to reach the water surface, and at 120-hpf successful larvae would inflate the swim bladder and swim freely. The observed development of early-life stage of zebrafish is shown in Fig. 2.4.

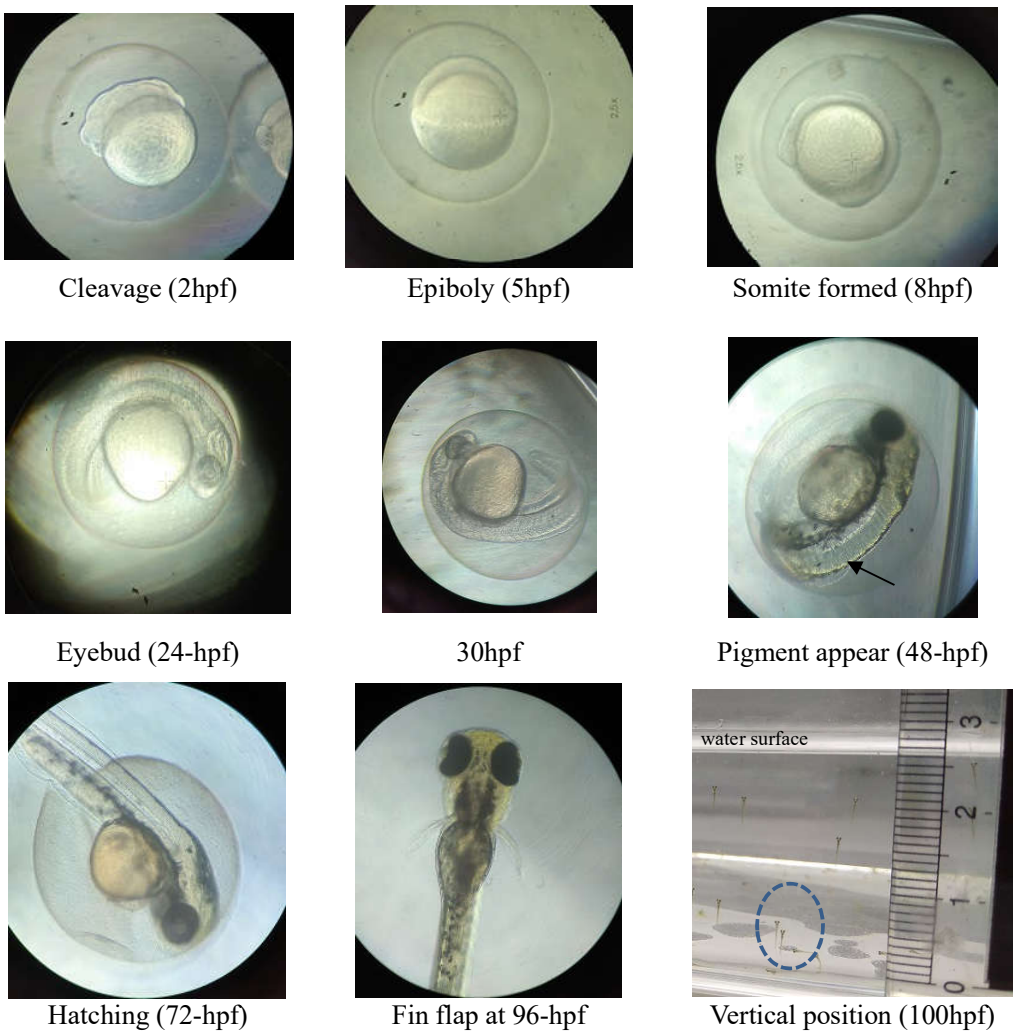

Fig. 2.4 Early-life stage of zebrafish (*Danio rerio*) at incubation temperature 26±1 ºC

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Chapter 3

Determination Organophosphorus Pesticides in River Water Sample using Solid Phase Extraction and Gas Chromatographic-Mass Spectrometer

3.1 Introduction

The beneficial of artificial pesticide usage to increase the productivity of agricultural commodity has also the adverse impact on increasing the potential risk to the environment, which later could affect to the human life. It will be getting worse when meet the unwise field application. The farmers face the risk of the harvest failure, so they tend to use the pesticide excessively to assure their investment (Mariyono, Kuntariningsih, & Kompas, 2018).

Based on several previous occasions in the world by their toxic effect to higher ecosystem level (Finizio & Villa, 2002), (Tadeo, 2008), some pesticides has been banned for any uses. It is also applied in Indonesia. The Indonesian government through it agricultural ministry banned several active ingredient in pesticide and regulated its limited usage (Rahmawati, Margana, Yoneda, & Oginawati, 2013),(Rossana & Pertiwi, 2006). Therefore, its residue still being monitored and the parameters appear in surface water standard. This surface water includes the river water, which mainly use as raw material for fishery, agricultural irrigation, recreation or even water treatment plant.

Recently, organophosphorus (ORP) pesticide is preferable used to replace the

organochlorine (ORC) pesticide that more persistent in the environment. The ORP is considered easy to degrade compare to ORC (Park, El-aty, Lee, & Song, 2006), (Chow, 2009).

In Book of Registered Pesticide and Permitted for Agriculture and Forestry in Indonesia (Ministry of Agriculture, 2016), there are 3207 registered pesticides, include some ORPs e.g. dimethoate, toclofos-methyl, chlorpyrifos, fenthion (MPP), fenitrothion (MEP), phenthoate (PAP), prothiofos, methidathion (DMTP), and pyraclofos. These ORPs have been used and distributed through the farm market, which formally is monitored by related institution. This implies to the occurrence of ORP in Indonesia, whether in soil or water body, and its relation to the risk has mentioned in previous studies (Sekiyama et al., 2015), (Supriyadi, Utami, Widijanto, & Sumani, 2015).

The latest Indonesian water standard regulation has accommodated the ORP parameter. This intends to cover the intensive agriculture activity, with two or even three times harvesting-period annually. The natural phenomenon as percolation or runoff open the chance the residue to wash-out and entering the water body. Since Indonesia, especially West Java Province has agricultural area almost 15% of national scope (BPS-Statistics Indonesia, 2019), risk assessment of this pesticide residue is important.

Cipeles River, West Java Province, which has particular water usage as fish farming at some area between upstream and downstream area (Hermawan, 2010), is one of Cimanuk River tributary (Salim & Kusuma, 2006). The main land-use of the surrounding area is agricultural land (Kementerian Pekerjaan Umum, 2010), (BPS-Statistics of Sumedang Regency, 2018). The monthly average rainfall of Sumedang District between year 2015-2017 was 44 mm at dry season and 493 mm at

wet season (BPS-Statistics of Sumedang Regency, 2016). This rainfall potentially washed-out the residue of agricultural product to enter the water environment(Nakano, Miyazaki, Yoshida, Ono, & Inoue, 2004), which later put the risk into the aquatic biota. Some previous studies have been conducted in Cipeles and Cimanuk River, but based on our best knowledge the data of pesticide occurrence is still unknown.

The detection of ORP compounds in the environmental water matrix becomes the challenge in research, mainly caused by their short half-life and easy to degrade. Previous studies have developed the preconcentration method of ORPs from water sample, where solid phase extraction (SPE) has been shown the good result (Barceló & Alpendurada, 1996), (Picó, Fernández, Ruiz, & Font, 2007), (Ruiz-Gil, Romero-González, Garrido Frenich, & Martínez Vidal, 2008). The preconcentration was using the C_{18} cartridge that suitable for many matrices (Ma et al., 2009). As the separation and detection method, gas chromatographic-mass spectrometer (GC-MS) has proved the good result (Ma et al., 2009), (Li, Gan, Peng, Huang, & Yu, 2010), (Vijaya Bhaskar Reddy et al., 2016). This study aims to determine the ORP concentration in water samples from several sampling locations simultaneously using the SPE coupled with GC-MS.

3.2 Method

3.2.1 Sample collection

Water samples were collected from 11 sampling stations at Cipeles River on late wet season of April 2018. Sample collection was based on USEPA Method for organophosphorus compound analysis (USEPA, 2007c). The surface water was collected using 1-L amber glass water samplers. The pH value was measured and

since for extraction of organophosphorus compound target could be held in any range of pH (USEPA, 2007b), pH adjustment step was not necessary. All samples were preserved in the cooler box between 4-6ºC prior to analysis.

3.2.2 Sample preparation

Upon arrival at the laboratory, the duplicate water samples were filtered through 0.45 µm cellulose acetate filter membranes Advantec® (Toyo, Japan) to remove particulate matter (Mmualefe, Torto, Huntsman-Mapila, & Mbongwe, 2009). Then each filtrate was extracted using SPE Sep-Pak® Vac 6 cc, C_{18} cartridge (Waters®, USA). This step conducted as soon as the sample collected to prevent loss of volatile pesticide compound.

Prior to extraction, the cartridge was conditioned using 5 mL methanol followed by 5-mL ultrapure water (Millipore, Bedford, USA) without leaving it dried-out thereafter (Vijaya Bhaskar Reddy et al., 2016). For sample extraction, the C_{18} cartridge Waters® 6 cc (USA) was carried-out under vacuum manifold (Visiprep®) within flowrate 4 $mL.min^{-1}$. A 250mL water sample extraction finished by washing the cartridge using 10 mL ultrapure water (Millipore, Bedford, USA) and dried within 15 minutes. For eluting the sample 3 mL acetone, 3 mL n-hexane, and 3 mL ethyl acetate (Wako®, Osaka, Japan) successively passed through the cartridge, then dried by gentle nitrogen gas stream, approximately 5 $L.min^{-1}$ during 1 hour until 0.25 mL. The remain solution was added by acetone p.a. until 1 mL, transferred carefully to 2 mL amber vial and stored at -30ºC prior to GC-MS injection. All the procedure was conducted in the fume chamber, as shown in the Fig. 3.1.

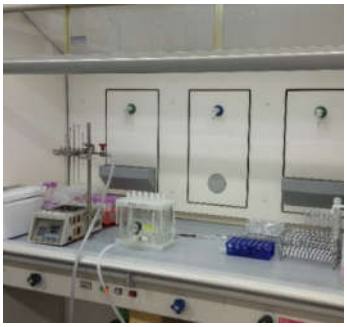

Fig. 3.1 Sample extraction using SPE vacuum manifold

3.2.3 GC-MS Analysis

Separation and detection were carried out using Shimadzu GC-MS QP2010 SE (Japan) equipped with an AOC-20i autosampler, as shown in Fig. 3.2. The nonpolar column DB-5MS (Agilent J&W) 60 m x 0.25 mm (maximum temperature 325^{o}C) was used. GC condition was set at injection temperature 250^{o}C, column temperature 60^{o}C (2 min), ramping temperature 30 ^{o}C.min^{-1} to 180^{o}C, 4^{o}C.min^{-1} to 280^{o}c hold 15 min. The injected volume was 1 μL, split less, using Helium (purity 99,99%) as the carrier gas with flowrate 1.0 mL.min^{-1}.

Fig. 3.2 Analysis using GC-MS QP2010 SE

The MS condition was performed in selected ion monitoring (SIM) mode. Mass-to-charge ratio (m/z) was set between 75 – 314 regarding the characteristic ion as shown in Table 3.1. The total analysis time was 46 minutes.

3.2.4 Quality assurance

Quality assurance was brought throughout the analysis. Calibration curve of mixed standard ORP (Wako®, Osaka, Japan) was performed at minimum five concentrations level between 10 - 200 μg.L^{-1} and coefficient of determination (R^2) > 0.99 was determined for each compound.

The compound target was identified regarding of the retention time, the signal to noise ratio, and the abundance ratio (Vijaya Bhaskar Reddy et al., 2016). The method detection limit (MDL) was regarding the lowest detected concentration that gave the signal to noise (S/N) ratio more than 3, thus the replicates of spiked blank samples were analyzed. The MDL was calculated using Eq. 3.1 (USEPA, 2016),

$$MDL = t_{(n-1,\ 1-\alpha=0.99)} x\ SD \qquad \text{Eq. 3.1}$$

where *t* the Student's t-value and a standard deviation estimate with n-1 degrees of freedom and SD is the standard deviation of the replicate spiked sample analyses. The relative standard deviation (RSD) of reproducibility also calculated, in spite of lower than 20%.

Surrogate chemical is recommended to be added in the sample for pesticide analysis (USEPA, 2007b) and several surrogate chemicals specifically have the characteristic which similar to the pesticide compound. The 2-Fluorobiphenyl as the surrogate on organophosphorus compound detection by GC/MS (USEPA, 2007a) in this study was added before the extraction procedure began. Triplicates of spike sample were carried out where the recovery of the surrogate calculated using Eq. 3.2 should between 70-130% (USEPA, 2007b).

$$Surrogate\ recovery\ (\%) = \frac{Concentration\ found}{Concentration\ added} x\ 100 \qquad \text{Eq. 3.2}$$

3.3 Result and Discussion

3.3.1 Determination method

The extraction of ORP compounds by SPE C_{18} cartridge was followed by separation and detection using GC-MS in this study. The elution step was applied using acetone, hexane, and ethyl acetate to allow the wide range extraction of the compound targets. Acetone and hexane have proven at good recovery for ORP compound as mentioned in some previous studies (Table 3.2). The mid-polar acetone and ethyl acetate could extract more soluble compound such as ethoprophos, thiometon and methidathion, while nonpolar hexane completed the elution of the target compounds from the SPE for most target compounds which have low solubility in water (Table 3.1). The triplicates surrogate recovery using the method in this study gave the means, standard deviation and RSD 70.91%, 8.68 %, and 12.24% respectively. The standard deviation of recovery was at the low accepted range, which might be caused by the loss of analyte during the dried-up in the fume chamber. Flowrate of preconcentration at 4.0 $mL.min^{-1}$ gave insignificant cause of low recovery since there is no different recovery shown at slightly changes of flowrate between 3.0-7.0 $mL.min^{-1}$ (Vijaya Bhaskar Reddy et al., 2016). At last, the RSD resulted below 20% as requested (USEPA, 2017), so the method was acceptable.

The chromatogram result of ORP standard injection at 200 $\mu g.L^{-1}$ was shown in Fig. 3.3. Fifteen compounds were detected in standard ORP that had been injected into GC-MS, on the SIM method. The compound target identified regarding of retention time and the S/N ratio, and composed into calibration curve of minimum five level concentrations. The calibration curve resulted the coefficient of determination R^2

Table 3.1 The properties of organic solvents and target compounds

Chemical	MW, g.mol^{-1}	m.p., ^{o}C [a)]	b.p., ^{o}C [a)]	Solubility[a),b)]	Characteristic Ion, m/z [c)]		Polarity index*
				mg/L	Primary Ion	Secondary Ion	
Solvent							
Methanol	32	-98 [d)]	65 [d)]	1000			5.1[d)]
Acetone	58	-95 [d)]	56 [d)]	1000			5.1[d)]
Ethyl acetate	88	-84 [d)]	78 [d)]	64x10^{3}			4.3[d)]
Hexane	86	-95 [d)]	69 [d)]	9.5			0.01[d)]
Target Pesticide							
Ethoprophos	242	-13	86-91	750	158	97; 126	
Phorate	260	< -15 [e)]	75-78	50	75	121; 97	
Thiometon	246	ND	110	200	88	125; 75	
Terbufos	288	-29.2 [e), d)]	69	5.07	103	231;97;125	
Etrimfos	292	-3.35	ND	0.04	292	181;125	
Dichlofenthion	315	ND	164-169	0.245	279	223;97;109	
Tolclofos-methyl	301	79	>100 [f)]	0.3-0.4	265	267, 125	
Chlorpyrifos	350	41.5-44[e)]	160 [g)]	1.4	97	197,199,314	
Fenthion (MPP)	278	7.5 [e)]	87	0.0075	278	125, 109, 93,169	
Isofenphos	345	< -12 [e)]	120	22.1	121	213	
Penthoate (PAP)	320	17.5	70-80	ND	274	121, 93, 125	
Methidathion (DMTP)	302	39.5 [e)]	>100 [f)]	187	145	85,93	
Butamifos	332	ND	422 [h)]	6.19 [h)]	286	200, 96	
Prothiofos	345	ND	110 [f),h)]	0.07 [h)]	141	75;97	
Sulprofos	322	-15	155-158	0.31	140	141;125	
Surrogate							
2-fluorobiphenyl	172	73.5	248		172	171, 170	

[a)]PubChem .https://pubchem.ncbi.nlm.nih.gov; [b)]at 20-25^{o}C, [c)](USEPA, 2017)(USEPA, 2007c)(Li et al., 2010) ; [d)](Anonim, n.d.); [e)](edited by Michael A.Kamrin, 2000); [f)]flash point; [g)]decomposes; [h)]PPDB. https://sitem.herts.ac.uk/aeru/ppdb/en/Reports/560.htm. ND=no data

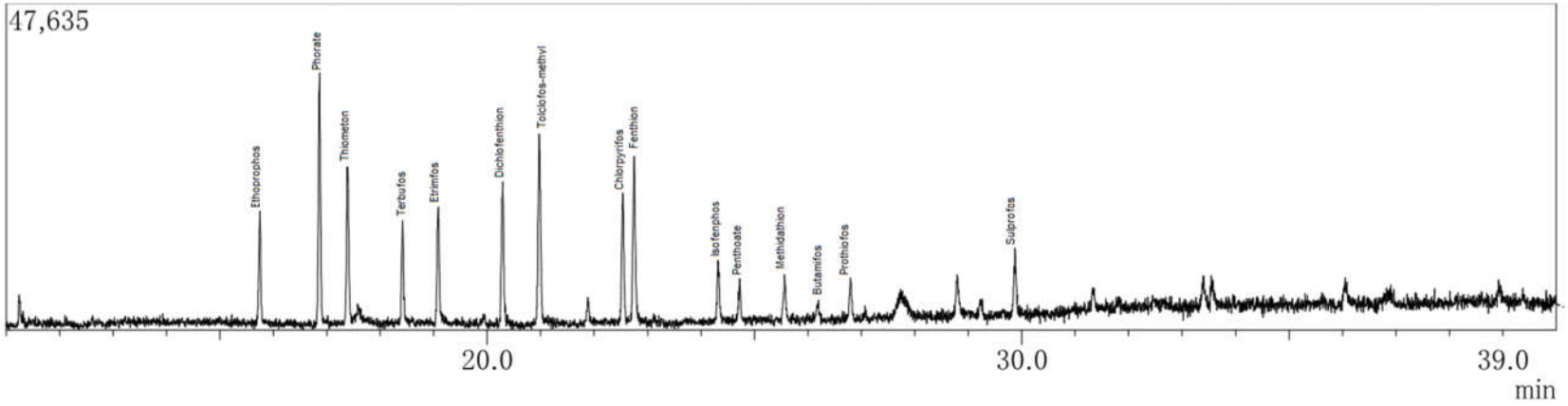

Fig. 3.3 GC-MS chromatogram obtained for injected 1 mL mixed standard ORP 200 mg.L^{-1} on SIM mode

Table 3.2 Elution solvent in SPE from various water sample

Target compound	Elution solvent	Sample	Ref.
ORP, carbamate	acetone, hexane, ethyl acetate	River water	(Tanabe et al., 2000)
ORP, aniline	ethyl acetate	Drinking water	(Aravinna & de Alwis, 2003)
ORP, ORC, carbamate, pyrethroid	acetone, n-hexane/acetate, n-hexane	Water sample	(Ruiz-Gil et al., 2008)
ORP	ethyl acetate	Underground water	(Ma et al., 2009)
ORP	methanol, DCM /acetonitrile	Water sample	(Vijaya Bhaskar Reddy et al., 2016)

above 0.99, except sulprofos at 0.9891, as shown in Table 3.3. At the chromatogram Fig. 3.3, low responses were shown from penthoate, butamifos, prothiofos and sulprofos.

The MDL from seven replicates was determined for each target compounds and gave the RSD as shown at Table 3.3. The MDL is quite high compare to other study but the RSD were all below 20%.

Table 3.3 Retention time, coefficient of determination on calibration curve**,** MDL and RSD for reproducibility each compound target

Compound	Retention Time, min	Calibration Curve R^2	MDL, μg,L^{-1} *(n = 7)*	RSD, %
Ethoprophos	15.75	0.9973	16.895	7.77
Phorate	16.87	0.9908	1.997	5.00
Thiometon	17.39	0.9954	4.459	8.99
Terbufos	18.42	0.9981	15.482	8.01
Etrimfos	19.09	0.9932	4.202	9.33
Dichlofenthion	20.29	0.9920	3.744	7.93
Tolclofos-methyl	20.97	0.9909	2.896	6.44
Chlorpyrifos	22.54	0.9967	12.203	5.73
Fenthion	22.75	0.9961	4.043	10.61
Isofenphos	24.32	0.9921	14.275	9.03
Methidathion	25.56	0.9926	14.592	13.00
Butamifos	26.20	0.9905	13.589	14.82
Sulprofos	29.88	0.9891	5.846	4.46

3.3.2 Application to real sample

The developed method was applied for water sample that collected from eleven sampling locations at agricultural area, from upstream to downstream area. Duplicate samples were determined with the surrogate that carried out during the determination. Detected pesticide compounds were depicted in Fig. 3.4.

On St. 1 at the upstream area, the terbufos, chlorpyrifos and thiometon were detected, at 0.93, 1.19, and 0.07 μg.L^{-1} respectively. At city area (St.4), chlorpyrifos

was detected at 0.49 µg.L^{-1} where below the chlorpyrifos concentration at upstream area. Chlorpyrifos also detected at downstream area, at St.10 on Cimanuk River, 0.28 µg.L^{-1}. The chlorpyrifos concentration here was lower than city area.

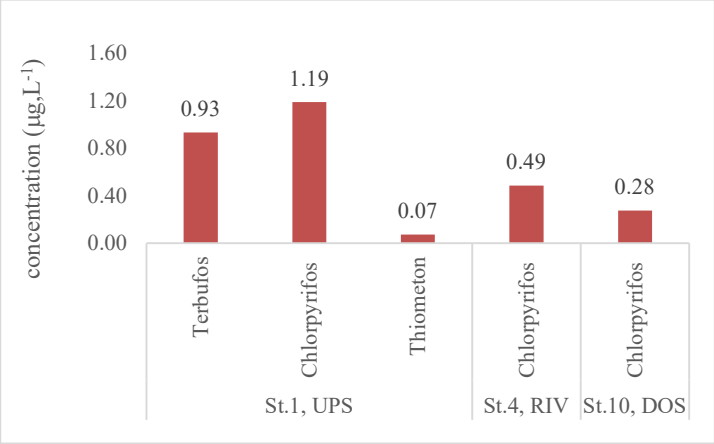

Fig. 3.4 Detected ORP compound in water sample

The pesticide compound that detected in ORP standard on this study mostly is insoluble until slightly soluble in water, which indicate by the solubility value below 100mg.L^{-1} (edited by Michael A.Kamrin, 2000). Regarding Table 3.1, chlorpyrifos and terbufos has 1.4 and 5.07 mg.L^{-1} for their solubility, respectively, which means slightly soluble in water. Chlorpyrifos tend to persist in soil and volatilization is the most possibility in loss of chlorpyrifos in water. The other chlorpyrifos characteristic is tend to accumulate in the sediment (Ccanccapa, Masiá, Navarro-Ortega, Picó, & Barceló, 2016). Meanwhile, terbufos hydrolyzed very rapid in water (edited by Michael A.Kamrin, 2000). It means chlorpyrifos and terbufos would be difficult to be found in water, compared to other compound that has higher water solubility.

Meanwhile thiometon is classified as soluble in water with their solubility 200 mg.L^{-1}. But in this study, the concentration was found 0.07 µg.L^{-1}, the smallest concentration between other ORP compound, even the sampling period was conducted

at the end of wet season when rain intensity is still high, 17 days.month^{-1} (BPS-Statistics of Sumedang Regency, 2019).

At the upstream area, the area where insecticides chlorpyrifos, terbufos, and thiometon were detected, was in the flowering stage of paddy field. The previous studies mentioned that the application of the pesticide commonly does at the initial stage of paddy growth and avoided during the flowering stage (Parveen & Nakagoshi, 2001)(Hashim et al., 2017). The occurrence of pesticides in the river body might be caused by previous period application that leach during rainy season, before the sampling was conducted. Thiometon that easily dissolved in water probably already dilute in concentration. Chlorpyrifos and terbufos that likely to attach in soil than leaching into the water last longer, but during wet season, the leaching also occurs. It also the reason chlorpyrifos is found at upstream, city and downstream area in the reduced concentration as the river flow. While terbufos that easily to convert to its metabolites (edited by Michael A.Kamrin, 2000) give the reason of small detection in the water. All these assumptions are taken when the same usage of pesticides is applied and without any other input source to the stream. The pesticide application usually depends on farmer habit on pesticide dosage and treatment of remain pesticide (Rahmianna, Suharsono, & Harnowo, 2015). If the concentration of the pesticides from St.1 already diluted, the chlorpyrifos which detected in St.4 and St.10 was emerged from the pesticide application activity around those sampling sites that washed out to the stream during rainy period. Chlorpyrifos occurrence is reasonable since chlorpyrifos is one of active compound that included between 328 registered active compounds of pesticide in Indonesia (Ministry of Agriculture, 2016). However, the concentration of detected ORP pesticides was below Indonesia regulation of class C for

fishery and farming, 100 μg.L^{-1}.

Regarding the aquatic acute 96 hours-LC_{50} toxicity level to rainbow trout (Helfrich, Weigmann, Hipkins, & Stinson, 2009), chlorpyrifos and terbufos are classified as super-extreme level as their LC_{50} 0.003 and 0.01 mg.L^{-1} respectively, while thiometon is moderate toxic level with the LC_{50} 8 mg.L^{-1} (Anonim, 1988). Since the ORP pesticide easily to degrade to its derivative compound, the study about its derivative detection is interesting to develop. The future study on ecological risk assessment of the detected ORP compounds or its derivative will give more comprehensive understanding.

3.4 Conclusion

The extraction, separation and detection method of ORP at study area has conducted simultaneously in this study. The recovery using multiple ORP standard is in acceptable range, 70.91%, and the coefficient of determination R^2 of each 13 compounds between 0.9891-0.9981. The detection on water sample gave result that chlorpyrifos was detected in the water sample from upstream (St.1), city area (St.4) and downstream (St.10), at 1.19, 0.49 0.28 μg.L^{-1}, respectively. While terbufos and thiometon were detected at the St.1 at upstream area, 0.93 μg.L^{-1} and 0.07 μg.L^{-1}, respectively. However, the concentration of detected ORP pesticides was below Indonesia regulation of class C for fishery and farming, 100 μg.L^{-1}. The occurrence of the ORP pesticide was influenced by the agricultural phase activity. The future study of ecological risk assessment of detected ORP compounds or its derivative might be interesting to conduct to give better understanding.

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Chapter 4

Distribution, Source Identification, and Assessment of Heavy Metal Pollution in the Surface and Pore Waters of Cipeles River, West Java, Indonesia

4.1 Introduction

As one of the six major rivers in West Java, Indonesia, the Cimanuk River plays an important role in supplying a source of water for irrigation, domestic uses, raw water for water supply companies, and fishing (Susilo and Sjafei 2006); (Sulaksana et al. 2013) (Kementerian Pekerjaan Umum 2010); (Sulthonuddin, Hartono, and Utomo 2018). As it flows downstream, many tributaries enter the Cimanuk River. In 2010, monitoring results for the Cimanuk River showed it had qualitatively exceeded the allowable standards for organic matter and some metals (Kementerian Pekerjaan Umum 2010). Previous studies have investigated the status of the Cimanuk River using different methods and concluded that the river was lightly to heavily polluted (Susilo and Sjafei 2006); (Sutriati 2011); (Sulthonuddin, Hartono, and Utomo 2018).

The Cipeles watershed is the largest subsystem of the Cimanuk watershed. The main river in the Cipeles watershed, the Cipeles River, shares its estuary with Cimanuk River in the Tomo region. For utilization, a previous study (Hermawan 2010) mentioned that some areas of the Cipeles River were appropriate places for aquatic life habitat. The land usage along the Cipeles River is divided into paddy fields, residences, non-paddy farm land, and forests (Salim and Kusuma 2006). Each subdistrict along the river

comprises a 12%–86% agricultural area, whereas the other 45%–88% of land usages include forests, residences, and trade and industry areas (BPS-Statistics of Sumedang Regency 2018).

Many effluents originating from population activities directly enter the Cipeles River, which causes ecological risks. Anthropogenic pollutants such as heavy metals contribute to polluting the river. Heavy metals are nondegradable and potentially enter the food chain and accumulate in the environment (Mustafa et al. 2019). Therefore, reducing the pollution from the source is a concern in addition to minimizing the risk to vulnerable receptors, such as aquatic organisms (Vardy et al. 2014) or agricultural plants. In this study, the water quality data with respect to heavy metals will be determined for use as a basic database. The water quality was then assessed using indices related to heavy metals in surface and pore waters.

4.2 Method

4.2.1 Sample collection

Water samples were collected in the late wet season in April 2018 at upstream, city, and downstream sites on the Cipeles River (Fig. 4.1) (107°49'18.6780"-108°08'00.83" E - 06°45'43.27"-06°53'51.3456" S). The sampling position was from a location in the river at approximately one-third of the river width. The surface water was collected using 1-L amber glass water samplers at half of river depth, while the pore water was collected from the top-layer sediment (0-5 cm) using thermoplastic scoops which are immediately stored in the clean 100-mL polypropylene plastic bottle. The samples were filled until the top of the lid to prevent the oxygen

presence, then preserved and handled based on the EPA Method 200.8 (1994) for dissolved metal elements, where the water was acidified using nitric acid until pH < 2 and put into a cool box prior to laboratory analysis. The in situ parameters, such as pH, dissolved oxygen (DO), and temperature measured using a water quality checker (Horiba, Kyoto, Japan), are listed in Table 1.1.

Considering Table 1.1 and the Indonesian Government Regulation for stream standard class III, a low DO value is shown, i.e. < 3 $mg.L^{-1}$, while the neutral pH ranges between 6–9.

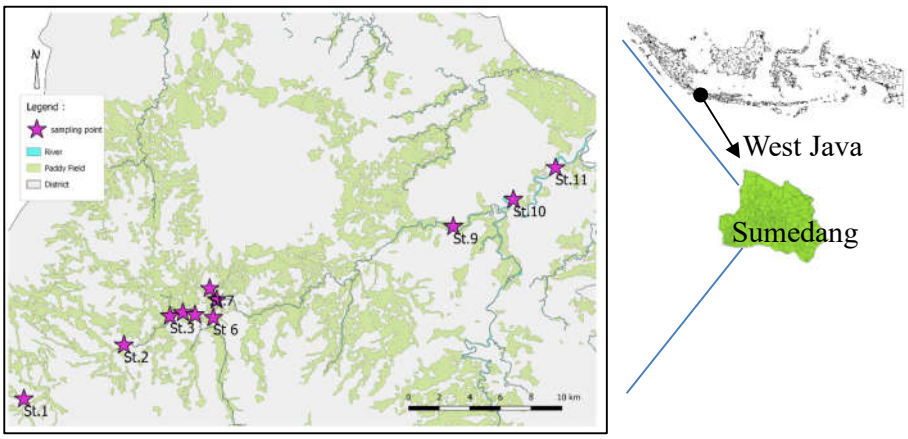

Fig. 4.1 Map of Sampling Location

4.2.2 Sample preparation

The water sample was prepared by filtering a 10-mL sample through a 0.45-micron cellulose acetate membrane filter Advantec® (Toyo, Japan) and placed in a 15-mL polypropylene centrifuge tube. Dilution was performed as required due to the prediction of a high concentration of heavy metals. One milliliter of nitric acid (Wako Pure Chemical, Osaka, Japan) was added to ensure the pH of the sample <2. Finally, the solution was mixed and homogenized thoroughly.

The heavy metals were analyzed using inductively coupled plasma mass spectrometry (X Series 2; Thermo Fisher Scientific, Waltham, MA, USA). A standard solution of target elements (Wako Pure Chemical, Osaka, Japan) was prepared in five concentrations: 0.1, 0.5, 1, 5, and 10 ppb. A calibration curve for target elements was established, where good linearity was performed, i.e., Mn (0.9999), Fe (0.9998), Zn (0.9998), Ba (0.9999), Cr (1.00), Co (0.9991), Cu (0.9999), and Pb (0.9975). The blank was also analyzed for background. The limit of detection (LoD) for each element was obtained from multiplying the standard deviation (SD) by Student's *t* value of ten replicates ($t_{(n=10)}$ = 2.821) (US EPA 1994). While the limit of quantification (LoQ) was obtained from 10 times SD.

4.2.3 Plotting the heavy metal concentrations and statistical analysis

The heavy metal concentrations at each sampling station were the input data for plotting a map overlaid over a land-usage map of Sumedang District (Badan Informasi Geospasial Republik Indonesia 2019) using QGIS (v. 3.4.) open-source software.

To analyze the correlation between heavy metals in surface and pore waters, analyses of variance were performed using regression analysis in Excel 2016 software (Microsoft, Redmond, WA, USA).

4.2.4 Heavy metal pollution index

Following the determination of heavy metal concentrations in the river water, a heavy metal pollution assessment could be performed to identify ecological concerns. Considering the multiple sources of pollutants that potentially enter the river water, this

assessment should accommodate various heavy metal elements. Initially developed for drinking water assessments, the heavy metal pollution index (HMPI) represents the total influence of heavy metals in drinking water (Mohan, Nithila, and Reddy 1996). Recently, an application of the HMPI was developed for streams or groundwater (Ojekunle et al. 2016); (Giri and Singh 2014). The HMPI equation uses a weighting scale for each element with respect to the ideal concentrations and the heavy metal standard as described in Eq. 4.1 and Eq. 4.2 (Giri and Singh 2014),

$$HMPI = \frac{\sum_{i=1}^{n} W_i Q_i}{\sum_{i=1}^{n} W_i} \qquad \textbf{Eq. 4.1}$$

$$Q_i = \frac{M_i - I_i}{S_i - I_i} x100 \qquad \textbf{Eq. 4.2}$$

where Q_i is the subindex value of the ith parameter; W_i is the unit weight of the ith parameter; and n is the number of concerned parameters. The value of Q_i of the parameter is calculated by Eq. (2), M_i is the measured heavy metal concentration, I_i is the ideal concentration, and S_i is the heavy metal standard that must be complied with.

The critical value was between 0 and 100, but the previous study modified the index classification into low for values <15, medium for values between 15 and 30, and high for values >30 (Edet and Offiong 2002).

4.3 Results and Discussion

Based on the heavy metals analysis results (Table 4.1), each element was plotted on the map (Fig. 4.2 and Fig. 4.3). The circle diameter represents the concentration,

which significantly differed between the macro and micro concentrations; therefore, the concentrations were illustrated in two parts to represent each concentration range.

Table 4.1 Heavy metals concentration on pore and surface water at each sampling station (ppb)

	Location	Mn	Fe	Ba	Zn	Co	Cu	Cr	Pb
PORE WATER									
Upstream	St.1, UPS	133,800.0	36,200.0	628.0	27.0	56.1	18.0	1.1	13.9
	St.2, UPS	173,800.0	100.0	6,371.0	172.0	114.5	322.2	29.1	94.1
	St.3, SUB	339,300.0	100.0	6,013.0	249.0	137.5	352.7	132.0	397.3
City center	St.4, RIV	388,700.0	4,000.0	7,930.0	976.0	1,683.0	110.0	1.0	243.7
	St.5, CITY	456,100.0	100.0	11,460.0	691.0	165.6	532.0	136.9	238.0
	St.6, FOOD	533,300.0	9,900.0	10,010.0	548.0	101.5	266.9	32.7	81.3
	St.7, FARM	36,300.0	37,600.0	1,866.0	136.0	206.2	55.2	1.4	77.8
	St.8, DOM	394,400.0	28,000.0	17,600.0	1,655.0	127.2	369.7	51.5	114.6
Downstream	St.9, JUNC	254.0	97.0	1,287.0	233.0	21.9	26.4	1.0	17.5
	St.10, DOS	5,800.0	14,600.0	549.0	32.0	55.2	20.2	1.0	13.7
	St.11, DOS	190,600.0	61,400.0	3,572.0	1,022.0	1,077.0	25.6	0.7	16.4
SURFACE WATER									
Upstream	St.1, UPS	134.0	799.0	12.3	5.6	1.1	3.4	0.4	1.0
	St.2, UPS	75.0	633.0	9.9	20.9	1.2	3.2	0.3	1.0
	St.3, SUB	89.0	719.0	9.0	8.8	1.4	3.5	1.3	4.0
City center	St.4, RIV	139.0	1,140.5	20.7	469.1	3.5	21.4	1.4	7.4
	St.5, CITY	109.0	1,234.0	22.4	24.6	1.6	5.2	1.4	2.4
	St.6, FOOD	90.0	634.0	8.7	55.6	1.0	2.7	0.3	0.8
	St.7, FARM	609.0	1,053.0	23.8	18.7	1.6	4.4	0.8	4.1
	St.8, DOM	86.0	1,193.0	18.6	175.7	1.3	3.7	0.5	1.2
Downstream	St.9, JUNC	95.0	1,322.0	32.5	8.9	1.7	5.7	0.7	2.7
	St.10, DOS	229.0	3,965.0	33.1	43.9	3.6	8.9	1.6	14.0
	St.11, DOS	162.0	2,246.0	27.2	4.8	2.9	7.2	1.0	3.6
Indonesian standard, class III		(-)	(-)	(-)	50	200	20	50	30
LoD		0.00583	0.02836	0.00758	0.06052	0.00535	0.00595	0.01484	0.00363
LoQ		0.02066	0.10053	0.02685	0.21452	0.01897	0.02111	0.05259	0.01287

4.3.1 Heavy metal concentrations in pore water

Compared to Fig. 4.2 and Fig. 4.3, the pore water consisted of higher heavy metal concentrations than the surface water. This higher concentration is caused by soil erosion sinking into the sediment, collecting abundant minerals and organic matter that it could release into the water phase depending on environmental conditions (Huang et al. 2017). When entering the water environment, heavy metals accumulated in the sediment by adsorption, complexation, precipitation, and other processes. When the pH, DO, or biogeochemical processes change, the accumulated metals may be released from the sediment by desorption, dissolution, oxidation, and also reduction, which later causes secondary pollutants in surface water (Nelson et al. 2019); (Zhang et al. 2019).

Heavy metals in pore water dominated by manganese (Mn), iron (Fe), and barium (Ba), were found mostly in the city center (St. 3 – St. 8). Manganese and iron occurred hundreds to thousand times higher compared to the other heavy metals, which means they were the dominant metals. In nature, manganese and iron occur in rock and soil (ATSDR 2012), which are present in the water environment as result of the oxidation–reduction of the chemical weathering process. Manganese commonly has relatively higher concentrations than other metals. Manganese would be replaced by iron through a later oxidation–reduction process (Othman et al. 2019). A previous study (Kementerian Pekerjaan Umum 2010) observed that the upper Cipeles River consists of laterite soil, while alluvial soil is found near the riverbank soil. Laterite soil is rich in iron (Jackson and Sherman 1953), while manganese has rich concentrations in alluvial soil. The oxidation–reduction process might be influenced by physicochemical characteristics, which allows the mineral to be released from the soil into the pore water.

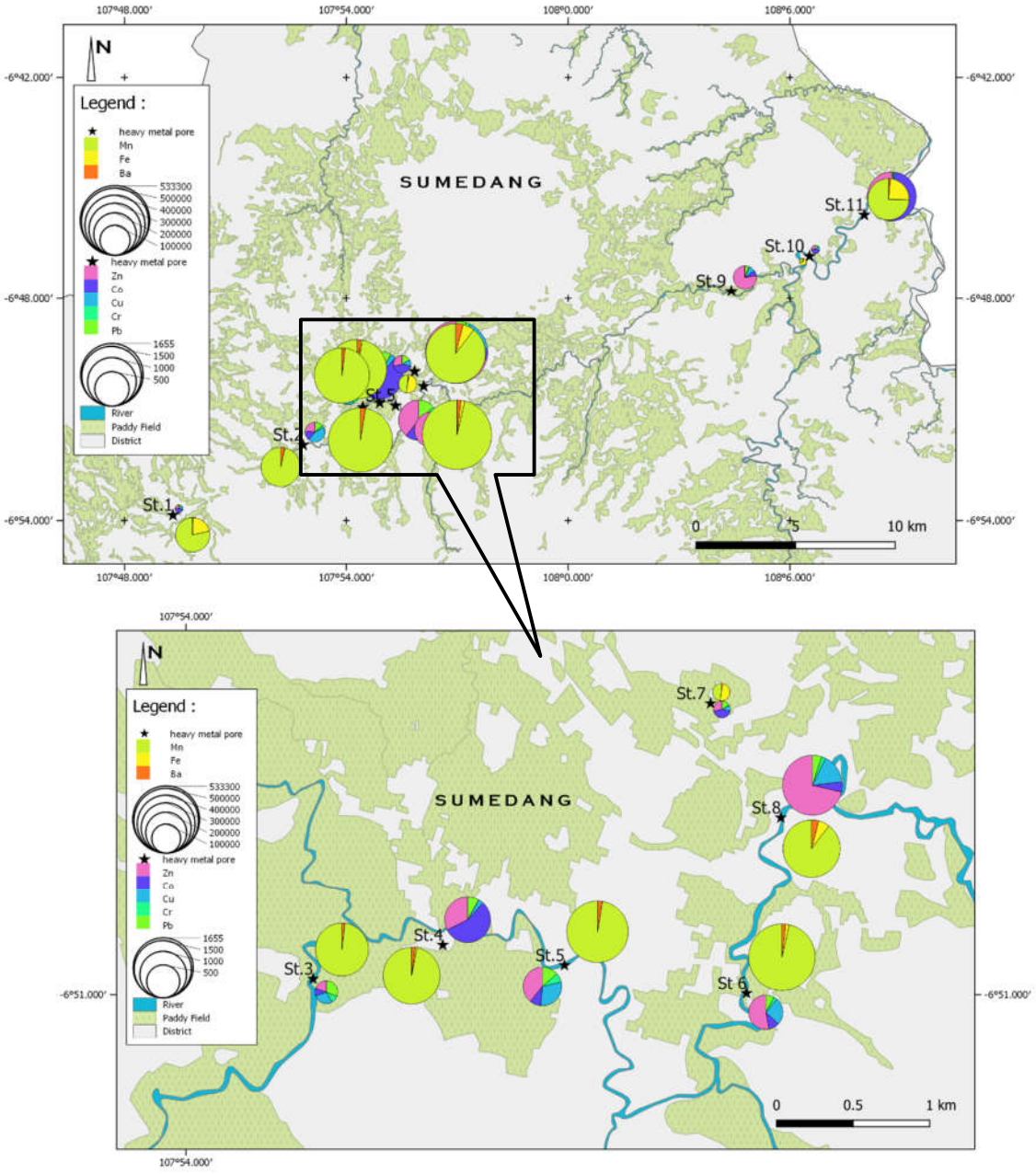

Fig. 4.2 Distribution of heavy metals in pore water (concentrations in ppb)

The other elements that were also found include zinc (Zn), cobalt (Co), and copper (Cu), followed by chromium (Cr) and lead (Pb). In a comparison with other studies of pore water from different locations, the city area has higher concentrations of

copper, lead, and zinc (Table 4.2). Only the chromium concentration was lower compared to Xiao River, where sewage and industrial wastewater effluent is the main source of pollutants (Zhu et al. 2016).

Table 4.2 Maximum concentrations of Cr, Cu, Pb, and Zn in this study and other areas

Sites	Maximum concentrations (ppb)				References
	Cr	Cu	Pb	Zn	
Xiao River, China	162.0	61.8	40.3	252.0	(Zhu et al. 2016)
Tagus Estuary, Portugal	-	10.7	28.5	915.0	(Santos-Echeandía et al. 2010)
Jiaozhou Bay, China	2.56	-	115.0	115.0	(Ye et al. 2011)
Cipeles River (upstream)	132.0	352.7	397.3	249.0	This study
Cipeles River (city)	136.9	532.0	243.7	1,655.0	This study
Cipeles River (downstream)	1.0	26.4	17.5	1,022.0	This study

4.3.2 Heavy metal concentrations in surface water

Fig. 4.3 shows that the heavy metals in the surface water were also dominated by iron and manganese. The manganese concentration in the water affected by the pH and redox conditions, where the solubility increases under low pH and anaerobic conditions (Thuong et al. 2015). The anaerobic conditions, as indicated by the low DO level of physicochemical characteristics in Table 1.1, allows the manganese to easily release into the water phase in the surface water.

Among the micro concentrations, zinc was present in major percentages, especially at St. 4, St. 7, and St. 10. The St. 4 and St. 7 sites are located in the city area, while St. 10 is located in a downstream area after the junction with Cimanuk River. Zinc usually occurs from natural phenomena and anthropogenic activities. Meanwhile, mining and heavy metals-related industries are not present in the study area. As an agricultural area, the usage of agrochemicals, such as fertilizer, could be considered as

the source of zinc. In Indonesia, organic and inorganic fertilizers are enriched with zinc, copper, cobalt, lead, iron, and manganese as micronutrients (Anonim 2011) (Anonim 2017).

The wash-out phenomena during wet season, when the sampling was performed, allowed those heavy metals caches to leach into the river body. Meanwhile, the high concentrations at St. 10 might be contributed from the Cimanuk River, which has a higher flow rate as the main river in the watershed. Indonesia's statistics yearbook for 2010–2016 notes that the flow rate of Cimanuk River at Sumedang-Tomo is between 24.5 – 429 $m^3.s^{-1}$, which is a strong flow rate.

The concentrations of barium also appear quite high compared to lead, copper, and chromium. In nature, barium is usually found in groundwater (ATSDR 2007). Considering the toxic effects that might be caused by barium compounds, especially soluble barium, the concentration of barium was determined. However, as well as iron and manganese, barium is not included in the mandatory list in the Indonesian regulations for stream standard class III for fishery, farming, and irrigation purposes. In addition, it does not contribute to the HMPI calculation results, except that the stream class standard becomes stricter. Iron, manganese, and barium concentrations must be below 300, 100, and 1000 ppb, respectively, if the surface water will be utilized as class I, i.e., raw water for water treatment plants.

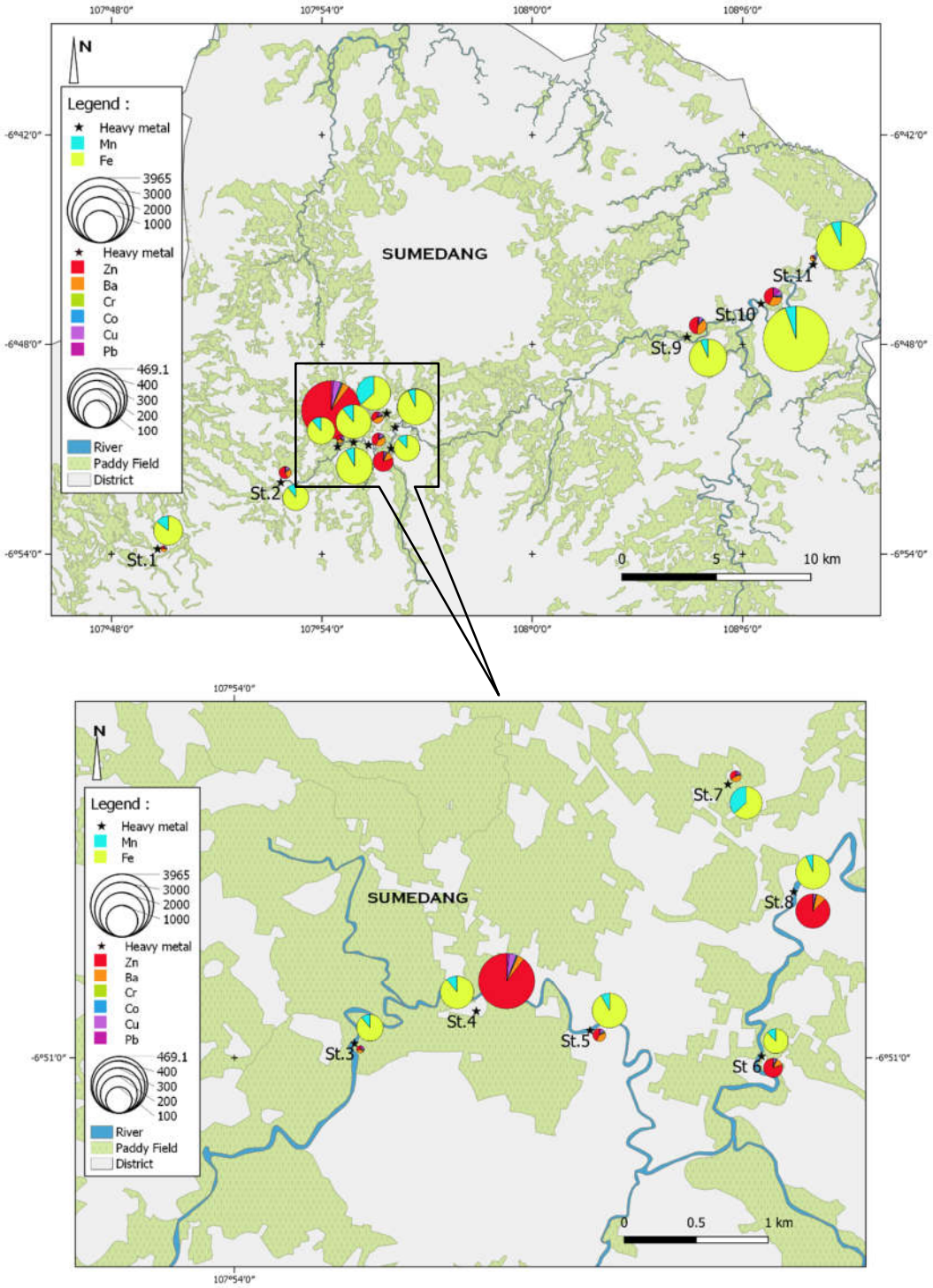

Fig. 4.3 Distribution of heavy metals in surface water (concentration in ppb)

Copper, one of the heavy metals that were detected in surface water, might be contributed by pesticide applications in agricultural areas (Giri and Singh 2014); (Zhao, Coles, and Wu 2015); (Ojekunle et al. 2016); (Zhou et al. 2016), e.g., copper-based herbicides (Helfrich L.A., Weigmann D.L.,Hipkins P. 2009). Fig. 4.3 shows that copper concentrations varied, but tended to increase where city areas, domestic uses, food industries, and farming activities were located and even decrease when the river flowed into downstream areas (St. 10 and St. 11).

However, the heavy metals in upstream pore and surface waters where only agricultural activity is performed show lower concentrations than the downstream area. Other activities in the city center might contribute to the higher heavy metal concentrations in the downstream area.

4.3.3 Heavy metal pollution index

The HMPI for each sampling location was calculated, considering the heavy metal concentrations in surface water, the standard, and the ideal concentrations that could be achieved. The HMPI also describes how the identified heavy metal concentrations polluted the water environment; however, the HMPI values in this case were compared to the standard and ideal target values.

In this study, the standards are the Indonesian government's regulations for stream standard classes I and III, and also the World Health Organization's (WHO) clean water standard. Considering the modified critical values by (Edet and Offiong 2002), a comparison with the standard is shown in Fig. 4.4.

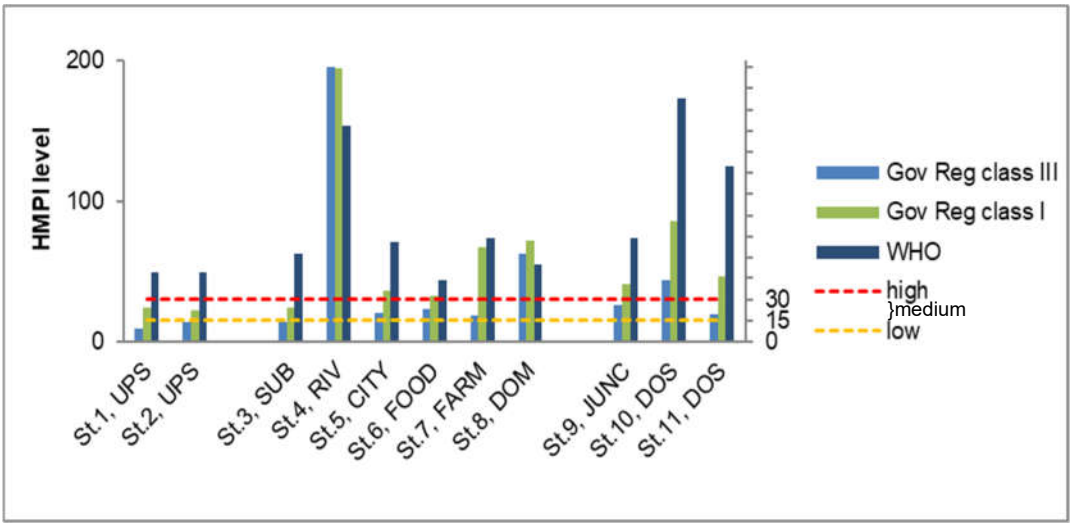

Fig. 4.4 Heavy metal pollution index

As shown by Fig. 4.4, the high index values at St. 4 are very significant. Using the standard for class III shows that St. 4 was classified as highly contaminated caused by the high zinc concentration found in the surface water sample (Fig. 4.3), where the zinc concentration standard only allows <50 ppb. Zinc is present in nature, but also occurs as an anthropogenic element, mainly from industrial discharge, domestic waste water, or washed-out fertilizer in soil loss from run off (ATSDR 2005). St. 4 was located in an agricultural area and the high concentrations in surface water was caused by the wash-out phenomena of agricultural products. This also occurred in St. 8, a sampling location near a domestic area, which showed a high zinc concentration leading to an increase in HMPI values.

At St. 11, the HMPI values decreased after increasing at St. 10. Based on the sampling location in Fig. 4.1, this station was located after the junctions with Cipeles River at St. 9 and Cimanuk River at St. 10. As represented by St. 10, the Cimanuk River has higher heavy metal concentrations in the surface water (Fig. 4.3). This input from Cimanuk River might contribute to the heavy metal concentrations at St. 10 through a

vertical water-mixing phenomenon, which is affected by many factors, such as flow rate, physical parameters, and riverbed characteristics (Chanson 2004). The strong flow rate of Cimanuk River at Sumedang-Tomo allows the heavy metals to flow horizontally rather than settle to the bottom of the river. After St. 10, the vertical water-mixing process is insufficient enough to allow the heavy metals to settle in the bottom sediment. Therefore, all heavy metal concentrations in the surface water reduced at St. 11, while the respective concentrations in the pore water increased.

In Fig. 4.4, the changes of indices from upstream to downstream area are significant, especially using standard class III. The HMPI worsens in category and is classified as highly polluted for all stations when the class level concerning health standard becomes stricter. For class III, when the pollution level of heavy metals showed medium to high levels, it may affect the aquatic biota if the river water was used for fisheries activities.

4.3.4 Relationship between heavy metals in surface and pore waters

Considering the overall results of spatial analysis, the heavy metals in the city center's pore and surface waters relatively increased compared with the upstream area. The heavy metals in sediment or pore water can be released into the surface water due to changes in environmental conditions (Vu et al. 2017), which adversely affects the water quality. A regression analysis shows the correlation between heavy metals in pore and surface waters in the study area (Table 4.3Table 4.3).

Some significant relationships between the heavy metal concentrations in pore water with that in surface water are shown in Table 4.3. In the city area, only Mn and Co showed a significant relationship between heavy metals in pore and surface waters.

Meanwhile in the downstream area, a significant relationship was shown for Ba and Zn. In the upstream area, all heavy metals in the surface water showed an insignificant relationship with pore water.

Barium and zinc in the downstream area tended to increase in surface water when the concentrations in pore water also increased and vice versa (Fig. 4.3). The peak concentration was obtained when the downstream area of the Cipeles River met the main river, Cimanuk River, at St. 10. It could be assumed that the heavy metal concentrations in the main river dominantly contributed to the sample at St. 10. Barium is a heavy metal not included in agricultural products; therefore, its occurrence in the surface water could be assumed to have dominantly come from pore water that originated from the original soil around the sampling site. Considering zinc, which is included as a micronutrient in fertilizer products, the residue of zinc products in the pore water is assumed to mix with the surface water. The flow rate in the downstream area and conjunction with Cimanuk River allowed a vertical water-mixing condition (Chanson 2004) or eased the release of zinc from the pore water (Li et al. 2013).

Table 4.3 Regression results for heavy metals in pore and surface waters

	Regression, R^2		
	Upstream	City	Downstream
Mn	0.2125	0.9234*	0.0007
Fe	0.7317	0.0004	0.0032
Ba	0.8984	0.0896	0.9795**
Zn	0.1362	0.2228	0.986**
Cu	0.000003	0.1706	0.8656
Pb	0.9544	0.3043	0.9553
Co	0.5759	0.9553*	0.0285
Cr	0.932	0.067	0.0272

Significance level: *$p < 0.05$; ** $p < 0.10$.

The washed-out phenomena in the city area might not apply for manganese and cobalt because the fluctuation of those concentrations in pore and surface waters was similar.

The heavy metal concentrations detected at the surface or in the sediment of the river was affected by the type and quantity of the pollutant source related to the land usage (Wang et al. 2019); (Othman et al. 2019), surrounding soil type (Othman et al. 2019), season and stream flow path(Nelson et al. 2019), and also physicochemical and biological processes in the river ecosystem itself (Zhang et al. 2019). There is a relationship between the quality of surface and pore waters, especially in the hyporheic zone. This porous zone is situated beside and under the river flow, where the mixing between surface water and upper ground water usually occurs (Nelson et al. 2019). The relationship between surface water, pore water, and groundwater becomes part of the hydrology cycle in the water environment. Those surface water and groundwater processes naturally occur biogeochemically powered by kinetic energy, pressure, and the chemical potential of water flows caused by hydraulic gradients and concentrations (Notodarmojo 2005).

The adsorption and desorption process of heavy metals between the sediment and the overlying water depends on several factors, such as pH, which later affects the fate of the heavy metals whether in surface water, into finer suspended solids, or removed to suspended solids in the surface sediment (Rahim et al. 2019); (Xie et al. 2018). The presence of iron and manganese oxide is another factor that affects the adsorption and desorption process (Maslukah 2013) in addition to salinity and DO concentrations (Atkinson, Jolley, and Simpson 2007).

In this study, heavy metal concentrations in pore water were higher than in surface water. When considering the study area, which consists of laterite soil, naturally containing iron, nickel, manganese, and cobalt (Lintjewas, Setiawan, and Kausar 2019), the wash-out phenomena became possible. Due to run off, the laterite soil entered the river water and settled down on the bottom or side areas along the river flow. The sample collection at the end of the wet season allows for a sufficient period for heavy metals to enter the river water.

In the domestic and city areas (i.e., St. 4–8), the iron and manganese concentrations were higher than in the downstream area (Fig. 4.2). The population activity contributed more heavy metals that sank into the sediment. As previously mentioned, more than 40% on average of the land use is paddy fields (BPS-Statistics of Sumedang Regency 2018); therefore, agricultural product residues concentrate in the city area. Not only limited to pesticides, the use of fertilizers with metal-rich contents may cause the occurrence of heavy metal concentrations in the river due to run off. For the downstream area in particular (i.e., St. 9–11), the concentrations of iron, manganese, and cobalt significantly increased with the river flow. This data proved the consistency of chemical heterogeneity in pore water with flow path (Nelson et al. 2019).

4.4 Conclusion

The heavy metal concentrations in pore and surface waters obtained from 11 sampling stations along the upstream, city area, and downstream areas of Cipeles River, Indonesia, were determined. A spatial analysis of the heavy metal concentrations simplified the evaluation of heavy metal distributions in each location. While the

dominant heavy metals, iron and manganese, were the results of run off from the laterite and alluvial soils in the study area, the presence of other micro concentrations of heavy metals helps indicate the source of pollutants. The increasing concentrations of heavy metals in the city center showed anthropogenic results of nonmining activities, such as fertilizer and pesticide usage in agriculture, which contributed to the heavy metal concentrations in the surface water. As a result, the heavy metal occurrence in the pore water was Mn>Fe>Ba>Co>Zn>Cu>Pb>Cr in the order of abundance, while in the order was slightly different for the surface water, i.e., Fe>Mn>Zn>Ba>Cu>Pb>Co>Cr. This result was also described by the HMPI calculation, which compared the metal concentrations in the sample with the water standard for class III, where the pollution level tends to increase from low levels in upstream areas to medium-to-high levels in city and downstream areas, respectively. Considering the water sample in this study, there was a significant correlation between heavy metal concentrations in pore and surface waters, especially for manganese and cobalt in the city area from agricultural product residues, while zinc and barium in the downstream area were dominantly contributed by the Cimanuk River. To improve our understanding of ecotoxicology, the correlation between these heavy metals at the pore and/or surface waters with the toxicity to the aquatic organisms will provide interesting results in further studies.

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Chapter 5

Lethal and Sublethal Effect on Early-life Stage of Zebrafish by Organophosphorus Phorate Exposure

5.1 Introduction

Fish Embryo Toxicity (FET) test using zebrafish *Danio rerio* have shown good correlation with the acute fish toxicity test on more than a hundred chemical substances, promising the way to 3Rs principle – Replace, Reduce, Refine - on toxicity testing (Lammer et al., 2009). Beside zebrafish as aquatic vertebrates, other fishes also use e.g. Japanese medaka (*Oryzias latipes*) and fathead minnow (*Pimephales promelas*)(Braunbeck & Lammer, 2006). Zebrafish has several advantages, i.e. their originality around Central Asia (Engeszer et al., 2007), readily maintainable, relatively inexpensive, and has transparent embryo (Braunbeck & Lammer, 2006)(Levin et al., 2004)(Hill et al., 2005)(Zhang et al., 2017). The latest will helps the observation of embryo development on the toxicity test. Further, the model using fish embryo shows its possibility for the integrative risk assessment of chemicals (Scholz et al., 2008), including pesticide.

The improved knowledge of pesticides, especially on the adverse effect of its persistence in the environment, emerges the awareness to alter pesticide usage. Organophosphorus (ORP) has less persistent and likely to use in recent agriculture to replace the previously banned organochlorine (ORC) (edited by Michael A.Kamrin,

2000),(Park et al., 2006). On the other side ORP, which classified as Acetylcholinesterase (AChE) inhibitors(Halim et al., 2018)(Sekiyama et al., 2015), is more toxic compare to ORC(edited by Michael A.Kamrin, 2000). Its occurrence in the water environment makes the aquatic organism vulnerable as the first receptor.

Phorate, also known as phosphorothioic acid, *O*,-diethyl *S*-(ethyl thio)methyl ester ($C_{17}H_{17}O_2PS_3$), is the ORP insecticide which commonly uses in agricultural activity. Phorate which has the chemical structure as shown in Fig. 5.1 is a highly toxic compound in EPA toxicity class I and classified as a Restricted Use Pesticide (RUP) (edited by Michael A.Kamrin, 2000). It has solubility 50 $mg.L^{-1}$, which categorized as slightly soluble in water.

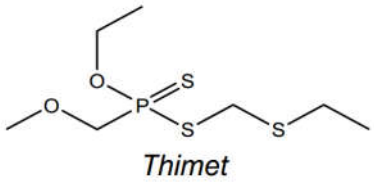

Fig. 5.1 Chemical structure of phorate, or commercially known as Thimet

The toxicity level of phorate to aquatic organisms, which expressed in LC_{50}, has been performed on previous studies, as listed in Table 5.1. Rainbow trout represent the cold-water fish, bluegill sunfish is the warm-water fish, meanwhile northern pike is freshwater fish at northern part likewise Canada, Ireland, and North America. USEPA has categorized the toxicity level for freshwater organisms, which the highest toxicity category is expressed on LC_{50} below 100 $\mu g.L^{-1}$ (Davy & Angier, 2008).

Fish embryo toxicity test using zebrafish *Danio rerio* has been developed and showed a positive correlation with fish acute toxicity test on several fish species

(Lammer et al., 2009). All the comparison was set on 48 hours or 96 hours acute to compare whether the lethal and sublethal effects. The fish embryo toxicity test using zebrafish showed the possibility to alter the fish acute toxicity test (Embry et al., 2010).

Table 5.1 96-hour LC_{50} of phorate on several fish

Aquatic organism	LC_{50}, $\mu g.L^{-1}$	Ref.
Northern pike (*Esox lucius*)	110	(edited by Michael A.Kamrin, 2000)
Channel catfish (*Ictalurus punctatus*)	280	(edited by Michael A.Kamrin, 2000)
Rainbow trout (*Onchorynchus mykiss*)	19,800	(Shophiya et al., 2016)
	13	(Wagner et al., 1996), CERC-USGS
	45	ECOTOX [a)]
Coho salmon (*Oncorhynchus kisutch*)	67.34±3.41	(Lavado et al., 2011)
Spot (*Leiostomus xanthurus*)	5	(FishBase, 1987)
Fathead minnow (*Pimephales p.*)	250	ECOTOX [b)]
Bluegill sunfish (*Lepomis macrochirus*)	1	(Wagner et al., 1996)
	2 - 4	CERC-USGS
	4.9	ECOTOX

[a)] https://cfpub.epa.gov/ecotox/search.cfm ,
[b)] https://www.cerc.usgs.gov/data/acute/multiselect.asp

After 96-hours post fertilization (96-hpf), the zebrafish larvae will continue to swim-up to the water surface on next 24 hours. This capability to swim-up to water surface has been proven as one stage of next stage of zebrafish larvae to survive. Once achieved to the surface, zebrafish larvae will inflate their swim-bladder to help them to control their buoyancy in the water (Lindsey et al., 2010). This phenomenon will deliver the other observation sublethal endpoint in toxicity test, beside hatching rate or the other visible endpoint. This recent study would determine the acute lethal and sublethal toxicity test of phorate exposure on zebrafish early-life stage on prolonged period.

5.2 Method

5.2.1 Material

Phorate standard and acetone solvent at analysis grade were obtained from Wako® (Osaka, Japan). The conductivity of zebrafish culture water was maintained using synthetic saltwater ReiSea®(Japan).

5.2.2 Zebrafish culture

Zebrafish cultures in the recirculation water system of multi-stages filtered water, i.e. filter pad, active carbon filter, biological filter and UV light disinfection. The reverse-osmosis (RO) water used as the make-up water. The water temperature maintains at 27$\pm$1°C, conductivity 0.5 $mS.cm^{-1}$, pH 6-7, recirculation flowrate at 3.0$\pm$0.5 $mL.s^{-1}$. The artificial photoperiod was set on light and dark 14h:10h. Zebrafish was obtained from local pet shop in Kyoto city and acclimated for one month in the culture water. During the acclimated and test, the fish were fed by dry flake-food twice and brine shrimp (*Artemia nauplii)* once a day. For collecting the eggs, the male and female (ratio 2:1) were place separately on chamber completed by mesh trap at the bottom of water tank before light off-set, as shown in Fig. 5.2. The barrier between male and female was release on breeding phase, when the light on-set, and let for 30 minutes. The eggs would be collected at the bottom of the tank. After rinsing with dilution water, the fertilized eggs selected under microscope on 20x magnification, then placed in each chamber test.

Fig. 5.2 Breeding chamber

5.2.3 Phorate exposure

The exposure test was carried-out on static, non-renewal, with the control and dilution water using zebrafish culture water. Each embryo was put into each well of non pyrogenic 24-well plate, where 4(four) wells for internal control using culture water. Two milliliter phorate on five level concentration of 10^0, 10^1, $10^{2,}$ $10^{3,}$ 10^4 $\mu g.L^{-1}$ was put in each well. The plate was incubated in 26±1°C on 120 hours, and observed every 24 hours under microscope Zeiss Axiovert25 100x, as shown in Fig. 5.3. One plate negative control, internal control, fertilization rate above 70%, and lethal rate of internal control under 10% were conducted to ensure the accuracy of the test.

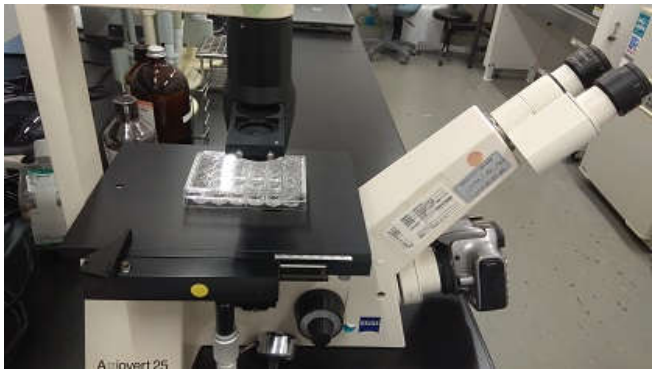

Fig. 5.3 FET observation under magnification 100x

5.2.4 Endpoint and Calculation

The endpoint of the acute test was defined as the lethal effect and sublethal effect. The sublethal effect was expressed in accumulated hatching rate and swim-up failure rate. Lethal rate was defined as the lethal embryo divided by the number of embryos in each plate, without internal control. Hatching rate was calculated based on total number of embryos successfully hatched divided by 20, while swim-up failure was calculated by the number of embryos that fail to swim-up at 120-hpf, including the lethal embryo, divided by 20, number of embryos in treatment plate. All the rates were multiplied by 100 to expressed in percentage.

The experiment was conducted in triplicate and the data was performed in mean and standard deviation. Statistical significance was determined using Two-way ANOVA where $P<0.05$ was considered as significantly different from the control. The LC_{50} was determined using Multi Probit Analysis (MPA) in Acute-to-Chronic Estimation (Ace v 2.0) with Time-Concentration- Effect Model (USEPA, 2003). The No Effect Concentrations were estimated using Accelerated Life Testing (ALT) also from the same software as MPA. Meanwhile the EC_{50} was determined using probit analysis by regression analysis in Microsoft Excel® v.2016 (Redmond, WA, USA).

5.3 Result and Discussion

5.3.1 LC_{50} on Phorate Exposure

Lethal effect was identified by coagulation of the embryo, lack of somite, non-detachment tail and lack of heartbeat, as mentioned in the procedure (OECD, 2013). Compare to normal embryo, the coagulated embryo appears milky white or dark under

microscope, as shown in Fig. 5.4(b).

Regarding Fig. 5.5, lethal effect had occurred in every level of concentration, since the first 24-hpf observation. It continued to occur until 72-hpf, and there is no significant increasing after 96-hpf or 120-hpf. It mainly caused that the survived embryo already levelled up into next stage, where the higher stage of embryo become less vulnerable (Hill et al., 2005). After 72-hpf, all the embryo already hatched, which will be discussed in next subchapter.

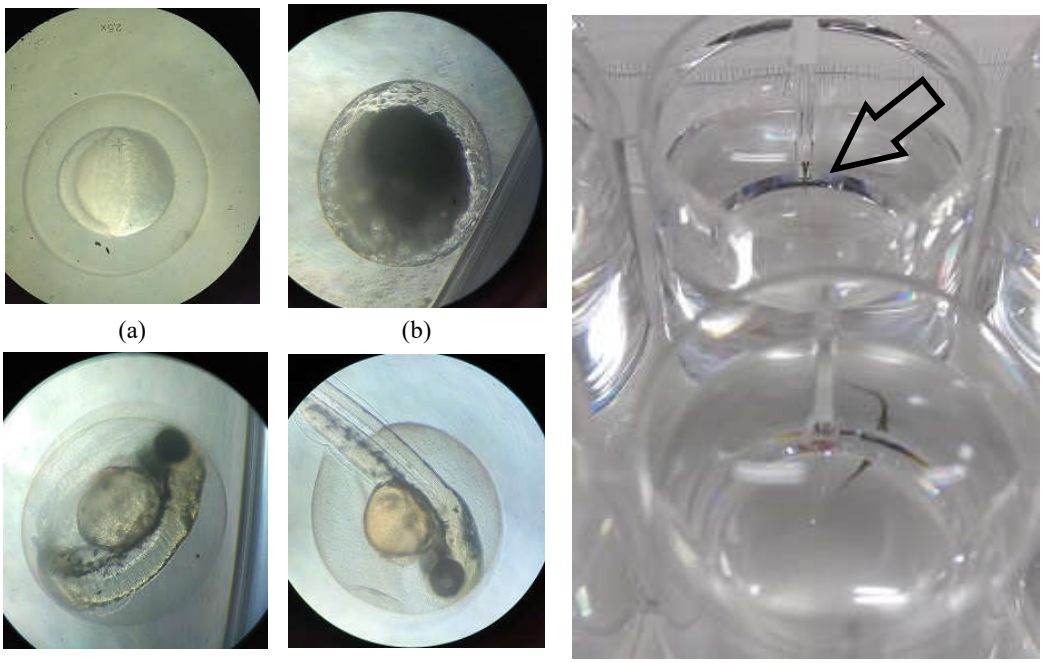

Fig. 5.4 The development of zebrafish embryo and larvae; (a) normal embryo at 5-hpf; (b) coagulated embryo; (c) at normal 48-hpf before hatching; (d) at normal after hatching; (e) at normal after 96-hpf, the larvae on vertical position to water surface (arrow)

While, compared to the control, even at 10^0 µg.L^{-1} was higher than 10^1 µg.L^{-1} and 10^2 µg.L^{-1}, the accumulation lethal rate significantly increased equally with the increasing of phorate concentration from 10^2 µg.L^{-1}, 10^3 µg.L^{-1} until 10^4 µg.L^{-1}. At

10^4 µg.L^{-1}, the lethal rate had achieved more than 50% of the total embryos in the test plate. Based on the significance analysis, there is a statistically significant difference between control and both treatment of phorate concentration and exposure time (two-way ANOVA, *P<0.05*) to the lethal effect.

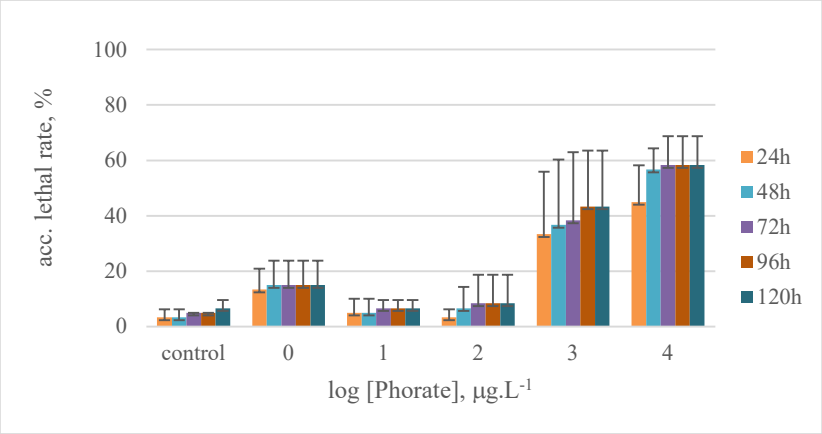

Fig. 5.5 Lethal effect on 120-hours test. Data was expressed in mean and standard deviation (*n=3*)

The lethal concentration on 50% population (LC_{50}) on recent phorate exposure was determined using the MPA, where the linear probit versus concentration in logarithmic scale was shown in Fig. 5.6. The MPA calculation was revealed at 4,537 µg.L^{-1} or about 4.54 mg.L^{-1} (see Appendix I.D.). Regarding previous study (Belanger et al., 2013), where the regression relationship between FET test-zebrafish and acute fish toxicity, i.e. log FET LC_{50} = (0.989 × log Fish LC_{50}) – 0.195; (n = 72 chemicals, r = 0.95, $p < 0.001$, LC_{50} in mg.L^{-1}), the FET LC_{50} in this study 4.54 mg.L^{-1} would result the Fish LC_{50} 7.27 mg.L^{-1}. This value is lower than LC_{50} on Rainbow trout (*Onchorynchus mykiss*) 19,800 µg.L^{-1} (Shophiya et al., 2016). But it is 25-30fold higher than LC_{50} of channel catfish (*Ictalurus punctatus)* or fathead minnow (*Pimephales p.)* in Table 5.1. It has revealed on the previous study that phorate was not embryotoxic to

rabbit until 1.2 mg/kg bw per day (FAO, 2005), the dose value that has caused maternal toxicity effect. This weaker toxicity on lethal effect also mentioned on previous study, where the insensitivity of early-life stage of zebrafish to the neurotoxicant became the reason. The oxygen supply on early-life stage of zebrafish is mainly provided through their skin, which is differ on adult fish. The acute lethal as the response on respiratory by signal interference of neurotoxicant would not show in early-life stage of fish (Klüver et al., 2015).

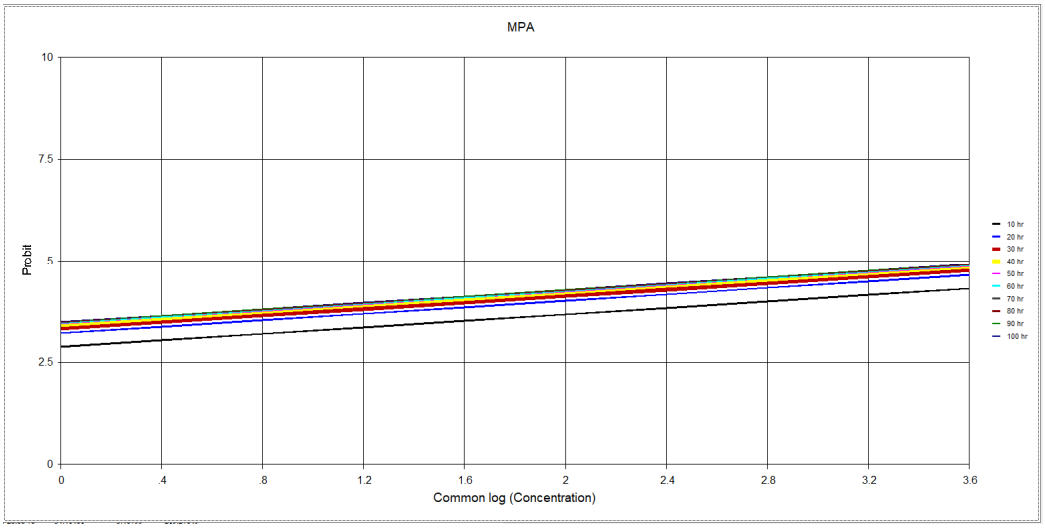

Fig. 5.6 Linear relationship between concentration and probit of phorate exposure for each exposure time

The chronic estimation on No Effect Concentrations by ALT, shown in Fig. 5.7, was determined as 10% mortality (LC_{10}), where the value for 30 days was 7.67 $\mu g.L^{-1}$, for 60 days was 4.98 $\mu g.L^{-1}$, and for 90 days was 3.87 $\mu g.L^{-1}$. The calculation was attached in Appendix I.E.

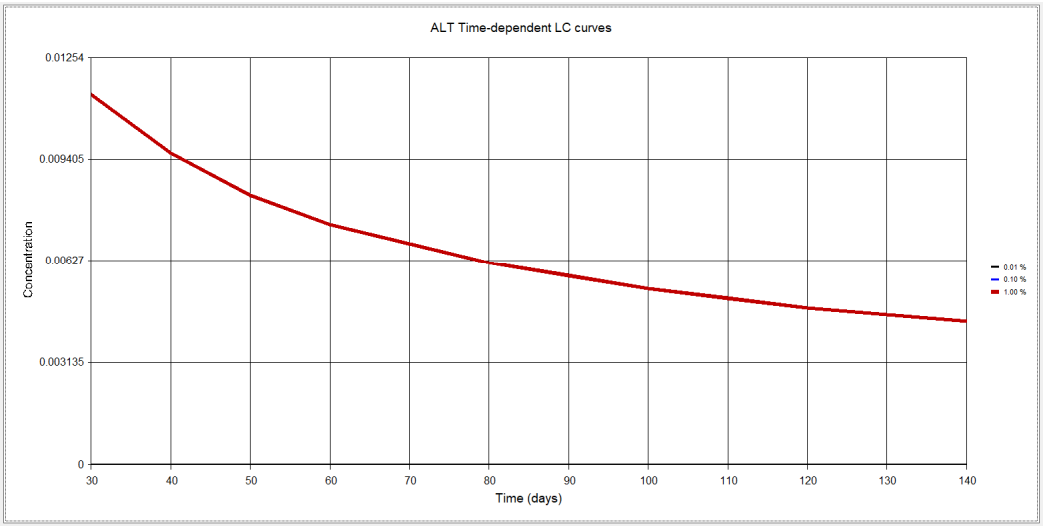

Fig. 5.7 Chronic estimation on NOEC

5.3.2 EC_{50} on Phorate Exposure

The EC_{50} endpoint in this study is divided into two effect, i.e., on hatching rate and swim-up failure.

In determining the effect of phorate exposure to hatching ability, the accumulation hatching rate was considered when the zebrafish embryo successfully hatch out from its chorion, as shown in Fig. 5.4(d). In general, the hatching ability also depends on temperature where the zebrafish embryo test plate was incubated. Usually, on 28°C the embryo begins to hatch at 48-hpf and delay until 72-hpf when the temperature as low as 24°C (Villamizar et al., 2012). In this study, where the culture temperature was set on 26±1°C, the embryos mostly hatching at 72-hpf, includes the control, as shown in Fig. 5.8. So, in this study, the temperature affects the hatching delay compare to the control. Similar to accumulated lethal rate, the hatching rate achieved their maximum value at 96-hpf and no additional hatching embryo in a prolonged period 120-hpf.

On phorate concentration 10^0 µg.L^{-1}, hatching rate was lower compare to the control. It might be caused by the lethal rate on this concentration also slightly higher compare to the control, 10^1 µg.L^{-1} and 10^2 µg.L^{-1}, which reduces the number of embryos that could successfully hatch on that concentration. Further, the hatching rate decreased when the phorate concentration increased to 10^3 µg.L^{-1} and also 10^4 µg.L^{-1}. The larvae in higher concentration found difficulty on hatching and dechorionated, even they showed normal formation, blood circulation, and heartbeat. Based on the significance analysis, there is a statistically significant difference between control and both treatment of phorate concentration and exposure time (two-way ANOVA, $P<0.05$) to the hatching rate.

The EC_{50} on the hatching rate showed on the concentration of phorate 9,746.01 µg.L^{-1}. It means the phorate exposure above 9,746.01 µg.L^{-1} will cause more than 50% zebrafish embryo's had adverse effect on hatching ability.

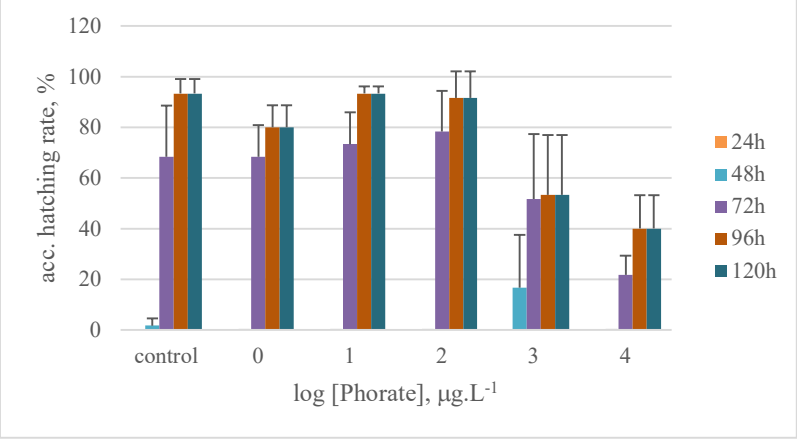

Fig. 5.8 Sublethal effect of hatching rate on 120-hours test. Data was expressed in mean and standard deviation ($n=3$)

While, the effect of phorate exposure to the zebrafish larvae swim-up failure was easy to observed after 96-hpf until 120-hpf, after vertical position along their way to the water surface, as depicted in Fig. 5.4(e). The swim-up failure rate raised in

percentage when the phorate concentration increased, as shown on Fig. 5.9. , even statistically insignificant (*P>0.005*). The previous study mentioned that certain ORP pesticide has affected the swimming behavior of zebrafish (Levin et al., 2004).

Base on Probit analysis at 120-hpf, the EC_{50} on the swim-up failure in this study was showed at phorate concentration 2.14 $\mu g.L^{-1}$. It means the phorate exposure above 2.14 $\mu g.L^{-1}$ would give the swim-up failure effect to more than 50% population. Compare to previous study, the EC_{50} on slowing zebrafish swimming activity was found at 100 $\mu g.L^{-1}$ of chlorpyrifos(Levin et al., 2004). The value from present study was lower from previous reference. Chlorpyrifos categorized as moderately toxic ORP with toxicity class II, less toxic than phorate which categorized as toxicity class I (edited by Michael A.Kamrin, 2000). If the assumption was taken in spite of neglecting the toxicity level of those pesticides, it may assume that embryo stage more vulnerable than the adult stage on receiving the effect of chemical exposure.

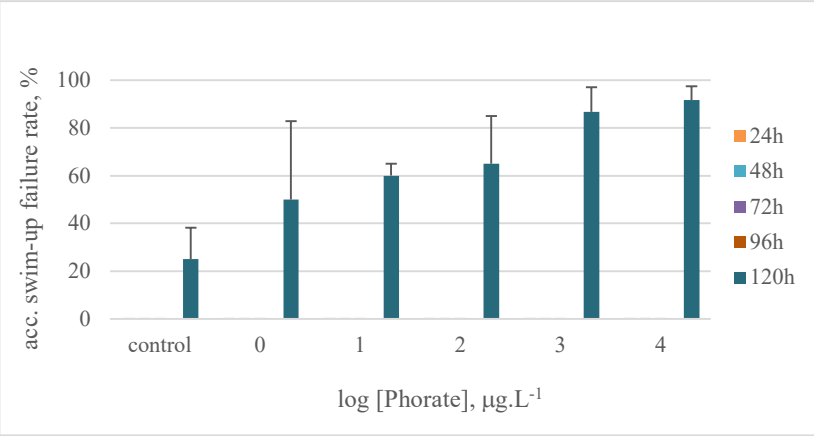

Fig. 5.9 Sublethal effect of swim-up failure rate on 120-hours test. Data was expressed in mean and standard deviation (*n=3*)

5.4 Conclusion

The determination of acute toxicity level on early-life stage of zebrafish induced by ORP phorate has been conducted. The increasing of phorate concentration and the exposure time give statistically significant difference with the control on lethal effect and sublethal hatching rate. The recent study revealed that the lethal LC_{50} was 4.54 $mg.L^{-1}$. The estimation of no effect concentration (NOEC) was found 7.67 $\mu g.L^{-1}$ at 30 days. While the sublethal EC_{50} on hatching rate and EC_{50} on swim-up failure was 9.746 $mg.L^{-1}$ and 2.14 $\mu g.L^{-1}$, respectively. The phorate concentration treatment affected into swim-up rate, even statistically not significant. Only the exposure time gave the significant difference to swim-up ability of zebrafish larvae, so the prolonged period 120-hpf were significant to be monitored as proposed. Rely on the result of lethal effect, the prolonged FET give other information about LC_{50} value of zebrafish early-life stage. Even the lethal effect endpoint was higher than the fish acute toxicity, however this prolonged FET could be used as the previous screening to fish acute toxicity to support the 3Rs principle.

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Chapter 6

Whole Effluent Toxicity Test of River Water Sample using Early-life stage Zebrafish

6.1 Introduction

The Whole Effluent Toxicity test (WET test) is a procedure to determine the toxicity level from effluent without concerning specific parameter as other chemical toxicity test. This method which is known as other term, e.g. Whole Effluent Assessment (OSPAR), Effluent Toxicity Test (in Canada), etc. (ECETOC, 2004) supports the environmental monitoring and aims to prevent the effluent directly discharge into the water environment without treatment. Some countries in Asia also started to implementing the procedure, e.g. Korea in 2011 and Taiwan in 2015, while Japan had published the draft testing on 2013 (Yamamoto, 2018).

USEPA has published the procedure in year 2000 (USEPA, 2000) as the modification of previous version and also modified the method for the short-term acute toxicity test (USEPA, 2002a) and estimating chronic toxicity test methods (USEPA, 2002b) for freshwater organisms. Acute test organisms are fish, daphnid, and green algae, while the chronic test applies on daphnid, mysid and fish. The test carries out on 24 or 96 hours depend on the objectives of the study. All the test requires minimum sample volume 15mL (for daphnids) and 1 L (for fish) at each concentration.

Fish Embryo Toxicity (FET) test was proposed on 2006 (Braunbeck & Lammer,

2006b) and published by OECD as the guidelines for chemical testing using zebrafish (*Danio rerio*) embryo (OECD, 2013). This test carried out using less chemical volume, only 2 mL on each concentration, so less consume of budget and also minimize the chemical waste. The usage of zebrafish embryo also gave another advantage which has shorter early-life stage compare to other organism that usually use for toxicity test. Zebrafish hatches in first 48-hpf, meanwhile nile tilapia 120-hpf and Japanese medaka in 9dpf (Iwamatsu, 2004). In spite of these advantages, FET test could be carried out on WET test.

On WET test, to understand the absolute toxicity of the sample, the culture water is used as the control and dilution water (USEPA, 2000). Therefore, the zebrafish culture water was monitored to ensure the quality of the control and dilution water. Since the zebrafish was found in freshwater nature (Engeszer et al., 2007), (Scholz et al., 2008), the culture water system was prepared in appropriate and similar condition, included its salinity. Zebrafish has optimum range of salinity, related with the conductivity, to keep the normal ammonia excretion from its body (Uliano et al., 2010). So, when mixing the field water sample with certain salinity of culture water, it was important to ensure the mixing water effect to development of zebrafish embryo, to prevent the bias of toxicity test result later.

The study area is an agricultural near domestic area where the agricultural product was entered into the environment during the activity. Non-mining industrial and food industry were existed there. The WET test was interesting to be conducted there since the source of pollutant was a non-point source.

The WET test usually conducted in 24 or 96 hours, depend on which lethal or sublethal endpoint will become the focus. In this study, the ability of hatching larvae

to swim-up to the water surface, was observed. It related with the possibility to survive on the later stage. This ability usually appears in 120 hours post fertilization (hpf). So, this study aims (i) to determine the lethal and sublethal level of salinity variation exposure on early-life stage zebrafish and (ii) to determine the lethal and sublethal level of field water sample exposure on early-life stage zebrafish at prolong-monitored period, 120-hpf.

6.2 Method

6.2.1 Zebrafish culture

Zebrafish was cultured in the recirculation water system using synthetic saltwater ReiSea® in filtered water and reverse-osmosis (RO) water as the make-up water. The water temperature maintained at 27±1°C, conductivity 0.5 $mS.cm^{-1}$, pH around 6, and the photoperiod light and dark 14h:10h. Zebrafish was obtained from local pet shop in Kyoto city and acclimated for one month in the culture water. During the acclimated and test, the adult fish consisted of female (0.65±0.1 gram) and male (0.50±0.1 gram), were fed by dry flake-food twice and brine shrimp (*Artemia nauplii)* once a day. For collecting the eggs, the male and female (ratio 2:1) were placed separately on chamber completed by mesh trap at the bottom of water tank before light off-set, as shown in Fig. 6.1. The barrier between male and female was release on breeding phase, when the light on-set, and let for 30 minutes. The eggs were collected at the bottom of the tank. After rinsing with dilution water, the fertilized eggs were selected under a microscope on 20x magnification, then placed in each chamber test.

Fig. 6.1 Breeding chamber

6.2.2 Whole Effluent Toxicity

The WET test was static, non-renewal, with the control and dilution water of zebrafish culture water. Each embryo was put into each well of 24-well plate, where 4(four) wells for internal control using culture water. Two milliliter sample was put in each well. The plate was incubated in 26±1°C during 120 hours and observed every 24hours under microscope Zeiss Axiovert25 100x. One plate of negative control, internal control, fertilization rate above 70%, and lethal rate of internal control under 10% were conducted to ensure the accuracy of the test.

The endpoint of the acute test was defined as the lethal rate and sublethal effect, expressed in accumulated hatching rate and swim-up failure rate. Lethal rate was defined as the lethal embryo divided by the number of embryos in each plate, without internal control. Hatching rate was calculated based on number of embryo in test chamber that successfully hatched, meanwhile the swim-up failure was calculated by the number of embryos that fail to swim-up at 120-hpf, including the lethal embryo, divided by number of embryos in each plate.

6.2.3 Sample Collection

Field water samples were collected on late wet season (April 2018) from eleven locations around the agricultural area in Cipeles River, the main tributary of Cimanuk River in West Java Province, Indonesia, as shown in Fig. 6.2. All the samples were imported under the permission of the Minister of Agriculture, Forestry and Fisheries, Japan in accordance with the Plant Protection Law.

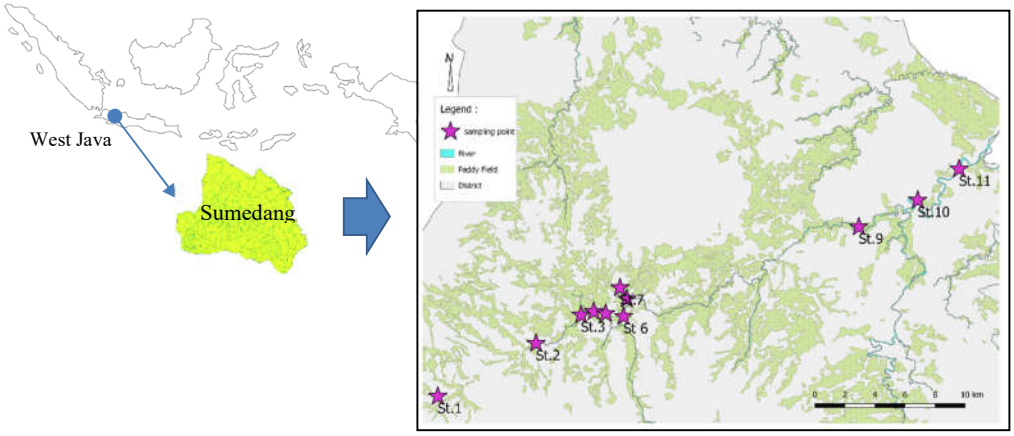

Fig. 6.2 Study Area, Cipeles River at Sumedang District

These water samples were conducted on dilution factor of 0%, 10%, 20%, 40%, and 80%, in two replicates. Dilution factor described the portion of total sample volume being replaced by zebrafish culture water. The selection of dilution water was based on this study objectives to determine the toxicity of the field water sample, so that the culture water was chosen as a dilution water in this study (USEPA, 2000).

6.2.4 Salinity

The field sample measurement resulted the salinity between 0.044 until 0.141 psu (practical salinity unit, equal with ‰), meanwhile the culture water had salinity 0.22 $\pm$ 0.02 psu. Since salinity of field sample lower than culture water, therefore, the

WET test on salinity variation must be included the lowest range of field sample salinity and also the mixture salinity between the field water sample and culture water on 0.04, 0.08, 0.13, 0.17, 0.22psu. Besides that, the test also observed the salinity effect on twice, three-times and four-times of the culture water salinity, i.e. 0.46, 0.71, and 0.96psu. Each salinity was conducted on five replicates. The result in graphic is presented in mean and standard deviation.

The stock 0.96psu-water was made by adding the synthetic seawater salt Rei-Sea II® (Japan) into RO water. Then, the salinity variation was prepared by diluting the stock water. Salinity was measured using conductivity meter Horiba, by converting the salinity value into conductivity, with regression $y = 0.0003x^{1.0614}$ ($R^2 = 0.9998$) where x is conductivity ($\mu S.cm^{-1}$) and y is salinity (psu).

Table 6.1 Salinity of the mixture between field samples and culture water at each dilution factor

	Salinity at each dilution factor, psu				
	0	10%	20%	40%	80%
Culture water	0.220 ± 0.02				
St.1, UPS	0.071	0.088	0.105	0.139	0.206
St.2, UPS	0.068	0.085	0.102	0.137	0.206
St.3, SUB	0.108	0.121	0.134	0.161	0.214
St.4, RIV	0.075	0.091	0.108	0.141	0.207
St.5, CITY	0.044	0.063	0.083	0.122	0.201
St.6, FOOD	0.081	0.097	0.113	0.145	0.208
St.7, FARM	0.141	0.151	0.161	0.181	0.220
St.8, DOM	0.050	0.069	0.088	0.126	0.202
St.9, JUNCT	0.090	0.105	0.120	0.150	0.210
St.10, DOS	0.083	0.099	0.114	0.146	0.209
St.11, DOS	0.076	0.092	0.109	0.141	0.207

UPS, upstream; SUB, suburban; RIV, river; FOOD, food industry; FARM, farming; DOM, domestic; JUNCT, junction; DOS, downstream

6.2.5 Statistical Analysis and Spatial Distribution of Toxicity Test

All variation treatment was compared with the control. The two-ways ANOVA was conducted on lethal and sublethal hatching rate on variance of salinity and exposure time. Otherwise the t-test two samples for equal variance was conducted on lethal and sublethal at specific 120-hpf exposure time. These statistical analyses were applied on Microsoft Excel 2016 data analysis tools. The *P-value<0.05* indicated the significant difference.

Geographical information system usually used for represent the phenomena by overlay analysis in order to easily understanding (Tiwari et al., 2015). For WET test result, the lethal toxicity effect from field water sample was plotted using QGIS v.3.4.

6.3 Result and Discussion

The study will discuss about the lethal and sublethal endpoint of WET test. Firstly, from the salinity variation and later the variation of field water sample.

6.3.1 Lethal Effect of Early-life Stage Zebrafish on Salinity Variation

The accumulated lethal rates were shown in Fig. 6.3. All the samples, compare to the control (culture water) at 0.22 psu, showed less than 10% lethal rate. Since one requirement of FET test is the lethality in control must be less than 10% (OECD, 2013), otherwise the test should be repeated, this study described that the lethal rate of mixed sample exist within the range of the limit test. However, statistically the salinity treatment had significant different than the control at 0.17, 0.46, 0.71, and 0.96 psu (t-test, $P < 0.05$).

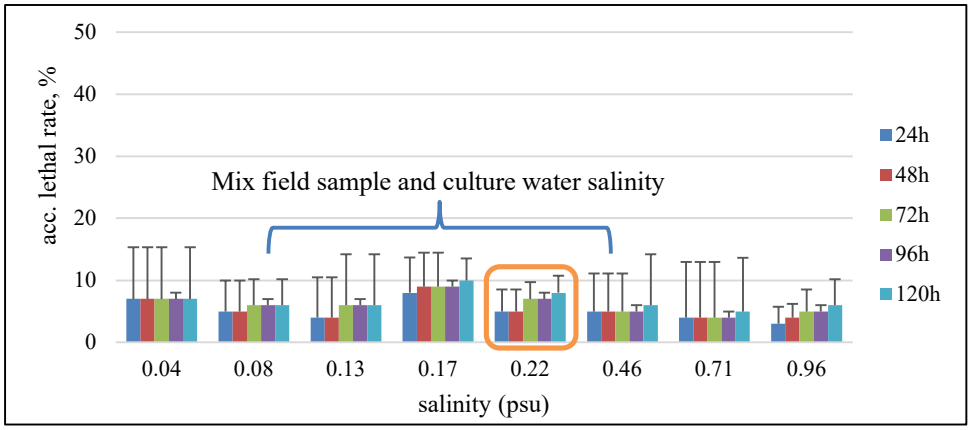

Fig. 6.3 Lethal effect on salinity variation at 120 hours test (*n=5*).
Salinity 0.22 psu was the control (orange bracket)

Base on Fig. 6.3, although the lethal rates were below 10%, the lethal effect increased after 48 hours. On that phase, the embryo already hatching, release from the chorion which had more stable environment. The lethality rate of salinity to zebrafish embryo is depend on the stage of the embryo was exposed (Ord, 2019).

6.3.2 Sublethal Effect of Early-life Stage Zebrafish on Salinity Variation

Sublethal effect was defined as accumulated hatching rate and accumulated swim-up failure rate. The hatching stage usually start at 48-hpf, thus continue at 72-hpf. Regarding Fig. 6.4, at 96-120-hpf mostly 89-94% embryo of salinity variation sample successfully hatched. Statistically, the salinity and exposure time treatment were not different than control (two-ways ANOVA, $P > 0.05$).

Previous study mentioned that zebrafish embryo could tolerated the salinity under 4‰ (psu), without further evidence on salinity lower than 2psu, and the embryo that incubated in salinity water 2psu had the hatching rate around 82% (Sawant et al., 2001). Compare to this study, the hatching rate is quite similar, between 89-94%. So,

the zebrafish survived to hatch on salinity as low as 0.04psu.

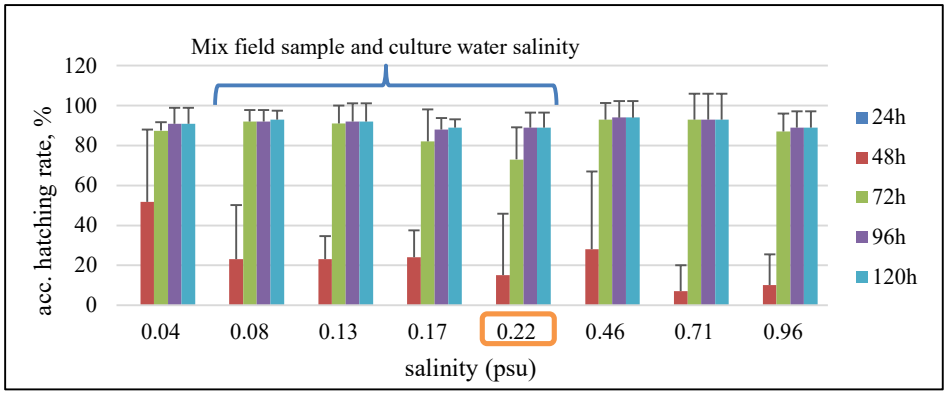

Fig. 6.4 Accumulation of hatching rate on 120-hpf test (*n=5*)

Another sublethal effect was the swim-up failure, where indicated by the hatching zebrafish larvae mostly stay at the bottom of the chamber on 120-hpf. Regarding Fig. 6.5, the swim-up failures were lower when the salinity was below 0.22psu, and tend to increase when the treatment of salinity was above 0.22psu. Statistically the swim-up failure at 120-hpf had significant difference with the control (t-test, $P < 0.05$). It means the salinity treatment effected to the swim-up failure. Since the mixture field sample and culture water salinity was between (0.063 – 0.220)psu (Table 6.1), later the field sample which has salinity above 0.22psu would be considered to have affected by salinity on it swim-up failure rate.

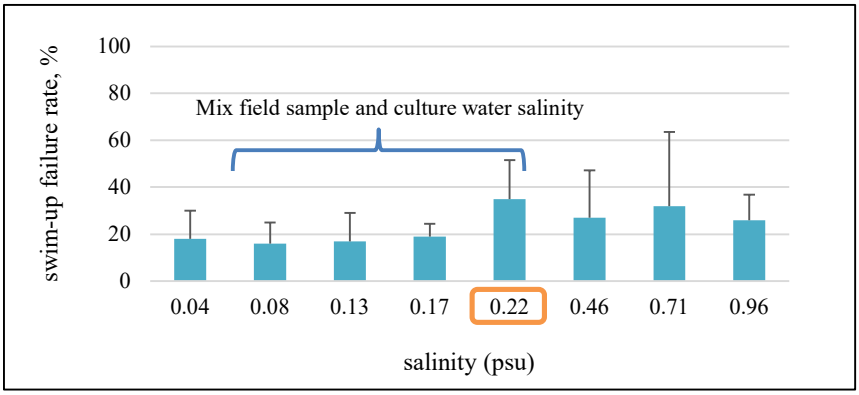

Fig. 6.5 Swim-up failure rate at 120-hpf (*n=5*)

6.3.3 WET test Lethal Effect on Field Sample

As the previous test on salinity variation, the lethal effect for field sample also express in accumulation lethal rate. In laboratory, the embryo showed one of the fact of became dark under microscope observation, lack of somite formation, tail non-detachment, or lack of heartbeat (OECD, 2013). As shown in Fig. 6.6, the lethal effect at each sampling station not linearly performed. Even statistically, significant different and higher than control (*t-test, $P<0.05$*), indicated by asterisk, was showed at the sample from St.1, St.4, and St.7 but the effect of dilution factor on the sample was not linearly performed in this result. When the dilution water was introduced into the field water, especially at 10% dilution, the lethal effect was increased. Thus, the lethal effect was decreased equal with the increasing of dilution factor. Theoretically, larger dilution factor would result smaller lethal rate, since the effluent being diluted in the environment ((Norberg-King et al., 2018). But in present study, the additional dilution water, which has salinity above field samples, seen affected the lethal rate. Regarding the salinity effect discussion on subchapter abovementioned, the salinity treatment at mixture range had significant different from the control at 0.17psu, the value which achieved when field sample was mixed at 80% dilution factor (Table 6.1). This evidence might be led the assumption by several factors since the effluent was consisted of total pollutant in sampling station which may interacted with the salinity in the dilution water.

The spatial plotting was depicted in Fig. 6.7, from the upstream area (St.1 - St.3), city and other activities area (St.4 - St.8), and downstream area (St.9 - St.11). At the city area (insert), highest lethal rate was shown on water sample comes from St.7 (near farming area), and also water sample from St.4 (near city area).

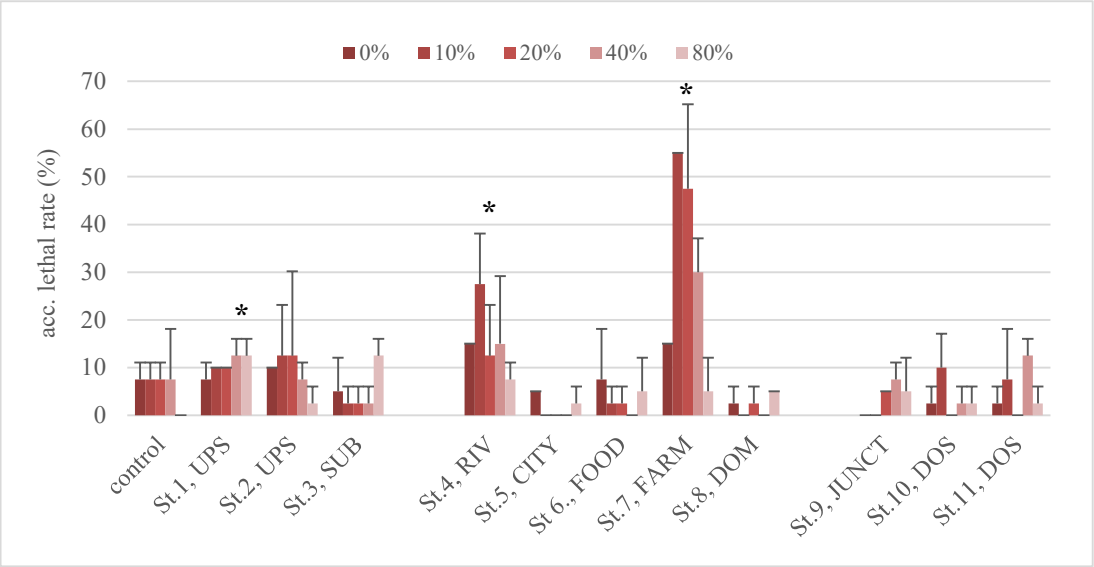

Fig. 6.6 Accumulation lethal rate at 120-hpf on variation of dilution factor (* indicated the statistically significant higher than the control; $p<0.05$)

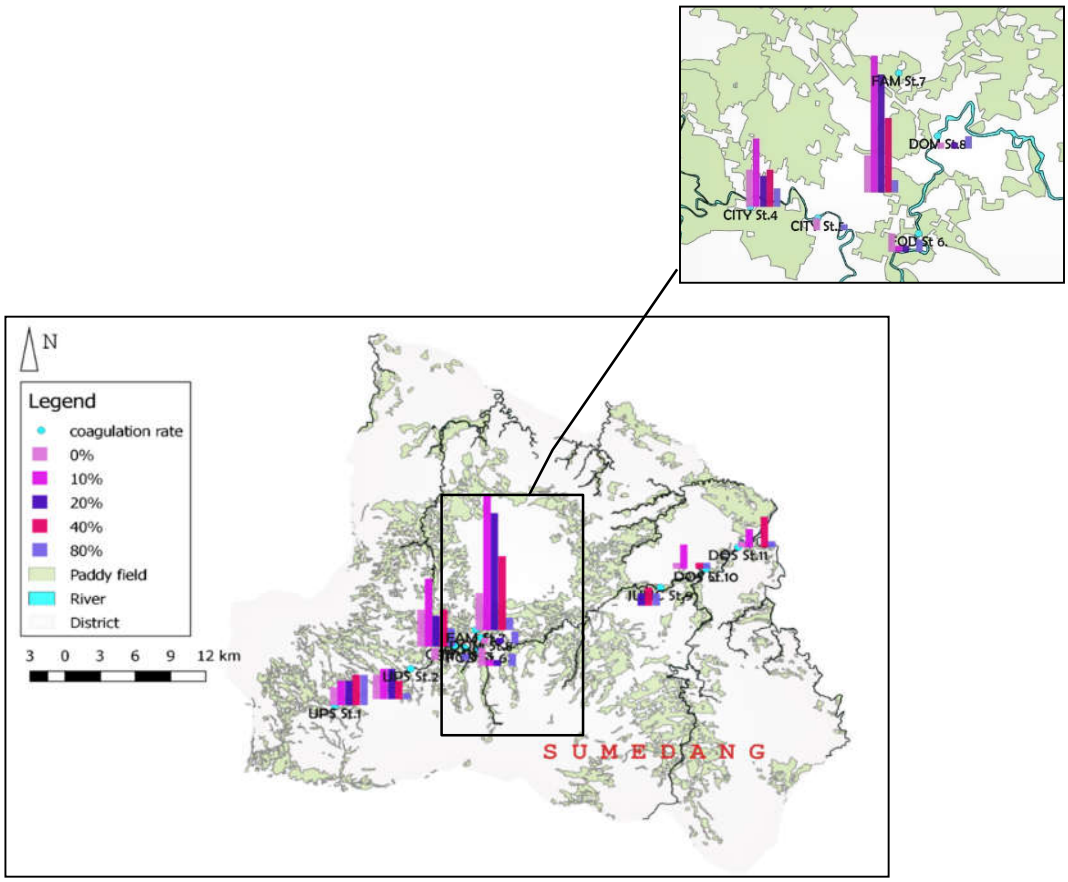

Fig. 6.7 Spatial plotting on lethal effect of field sample

Based on Fig. 6.6, the upstream area had the lethal rate maximum 12.5%, thus the lethal concentration that cause more than 50% lethal of embryos (LC_{50}) was shown by sample from St.7 10% dilution.

The salinity variation had significant effect to the lethality as discussion abovementioned. So that, the correlation analysis was carried out between the salinity of the mixture and lethality rate from each water sample. Based on Table 6.2, the correlation was varied between one water sample and another. Positive correlation indicated the increasing of salinity equal to increasing of the lethal rate, which in this case was shown at water sample from St.1, St.3, St.8, and St.9. Inversely, negative correlation indicated that the increasing of salinity was followed by decreasing of the lethal rate, which shown at water sample from St.2, St.4, and St.7. The other sample from St.5, St.6, St.10, and St.11 had insignificant correlation between its salinity and lethal rate.

Table 6.2 Correlation coefficient between the lethal rate and salinity mixture

Sampling station	R
St.1, UPS	0.85
St.2, UPS	-0.90
St.3, SUB	0.78
St.4, RIV	-0.64
St.5, CITY	-0.09
St.6, FOOD	-0.14
St.7, FARM	-0.55
St.8, DOM	0.57
St.9, JUNCT	0.65
St.10, DOS	-0.26
St.11, DOS	0.04

6.3.4 WET Test Sublethal Effect on Field Sample

The sublethal endpoint for field sample WET test in this study were (a) hatching rate and (b) swim-up failure of zebrafish. The hatching rate from each the sampling station was compared to the control, as shown in Fig. 6.8. Hatching rate which significant different from the control was shown by the water sample from St.4, St.5, and St.8. However, the hatching rate from sample St.5 and St.8 reached almost 100%, which means all embryos successfully hatched at the observation time. The concern especially addressed to the sample St.4, it seemed the compound(s) that contained in the sample suppressed the hatching ability of the embryos, so the embryo failed to hatch. It proved when the dilution factor increased the hatching rate also increased.

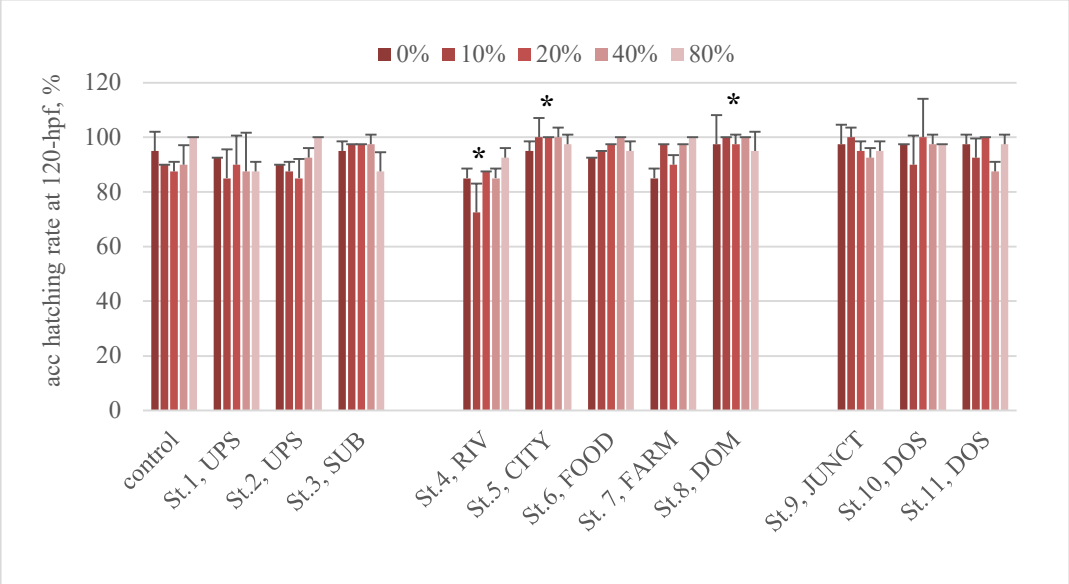

Fig. 6.8 Hatching rate at 120-hpf on each dilution factor (* indicated the statistically significant from the control)

The other sublethal point was the accumulation of swim-up failure, as shown in Fig. 6.9. Statistically, compare to the control, the water samples indicated by asterisk,

from St.1, St.5, and St.7 had significant different higher rate (*t-test, p<0.05*). The sample from St.8 until St.11 had lower swim-up failure.

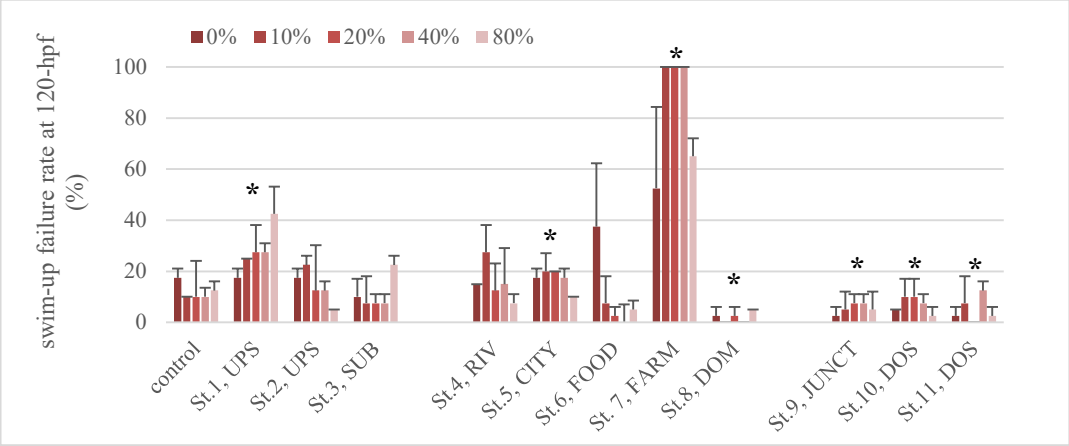

Fig. 6.9 Accumulation swim-up failure rate at 120-hpf on each dilution factor (* indicated the statistically significant from the control)

Regarding the lethal rate result mentioned in previous section, where the water sample from St.4. and St.7 indicated increasing than the control of lethal rate, the highest the swim-up failure rate at 120-hpf also shown by the water sample from St.7. The embryos in sample St.7 succeed to hatch but at 120-hpf fail to swim-up and showed the lethal effect. The salinity effect from dilution water might influence, but not significantly high as the water samples indicated.

From this WET test lethal endpoint, it can conclude that the sample from St.7 with 10% dilution showed LC_{50}, and it reduced since the dilution factor become higher. The other water sample has the lethal concentration less than 15%, especially at downstream area.

As previous study mentioned that hatching rate is not endpoint, it is possible the other sublethal endpoint like failure to swim-up to become consideration on acute test.

Extension period of the acute test into 120-hpf significantly affect the prediction of further toxicity level.

Proposed by previous research (Braunbeck & Lammer, 2006a), WET test using zebrafish embryo is possible to be applied. This will be an alternative to replace the fish acute toxicity test which require larger volume of water sample and high-level stage of testing organism. Around 1-2 L water sample minimum should be prepared for acute static non-renewal test using *Daphnia magna*, fathead minnow, or mysid, and 20-L minimum using *rainbow trout* (USEPA, 2002a).

6.4 Conclusion

WET test to determine the lethal and sublethal level to zebrafish embryo on salinity variation and field water sample has conducted. Salinity treatment has significant effect to lethal rate and swim-up failure, but not significant to hatching rate. Above salinity 0.22psu, the swim-up failure on zebrafish larvae was slightly increase. This study also revealed that zebrafish larvae could survived on salinity as low as 0.04 psu.

From this WET test lethal endpoint, it can conclude that the sample from St.7 with 10% dilution showed LC_{50}. The other water sample has the lethal concentration less than 15%, especially at downstream area.

The prolonged 120-hour acute test could be considered in spite to observe the sublethal effect on swim-up failure. This sublethal effect is easy to observe using simple microscope, being the advantage to be adapt in developing countries. This research only the approaching method to field condition, WET test using local aquatic organism

may be developed to get better conclusion.

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Chapter 7

Ecological Risk Assessment Estimation

7.1 Introduction

The pesticides have been worldwide applied to support the agricultural activities, likewise in Indonesia which country is successfully increased the intensification on National Rice Production Program in 1970-1980 (Rahmianna et al., 2015). Later on, the adverse effect to natural environment and reducing the quality of human health had changed the point of view in pesticide application. The global toxicity issues in previous banned pesticide become the consideration on deciding the regulation, especially which related with agricultural activities. In Indonesia, under Ministry of Agriculture Decree on 2001 where the 37 pesticides were prohibited to be used, and in 2007 had decided 36 prohibited pesticides for all-scope purpose, 2 prohibited household-insecticides and also 5 restricted pesticides. The distribution of pesticide must be registered, where the registration period is controlled by public-accessible website(Ministry of Agriculture Republic of Indonesia, 2019).

The organophosphorus (ORP) pesticide which less persistence in the environment compare to banned organochlorine (ORC)(Stoytcheva & Zlatev, 2011) and efficient as insecticide (X. Li et al., 2010) become the alternative usage on pesticide. This change also happened in Indonesia. The previous study on ORP determination in Indonesia has revealed it occurrence in several matrices, in soil(Supriyadi et al., 2015), surface water (Isworo et al., 2015), and also in blood (Sekiyama et al., 2015)(Halim et al., 2018).

However, the ORP residue occurrence in the water environment and agricultural product has put the risk to ecological and human health. As the Acetylcholinesterase (AChE) inhibitor, this kind of neurotoxicants will interrupt the neuro system, where the symptoms may include incoordination, loss of reflexes, weakness and fatigue (edited by Michael A.Kamrin, 2000). The symptoms depend on the dose and exposure time.

Among ORP pesticides, chlorpyrifos was detected in water samples in Indonesia (Chapter 3). From several sampling stations, chlorpyrifos detected on upstream, city area, and downstream. Otherwise, two other pesticides also detected at upstream area, i.e. terbufos and thiometon, even at lower concentration compare to chlorpyrifos. These pesticides have adverse effect as their occurrence in water environment, especially for aquatic organism. In the case of chlorpyrifos, the previous study has investigated that zebrafish embryo exposed with 0.039 $mg.L^{-1}$ showed the effect on locomotor activity on the first 24-26 hour (Selderslaghs et al., 2010).

Concerning the other parameter than ORP pesticides, ammonia and heavy metal measurement in previous chapters also showed significant increasing (Chapter 1 and Chapter 4). Acute toxicity of ammonia to 29 freshwater fish species was reported between range 0.88 to 4.6 $mg.L^{-1}$ (Australian Government Initiative, 2000) whereas the short term hazardous concentration Zn for freshwater organism at pH 7 was 230.6 $\mu g.L^{-1}$ (X. F. Li et al., 2019).

In this study, the risk characterisation on acute toxicity would be carried out regarding the detected compounds in water sample from one area in West Java Province, Indonesia, i.e. nitrogen-ammonia, zinc, and ORP pesticides. The risk characterization calculation result would be expressed in ratio, which compare to standard Levels of Concern (LOC) and would be useful for recommendation of the risk management.

7.2 Method

Risk assessment regarded the effect characterization and risk characterization. For risk characterization, the risk quotient approach calculation is the most common used (USEPA, 2017). The risk quotient (RQ) is the ratio between the estimation of exposure to the estimation of toxicity. Concentration of exposure was obtained from detected ORP pesticides (Chapter 3), ammonia and zinc in the surface water sample at agricultural area in West Java Province. On calculation the acute risk on aquatic organism, the lowest tested EC_{50} or LC_{50} is used as the endpoint. Data of LC_{50} and EC_{50} for effect characterization of pesticides was collected from previous studies, ECOTOX (https://cfpub.epa.gov/ecotox/search.cfm) and also the Acute Toxicity Database by Columbia Environmental Research Center – United State Geological Survey USGS (https://www.cerc.usgs.gov/data/acute/multiselect.asp). The effect was assumed as the adverse effect on standard freshwater test fish, which considering the study area of the water sample was utilized also as aquatic life habitat (Hermawan, 2010). The zebrafish (*Danio rerio*) data also collected on this study based on recent studies that revealed zebrafish has been used as predicting model vertebrate of acute toxicity of chemical testing.

The effect was defined as lethal and sublethal on early-life stage and adult fish, to recognize the impact of the exposure. The calculation of acute RQ in fish or other aquatic animal obtain by the equation Eq. 7.1 (USEPA, 2017):

$$RQ_{acute} = \frac{concentration\ of\ environment\ exposure}{LC_{50} or\ EC_{50}} \qquad \textbf{Eq. 7.1}$$

The RQ was compare with Level of Concern (LOC) to interpret the risk. Based on toxicity of the compound, the LOC value was categorized, where LOC above 0.5 for acute high risk, above 0.1 for acute restricted use, above 0.05 for acute endangered species, and above 1.0 for chronic were indicated concern of the risk.

7.3 Result and Discussion

7.3.1 Effect Characterization

Chlorpyrifos

Chlorpyrifos ($C_9H_{11}Cl_3NO_3PS$, CAS no. 2921882; MW 350.59 $g.mol^{-1}$) is one of the most insecticides used globally (Levin et al., 2004)(Richendrfer et al., 2012). It could be absorbed into the body by inhalation, skin exposure, or accidentally ingested. The acute and sub-chronic effect might appear by the effect of AChE inhibition, includes the aquatic organism. Chlorpyrifos toxicity to fish may be related to water temperature. The 96-hour LC_{50} for chlorpyrifos is 0.009 mg/L in mature rainbow trout, 0.098 mg/L in lake trout, 0.806 mg/L in goldfish, 0.01 mg/L in bluegill, and 0.331 mg/L in fathead minnow (edited by Michael A.Kamrin, 2000).

The recently developed protocol on zebrafish embryo was defined as the less harmful for the testing organism (Lammer et al., 2009). Therefore, the fish embryo toxicity test was applied on several chemicals likewise the other fish test (Belanger et al., 2013), includes chlorpyrifos, as listed in Table 7.1.

The chlorpyrifos concentration that caused 50% lethal of sample population (LC_{50}) depended on the type of fish. Regarding Table 7.1, goldfish and bluegill were reported more sensitive on receiving the effect of chlorpyrifos, compare to fathead minnow and Japanese medaka, as mentioned in the previous study (Giddings et al.,

2014). Whereas the LC_{50} on zebrafish embryo was higher than LC_{50} value on the fish toxicity test. The insensitivity of the embryo was assumed influence by incomplete development as well as in the adult fish, which indicated the sign of lethal effect (Klüver et al., 2015). Meanwhile, the concentration that exhibit the effect on 50% of sample population (EC_{50}) was depend on exposure type and duration of exposure. The chlorpyrifos 0.039 $mg.L^{-1}$ already affected the locomotor activity on first 24-26 hpf exposure (Selderslaghs et al., 2010). The hatching ability was affected on 9.62 $mg.L^{-1}$ exposure at 96 hpf (Chow, 2009), while the alteration of swimming behaviour initially observed 0.18 $mg.L^{-1}$ at 96 hpf (Selderslaghs et al., 2010) or 0.1 $mg.L^{-1}$ at 144 hpf (Levin et al., 2004).

Table 7.1 Toxicity value for chlorpyrifos on several freshwater trophic level

Species	Effect	Type	Means, $ug.L^{-1}$	Test Duration	Exposure Type	Ref.
Adult Fish						
Bluegill (*Lepomis macrochirus*)	Mortality	LC_{50}	1.7*	96 h	S	a)
Bluegill (*Lepomis macrochirus*)	Mortality	LC_{50}	2.4	NR	S	b)
Bluegill (*Lepomis macrochirus*)	Mortality	LC_{50}	10	96 h	NR	c),d), o)
Bluegill (*Lepomis macrochirus*)	Mortality	LC_{50}	6.5	96 h	NR	e)
Goldfish (*Cyprinus carpio*)	Mortality	LC_{50}	8	96 h	S	d)
Fathead minnow (*Pimphales promelas*)	Mortality	LC_{50}	170	NR	S	b)
Fathead minnow (*Pimphales promelas*)	Mortality	LC_{50}	331	NR	NR	c)
Fathead minnow (*Pimphales promelas*)	Mortality	LC_{50}	207	96 h	F	d)
Fathead minnow (*Pimphales promelas*)	Mortality	LC_{50}	155*	96 h	NR	e)
Japanese medaka (*Oryzias latipes*)	Mortality	LC_{50}	250*	48 h	R	d)
Sheephead minnow (*Cyprinodon v.*)	Mortality	LC_{50}	205	96 h	NR	e)
Western mosquitofish (*Gambusia a.)*	Mortality	LC_{50}	425*	96 h	NR	e)
Zebrafish (*Danio rerio*)	Swimming	EC_{50}	220*	24 h	NR	f)
Embryo/Larvae						
Zebrafish (*Danio rerio*)	Mortality	LC_{50}	430	240 h	S	g)

Species	Effect	Type	Means, ug.L^{-1}	Test Duration	Exposure Type	Ref.
Zebrafish (*Danio rerio*)	Mortality	LC_{50}	3,050*	96 h	NR	h)
Zebrafish (*Danio rerio*)	Mortality	LC_{50}	6,250	144 h	S	i)
Zebrafish (*Danio rerio*)	Mortality	LC_{50}	3,506	144 h	S	j)
Zebrafish (*Danio rerio*)	Mortality	LC_{50}	280	96 h	R	k)
Zebrafish (*Danio rerio*)	Swimming	EC_{50}	100	144 hpf	NR	l)
Zebrafish (*Danio rerio*)	Deformation	EC_{50}	10	120-hpf	S	g)
Zebrafish (*Danio rerio*)	Behaviour	EC_{50}	250	2 h	S	g)
Zebrafish (*Danio rerio*)	Hatching	EC_{50}	9,620	96 hpf	NR	h)
Zebrafish (*Danio rerio*)	Swimming	EC_{50}	180*	96 hpf	S	i)
Zebrafish (*Danio rerio*)	Locomotor act.	EC_{50}	39	24-26 hpf	S	i)
Zebrafish (*Danio rerio*)	Swim speed	EC_{50}	3.5	168 hpf	NR	m)
Zebrafish (*Danio rerio*)	Developmental	AC_{50}	3,156	96 hpf	S	n)
Algae						
Bacillariophyceae	diversity	NOEC	2,000*	42 days	L	p)
Algae	abundance	LOEL	35	42 days	S	p)
Daphnids						
Daphnia magna (juvenile)	Immobile	EC_{50}	0.74	2 days	S	p)
Daphnia magna (neonate)	Immobile	EC_{50}	0.21	4 days	NR	p)
Daphnia magna	Mortality	LC_{50}	0.82*	4 days	S	p)

*indicate the value for RQ calculation. a)CERC USGS; b)(USEPA, 1986), c)(edited by Michael A.Kamrin, 2000), d)(Giddings et al., 2014), e)(Munn & Gilliom, 2001), f)(Tilton et al., 2011), g)(Kienle et al., 2009), h)(Chow, 2009), i)(Selderslaghs et al., 2010), j)(Dishaw et al., 2014), k)(Wang et al., 2017), l)(Levin et al., 2004), m)(Richendrfer et al., 2012), n)(Padilla et al., 2019), o)(Helfrich et al., 2009), p)ECOTOX. Exposure type: S=static, R=renewal, F=flow through, L=lentic. NR=not reported

Terbufos

Terbufos is another ORP that detected in surface water at agricultural area in West Java Province. Terbufos (CAS no. 13071-79-9) is extremely toxic to fish and aquatic invertebrates. The reported 96-hour LC_{50} for terbufos in *Daphnia magna* and a freshwater invertebrate, was 0.31 ug.L^{-1} and 0.2 ug/L, respectively (edited by Michael A.Kamrin, 2000). However, terbufos had moderate potential of bioconcentration (edited by Michael A.Kamrin, 2000). The toxicity values for terbufos on several

trophic level was listed in Table 7.2.

Table 7.2 Toxicity value for terbufos on several aquatic trophic level

Species	Effect	Type	Means, ug.L^{-1}	Test Duration	Exposure Type	Ref
Adult Fish						
Bluegill (*Lepomis macrochirus*)	Mortality	LC_{50}	3.2	24 h	S	a), b)
Bluegill (*Lepomis macrochirus*)	Mortality	LC_{50}	1.8*	96 h	S	a), b)
Bluegill (*Lepomis macrochirus*)	Mortality	LC_{50}	4	96 h	NR	c)
Bluegill (*Lepomis macrochirus*)	Mortality	LC_{50}	1.7	96 h	NR	d)
Sheephead minnow (*Cyprinodon v.*)	Mortality	LC_{50}	4*	96 h	NR	d)
Fathead minnow (*Pimphales promelas*)	Mortality	LC_{50}	390*	96 h	S	a), b)
Fathead minnow (*Pimphales promelas*)	Mortality	LC_{50}	150	96 h	NR	d)
Embryo/juvenile						
Bluegill (*Lepomis macrochirus*)	Mortality	LC_{50}	0.77*	96 hpf	S	b)
Bluegill (*Lepomis macrochirus*)	Mortality	LC_{50}	12.3	96 hpf	S	b),+
Sheephead minnow (*Cyprinodon v.*)	Mortality	LC_{50}	3.4*	96 h	S	e)
Fathead minnow (*Pimphales promelas*)	hatching, abnormal dev.	NOEC	4.18*	until hatch	F	b)
Fathead minnow (*Pimphales promelas*)	growth (weight)	NOEC	2.03	32 d	F	b)
Algae						
Algae	abundance	NOEC	10*	4 days	NR	b)
Dapnids						
Ceriodaphnia dubia (neonate)	Immobile	EC_{50}	0.074	ug.L^{-1}	4 days	b)
Daphnia magna	Immobile	EC_{50}	0.4*	ug.L^{-1}	2 days	b)
Daphnia magna	Immobile	NOEL	0.18*	ug.L^{-2}	2 days	b)

*indicate the value for RQ calculation. [a)]CERC USGS; [b)]ECOTOX, [c)](Helfrich et al., 2009), [d)](Munn & Gilliom, 2001), [e)](Brecken-Folse et al., 1994), [+]technical purity 15% . Exposure type: S=static, F=flow through, NR=not reported

Thiometon

Differ from previous ORPs, thiometon (CAS no. 640-15-3) had limited data of toxicity value on fish. Several data only reported on LT_{50}, i.e. time that caused the 50% lethality of testing organism after exposed of certain chemical, or effect concentration.

The toxicity values for thiometon on several aquatic trophic level that exposed in freshwater media were listed in Table 7.3 .

Table 7.3 Toxicity value for thiometon on several aquatic trophic level

Species	Effect	Type	Means, $mg.L^{-1}$	Test Duration	Exposure Type	Ref.
Adult Fish						
Western mosquitofish (*Gambusia affnis)*	Mortality	NR	8	8 h	R	a)
Rainbow trout (*Oncorhynchus mykiss)*	Mortality	LC_{50}	8	96 h	NR	b)
Rainbow trout (*Oncorhynchus mykiss)*	Cell changes	EC0	50	24 h	NR	a)
Embryo/juvenile						
Western mosquitofish (*Gambusia affnis)*	Mortality	LT_{50}	2	18 h	R	a)
Algae						
Desmodesmus subspicatus	histology	EC_{50}	8.4	3 days	NR	a)
Daphnids						
Daphnia magna	equilibrium	EC_{50}	42	3 days	NR	a)

a)ECOTOX, b)(Anonim, 1988). Exposure type: R=renewal, NR=not reported

These data of toxicity values would be useful for estimating the risk quotient (RQ) of each compound on different sampling sites, which will be discussed in next subchapter.

7.3.2 Risk Quotient calculation

In Technical Overview of Ecological Risk Assessment (USEPA, 2017), RQ for acute toxicity for aquatic animals was regarded the lowest tested EC_{50} or LC_{50} from acute toxicity test, whereas the chronic toxicity was used the lowest NOAEC from early life-stage test. Later, the result of RQ calculation will be compared to the LOC, which has been agreed to be used on analysing the potential risk.

To calculate the RQ, LC_{50} or EC_{50} value was chosen at 96 h exposure time, except on chlorpyrifos exposure on *Oryzias l.* at 48 h and *Danio r.* sublethal effect at 24 h, as indicated by asterisk in Table 7.1. The NOEC (no observed effect concentration) of

terbufos exposure on *Pimphales promelas* also used on sublethal calculation of hatching effect (Table 7.2). For thiometon, since the toxicity data was limited, all the assumption was taken in respect each special condition, as listed in Table 7.3.

The RQs calculation of ORPs were shown in Table 7.4- Table 7.6, while the RQ calculation of ammonia and zinc was listed in Table 7.7.

Table 7.4 Acute and chronic RQ value of ORPs on algae and daphnids

Location	Compound	Acute		Chronic	
		Algae	Daphnid	Algae	Daphnid
St.1, UPS	Chlorpyrifos		**1.45083**	0.00059*	
	Terbufos		**2.33297**	0.09332	**5.18438**
	Thiometon	0.00001	0.00178		
St.4, RIV	Chlorpyrifos		**0.59546**	0.00024	
St.10, DOS	Chlorpyrifos		0.33698	0.00014	

*on 42 days. Bold numbers indicate RQ>LOC

Table 7.5 Acute lethal RQ value of ORPs on of several adult fish and fish embryo/juvenile

Location	Compound	Lethal on adult fish				Lethal on fish embryo/juvenile			
		Lepomis m.	*Pimphales p.*	*Oryzias l.*	*Gambusia a.*	*Lepomis m.*	*Cyprinodon v.*	*Danio r.*	*Gambusia a.*
St.1, UPS	Chlorpyrifos	**0.69981**	0.00768	0.00476	0.00280			0.00019	
	Terbufos	**0.51844**	0.00239			**1.21193**	**0.27447**		
	Thiometon				0.00001*				0.00004**
St.4, RIV	Chlorpyrifos	0.28722	0.00287	0.00195				0.00016	
St.10, DOS	Chlorpyrifos	0.16254	0.00163	0.00111					0.00009

*mortality on 8h; **mortality on 18h. Bold numbers indicate RQ>LOC

Table 7.6 Acute sublethal RQ value of ORPs on of several adult fish and fish embryo/juvenile

Location	Compound	Sublethal on adult fish		Sublethal on fish embryo/juvenile	
		Danio r.	*Oncorhynchus m.*	*Lepomis m.*	*Danio r.*
St.1, UPS	Chlorpyrifos	0.00541			0.00661
	Terbufos			**0.22325**	
	Thiometon		0.000001*		
St.4, RIV	Chlorpyrifos	0.00222			0.00271
St.10, DOS	Chlorpyrifos	0.00126			0.00154

*effect on 24h. Bold numbers indicate RQ>LOC.

Table 7.7 Acute RQ of concerned compounds on freshwater fish

Sampling station	Ammonia (NH_3)	Zn*
St.1	0.30	0.02
St.2	0.03	0.09
St.3	0.05	0.04
St.4	0.25	**2.04**
St.5	0.17	0.11
St.6	0.10	0.24
St.7	**1.69**	0.08
St.8	0.10	**0.76**
St.9	0.10	0.04
St.10	0.06	0.19
St.11	0.08	0.02

*on *Danio rerio*. Bold numbers indicate RQ>LOC

7.3.3 Risk Characterization

The RQ comparison to LOD was assumed in the worst case, where the pesticide categorized as the high-risk compound. Even in this study, chlorpyrifos (General Use Pesticide, (edited by Michael A.Kamrin, 2000) and thiometon categorized as moderate toxic class II. The exception was in the case of terbufos, the Restricted Used Pesticide, which has the LOC 0.1, tighter than LOC on acute high risk 0.5 (USEPA, 2017). The

following risk description also assumed the exposure at single chamber without neither other input nor output from the chamber.

At upstream area St.1, terbufos showed the RQ higher than LOC on acute lethal effect on adult *Lepomis m*., juvenile *Lepomis m.*, juvenile *Cyprinodon v.*, and also the sublethal effect on juvenile of *Lepomis m*. (Table 7.5). Terbufos also caused the acute and chronic risk on daphnids (Table 7.4). This mostly influenced by the restricted-use status of terbufos. The RQ calculation on chlorpyrifos also resulted in the value higher than LOC on acute lethal on daphnids and adult *Lepomis m.* The latest was the most sensitive fish that use in this study.

For St.4, RQ calculation was higher than LOC when chlorpyrifos exposure was compared to the acute lethal effect of daphnids, i.e. 0.595 (Table 7.4), and also when zinc environment exposure compared to short-term hazardous of freshwater organism, i.e. 2.04 (Table 7.7) .

At St.7, the RQ calculation also showed the value higher than LOC, i.e. 1.69, when comparing ammonia to acute toxicity of fish (Table 7.7). Whereas at St.8 the RQ higher than LOC, i.e. 0.76, was shown caused by zinc concentration exposure which is higher than short-term hazardous of freshwater organism. At St.10, all RQ calculations resulted in lower than LOC (<0.5 for chlorpyrifos). However, the RQ on adult *Lepomis m.* by chlorpyrifos exposure indicated more attention should be made to reduce the effect on further exposure.

Based on the abovementioned RQ results and the influence on local aquatic organisms, ORPs detected in the water samples from St.1 and St.4 put the acute lethal and sublethal effect to the local aquatic organisms that have sensitivity as early-life stage *Lepomis m.* and also lower trophic levels, such as daphnids. Since terbufos and

chlorpyrifos were hydrophobic with Kow 50,000 and 33,000 (edited by Michael A.Kamrin, 2000) respectively, they potentially tend to bioaccumulate in the cells of biota (Oost et al., 2003). Therefore, more attention should be addressed to minimize this risk.

Ammonia which detected in sample St.4 and St.8, and zinc that detected in sample St.7, also resulted the level of concern to short-term hazard or acute lethal effect to freshwater organisms.

In the case of *Danio rerio*, the species that proposed as the organism model for predicting the toxicity of chemical on higher vertebrate, even its adult showed insensitivity to lethal effect by ORP chlorpyrifos exposure but either its adult or early-life stage indicates more sensitivity to sublethal effect. Regarding Table 7.1, in the case of effect on swimming already occurred when the embryo of *Danio rerio* was exposed to 100 - 180 $ug.L^{-1}$ on 96-hpf until 144hpf, affect the locomotor activity showed on 39 $ug.L^{-1}$ exposure at first 24-26 hpf, deformation started to occur on 10 $ug.L^{-1}$ exposure at 120-hpf, and alteration on swimming speed already indicated as low as 3.5 $ug.L^{-1}$ exposure at 168 hpf. All the result enriched the possibility of fish embryo toxicity test implementation despite of 3R effort on fish acute toxicity test.

Local aquatic organisms that have their habitat in Cipeles River were possible to use as the testing organism, due to closer risk-related with the problem source. Hermawan (2010) mentioned that the dominant native fish which is locally called genggehek (*Mystacoleucus marginatus*) and seseren (*Cyclocheilichthys sp.*) found almost in every monitoring period. Those local fishes shared the same Cyprinidae family with fathead minnow (*Pimphales promelas*) and zebrafish (*Danio rerio*). Thus, the correlation might be developed to estimate the toxicity level.

All effort concerning the risk characterization result, especially on upstream area, might be supported by many related stakeholders, i.e. agricultural office on socialization on pesticide usage, control the pesticide distribution, together with the cooperation and farmer-union to continuously educate about the importance of Good Practice on making the spraying dosage, the proper period of spraying, and the importance of safety guard equipment usage. It is all to minimize the adverse effect on farmers themselves, nearby relatives, and the environment.

7.4 Conclusion

Risk characterization on detected ORP at the surface water sample on the study area has conducted in this study. Regarding the risk quotient ratio on three trophic levels of aquatic organisms, the station on the upstream area, represent by St.1, has higher concern compared to the other sampling stations, at the city center (St.4) and downstream area (St.10). The ORPs detected in those water samples might put not only the acute lethal-sublethal effect to the local aquatic organisms that have sensitivity as early-life stage *Lepomis m.* but also the chronic effect on the lower trophic level, such as daphnids. The concern also should be addressed to the area of St. 4, St.7, and St.8 considering the ammonia and zinc detected there. The adoption of risk assessment using local aquatic organisms in Cipeles River is possible to develop in further study to describe closer risk-related with the problem source. This risk characterization result recommended a good practical effort, especially in the upstream area.

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Chapter 8

General Conclusion and Future Recommendation

8.1 General Conclusion

The ecological risk assessment on a river water on agricultural area in West Java Province, Indonesia has been conducted. In respect to nonpoint-source pollutant that might enter to a river water, the comprehensive assessment would better to be conducted than single exposure.

The problem formulation established by determining the organophosphorus (ORP) pesticide and inorganic heavy metals in the representative field water samples. Thus, the effect characterization, especially on ORP pesticide, has conducted. By introducing the Whole Effluent Toxicity (WET) test using the early-life stage zebrafish toxicity test, the exposure characterization was studied. This acute toxicity test conducted in the prolonged period to detect the other sublethal effect than previous studies. On the other point of view, the heavy metal pollution index (HMPI) was also analyzed to describe the pollution level with respect to heavy metal. Later on, risk characterization by Risk Quotient ratio (RQ) was determined to indicate the level of concern of the detected ORPs that should be made.

The extraction, separation and detection method of ORP at field water samples has conducted simultaneously in this study. The recovery using multiple ORP standards is in acceptable range, 70.91%, and the coefficient of determination R^2 of each 13 compounds between 0.9891-0.9981. The detection on water sample gave result that chlorpyrifos was detected in the water sample from upstream (St.1), city area

(St.4) and downstream (St.10), at 1.19, 0.49, and 0.28 $\mu g.L^{-1}$, respectively. While terbufos and thiometon were detected at the St.1 at upstream area, 0.93 $\mu g.L^{-1}$ and 0.07 $\mu g.L^{-1}$, respectively. However, the concentration of detected ORP pesticides was below Indonesia regulation of class C for fishery and farming, 100 $\mu g.L^{-1}$.

The heavy metal concentrations in pore and surface waters obtained from 11 sampling stations along the upstream, city area, and downstream areas of Cipeles River, Indonesia, were determined. A spatial analysis of the heavy metal concentrations simplified the evaluation of heavy metal distributions in each location. While the dominant heavy metals, iron and manganese, were the results of run off from the laterite and alluvial soils in the study area, the presence of other micro concentrations of heavy metals helps indicate the source of pollutants. The increasing concentrations of heavy metals in the city center showed anthropogenic results of non-mining activities, such as fertilizer and pesticide usage in agriculture, which contributed to the heavy metal concentrations in the surface water. As a result, the heavy metal occurrence in the pore water was Mn>Fe>Ba>Co>Zn>Cu>Pb>Cr in the order of abundance, while in the order was slightly different for the surface water, i.e., Fe>Mn>Zn >Ba>Cu>Pb>Co>Cr. This result was also described by the HMPI calculation, which compared the metal concentrations in the sample with the water standard for class III, where the pollution level tends to increase from low levels in upstream areas to medium-to-high levels in city and downstream areas, respectively. Special attention was addressed at St.4, which has high concentration of zinc, which later influenced the HMPI calculation. Considering the water sample in this study, there was a significant correlation between heavy metal concentrations in pore and surface waters, especially for manganese and

cobalt in the city area from agricultural product residues, while zinc and barium in the downstream area were dominantly contributed by the Cimanuk River.

The determination of acute toxicity level on early-life stage of zebrafish (*Danio rerio*) induced by ORPs has also introduced in this study. In this case, phorate was chosen as the example of ORP exposure. The increasing of phorate concentration and the exposure time give statistically significant difference with the control on lethal effect and sublethal hatching rate, meanwhile the phorate concentration treatment did insignificant effect into swim-up rate. Only the exposure time gave the significant difference to swim-up ability of zebrafish larvae. The recent study has also revealed that the lethal LC_{50} was 4.54 $mg.L^{-1}$. NOEC was found 7.67 $\mu g.L^{-1}$ at 30 days. While, the sublethal EC_{50} on hatching rate and EC_{50} on swim-up failure was 9.75 $mg.L^{-1}$ and 2.14 $\mu g.L^{-1}$, respectively. Rely on the result of lethal effect, the FET give other information about LC_{50} value of zebrafish embryo toxicity. Even the lethal effect endpoint was higher than the fish acute toxicity, however this FET could be used as the previous screening to fish acute toxicity to support the 3Rs principle (Reduction, Refinement and Replacement).

Based on acute toxicity test using early-life stage zebrafish result, the WET test to determine the lethal and sublethal level using early-life stage zebrafish on salinity variation and field water sample was studied. Salinity treatment, represent the salinity of culture water, has significant effect to lethal rate and swim-up failure, but not significant to hatching rate. Above salinity 0.22psu, the swim-up failure on zebrafish larvae was slightly increase. The zebrafish larvae survive on salinity as low as 0.04 psu.

The lethal effect showed significant increase when embryos of zebrafish were exposed to water samples from St.4 and St.7. From WET test lethal endpoint, it can

conclude that LC_{50} from St.7 was at 10% dilution. The other water samples have the lethal concentration less than 15%, especially at downstream area.

Risk characterization on detected ORP at the surface water sample on the study area has conducted in this study. Regarding the risk quotient ratio on three trophic levels of aquatic organisms, the station on the upstream area, represent by St.1, has higher concern compared to the other sampling stations, at the city center (St.4) and downstream area (St.10). The ORPs detected in those water samples potentially risk not only the acute lethal-sublethal effect to the local aquatic organisms that have sensitivity as early-life stage *Lepomis m.* but also the chronic effect on the lower trophic level, such as daphnids. The adoption of risk assessment using local aquatic organisms in Cipeles River was possible to develop in further study to describe closer risk-related with the problem source. This risk characterization result recommended a good practical effort, especially in the upstream area.

Comparison between WET test result and RQ ratio allowed several conclusions. WET test revealed the high lethal rate of sample from St.4 might be caused by high concentration of zinc. RQ calculation already proved it (Chapter 7). Meanwhile, sample from St.7 might be caused by other compound than heavy metals and ORP, since the ORPs in this station was not detected. The presence of high ammonium ion as detected in preliminary study might cause high lethality rate in sample St.7. The high concern based on RQ ratio by detected ORPs pesticide from St.1 sample did not appeared at WET test result. It indicated that the early-life stage *Danio rerio* showed it insensitivity on ORPs. But later on, together with other researcher studies, the adjustment factor between early-life stage and adult test organisms could became the approachment method.

The correlation between WET lethal rate and concerned-compounds was shown in Table 8.1. The ORPs of chlorpyrifos and terbufos, both the ORPs pesticide gave the high LOC, ammonia, and zinc were correlated with WET test lethal rate even mostly correlated on 80% dilution factor, except ammonia. Ammonia had strong correlation to lethal rate on 0%, 10%, 20%, and 40%. Zinc had increase correlation to lethal rate on non-diluted field sample (0% dilution factor). Chlorpyrifos and terbufos showed correlation with lethal rate on 80% dilution factor on daphnids and *Lepomis m.*, meanwhile had less correlation with juvenile *Danio r.*.

Table 8.1 Correlation analysis between RQ of concerned compound and lethal rate on WET test (at different dilution factor)

	NH_3	Zn	CRP-Daphnids	TBF-Daphnids	CRP - *Lepomis m.*	CRP-*Danio r.*	TBF-*Lepomis m.*	0%	10%	20%	40%	80%
NH_3	1.00											
Zn	-0.06	1.00										
CRP-Daphnids	0.01	0.21	1.00									
TBF-Daphnids	0.02	-0.17	0.92	1.00								
CRP -*Lepomis m.*	-0.02	0.19	0.98	0.90	1.00							
CRP-*Danio r.*	-0.05	0.46	0.88	0.69	0.93	1.00						
TBF-*Lepomis m.*	0.02	-0.17	0.92	1.00	0.90	0.69	1.00					
0%	**0.61***	**0.46**	0.28	0.06	0.22	0.32	0.06	1.00				
10%	**0.89***	0.23	0.10	-0.03	0.09	0.18	-0.03	0.81	1.00			
20%	**0.95***	0.03	0.07	0.03	0.02	0.01	0.03	0.74	0.93	1.00		
40%	**0.84***	0.10	0.25	0.16	0.20	0.21	0.16	0.66	0.91	0.88	1.00	
80%	0.02	0.09	**0.65***	**0.61***	**0.59***	0.48	**0.61***	0.17	0.00	0.06	0.10	1.00

Bold numbers indicated strong correlation. **Pvalue<0.05*. CRP=chlorpyrifos; TBF=terbufos.

Regarding the correlation analysis, the WET test using early-life stage of *Danio rerio* and prolonged 120-hpf monitoring time gave support on the toxicity level determination both lethal and sublethal effect, regardless the exact compound consisted in the sample. It supports on reducing the volume of water sample, sample transport, and chemical reduction.

8.2 Further recommendation

To improve our understanding of ecotoxicology, the correlation between heavy metals at the pore and/or surface waters with the toxicity to the aquatic organisms will provide interesting results in further studies.

The prolonged 120-hour acute test could be considered in spite to observe the sublethal effect on swim-up failure. This sublethal effect is easy to observe using simple microscope, being the advantage to be adapted in developing countries. This research only the approaching method to the field condition, WET test using local aquatic organism may be developed also to get better conclusion.

According to maintain the surface water quality, the integrated pest management application and knowledge sharing from related stakeholders should be conducted. It also in order to minimize the chemical exposure to water body, healthier agricultural product, and better life quality of the farmers.

Appendix I

Statistic difference analysis, two-way ANOVA

A. Lethal Effect

Exposure time	Concentration, ug.L^{-1}					
	control	1	10	100	1000	10000
24h	3.3	13.3	5.0	3.3	33.3	45.0
48h	3.3	15.0	5.0	6.7	36.7	56.7
72h	5.0	15.0	6.7	8.3	38.3	58.3
96h	5.0	15.0	6.7	8.3	43.3	58.3
120h	6.7	15.0	6.7	8.3	43.3	58.3

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
24h	6	103.33	17.2222	316.296
48h	6	123.33	20.5556	465.185
72h	6	131.67	21.9444	469.352
96h	6	136.67	22.7778	506.296
120h	6	138.33	23.0556	494.907
control	5	23.333	4.66667	1.94444
1	5	73.333	14.6667	0.55556
10	5	30	6	0.83333
100	5	35	7	4.72222
1000	5	195	39	18.8889
10000	5	276.67	55.3333	33.8889

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	136.111	4	34.0278	6.34715	0.00182	2.8661
Columns	11153	5	2230.59	416.069	1.7E-19	2.7109
Error	107.222	20	5.36111			
Total	11396.3	29				

all *P-value<0.05*, so the null hypothesis could be rejected

B. Sublethal Effect on Hatching

Exposure time	Concentration, ug.L^{-1}					
	control	1	10	100	1000	10000
24h	0.0	0.0	0.0	0.0	0.0	0.0
48h	1.7	0.0	0.0	0.0	16.7	0.0
72h	68.3	68.3	73.3	78.3	51.7	21.7
96h	93.3	80.0	93.3	91.7	53.3	40.0
120h	93.3	80.0	93.3	91.7	53.3	40.0

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
24h	6	0	0	0
48h	6	18.3333	3.055556	44.907
72h	6	361.667	60.27778	438.24
96h	6	451.667	75.27778	533.8
120h	6	451.667	75.27778	533.8
control	5	256.667	51.33333	2229.7
1	5	228.333	45.66667	1760.6
10	5	260	52	2320
100	5	261.667	52.33333	2311.9
1000	5	175	35	627.78
10000	5	101.667	20.33333	400.56

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	34959	4	8739.815	47.982	6E-10	2.8660814
Columns	4110.7	5	822.1481	4.5136	0.006	2.7108898
Error	3643	20	182.1481			
Total	42713	29				

all *P-value<0.05*, so the null hypothesis could be rejected

C. Sublethal Effect on Swim-up Failure

Exposure time	Concentration, ug.L^{-1}					
	control	1	10	100	1000	10000
24h	0.0	0.0	0.0	0.0	0.0	0.0
48h	0.0	0.0	0.0	0.0	0.0	0.0
72h	0.0	0.0	0.0	0.0	0.0	0.0
96h	0.0	0.0	0.0	0.0	0.0	0.0
120h	25.0	50.0	60.0	65.0	86.7	91.7

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
24h	6	0	0	0
48h	6	0	0	0
72h	6	0	0	0
96h	6	0	0	0
120h	6	378.333	63.056	601.57
control	5	25	5	125
1	5	50	10	500
10	5	60	12	720
100	5	65	13	845
1000	5	86.6667	17.333	1502.2
10000	5	91.6667	18.333	1680.6

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	19084.8	4	4771.2	39.656	3E-09	2.8661
Columns	601.574	5	120.31	1	0.443	2.7109
Error	2406.3	20	120.31			
Total	22092.7	29				

P-value for rows < *0.05*, so the null hypothesis could be rejected; the exposure time is statistically significant difference.

P-value for column > *0.05*, so the null hypothesis could not be rejected; the concentration treatment is statistically insignificant difference.

D. Multi Factor Probit Analysis on Lethal Rate

Multi Factor Probit Analysis

Iteration	Intercept	Concentration	Time
0	3.72996	0.15664	-6.31781
1	3.55827	0.17166	-6.68600
2	3.54825	0.17245	-6.67150
3	3.54865	0.17236	-6.66657
4	3.54861	0.17237	-6.66685
5	3.54862	0.17237	-6.66683
6	3.54862	0.17237	-6.66683

CONVERGENCE CRITERIA IS MET AFTER 6 ITERATION(S)

CHI-SQUARE STATISTIC IS 35.55932

CRITICAL VALUE WITH DF = 22 ALPHA= 0.0500 IS 33.92401

VARIANCE COVARIANCE MATRIX

	Intercept	Concentration	Time
INTERCEPT	0.02833	-0.00251	-0.54119
Concentration	-0.00251	0.00046	-0.00385
TIME	-0.54119	-0.00385	30.33229

SINCE CHI-SQUARE TEST FOR HETEROGENEITY OF DISCREPANCIES IS SIGNIFICANT,
ALL VARIANCES ARE MULTIPLIED BY A HETEROGENEITY FACTOR.
FIDUCIAL LIMIT WILL BE COMPUTED USING T-DISTRIBUTION INSTEAD OF NORMAL.

ADJUSTED VARIANCE COVARIANCE MATRIX

	Intercept	Concentration	Time
INTERCEPT	0.04579	-0.00407	-0.87474
Concentration	-0.00407	0.00075	-0.00622
TIME	-0.87474	-0.00622	49.02707

NOTE : HETEROGENEITY FACTOR IS 1.61633273380606

INFINITE Days

Prob(Probit)	Concentration	Lower 95.00% Limit	Upper 95.00% Limit
0.01 %	0.00000	0.00000	0.00025
0.10 %	0.00007	0.00000	0.00421
1.00 %	0.00622	0.00002	0.13432
5.00 %	0.32481	0.00648	3.30076
10.00 %	2.67575	0.12059	20.11455
20.00 %	34.41401	3.40799	219.27049
30.00 %	217.06005	30.34898	1533.76325
40.00 %	1046.04846	163.65310	9687.28563
50.00 %	4537.64487	687.48878	62025.96352

E. Accelerated Life Testing to determine No Effect concentration

Acute to Chronic Estimation
Accelerated Life Testing

Parameter	Estimate	95.00% Lower limit	95.00% Upper limit
AA	13344.9675879	2624.3794478	24065.5557281
B	0.3610632	0.2337757	0.4883507
C	0.2247682	0.0000000	0.9188796
A	0.0323965	0.0000000	0.0700106
C/B	0.6225174	0.0000000	2.6255487

INTEPRETATION: AA--measure of initial toxic strength;B--measure of mode of concentration-response; C--measure of mode of time-response; A=(1/AA)**B; C/B--measure of domination between concentration and time.

Maxiuum likelihood estimates for 'No-effect' concentrations

30-DAYS

Mortality	Concentration	Standard Error	95.00% lower limit	95.00% upper limit
0.01%	0.00000	0.00000	0.00000	0.00000
0.05%	0.00000	0.00001	0.00000	0.00003
0.10%	0.00001	0.00008	0.00002	0.00019
0.50%	0.00166	0.00658	0.00162	0.01457
1.00%	0.01144	0.04191	0.01039	0.09359
5.00%	1.04472	3.16680	0.80572	7.25153
10.00%	7.67050	21.33824	5.49918	49.49269

60-DAYS

Mortality	Concentration	Standard Error	95.00% lower limit	95.00% upper limit
0.01%	0.00000	0.00000	0.00000	0.00000
0.05%	0.00000	0.00001	0.00000	0.00002
0.10%	0.00001	0.00006	0.00001	0.00014
0.50%	0.00108	0.00493	0.00119	0.01074
1.00%	0.00743	0.03183	0.00775	0.06982
5.00%	0.67858	2.50600	0.62113	5.59025
10.00%	4.98227	17.24307	4.30867	38.77807

90-DAYS

Mortality	Concentration	Standard Error	95.00% lower limit	95.00% upper limit
0.01%	0.00000	0.00000	0.00000	0.00000
0.05%	0.00000	0.00000	0.00000	0.00001
0.10%	0.00001	0.00005	0.00001	0.00011
0.50%	0.00084	0.00413	0.00099	0.00895
1.00%	0.00577	0.02689	0.00649	0.05848
5.00%	0.52721	2.15488	0.52785	4.75070
10.00%	3.87086	14.95306	3.68648	33.17832

Remark: You did not input long-term exposure.

F. Probit analysis on Hatching Rate

(1) Hatching rate on phorate exposure 1-10,000 ug.L^{-1}

Exposure time	Phorate concentration, ug.L^{-1}					
	control	1	10	100	1000	10000
24h	0.00	0.00	0.00	0.00	0.00	0.00
48h	1.67	0.00	0.00	0.00	16.67	0.00
72h	68.33	68.33	73.33	78.33	51.67	21.67
96h	93.33	80.00	93.33	91.67	53.33	40.00
120h	93.33	80.00	93.33	91.67	53.33	40.00

(2) Conversion hatching rate at 120-hpf on log 10[phorate exposure] based on Probit transformation table

	log10 concentration, ug.L^{-1}				
Time exposure	0	1	2	3	4
120h	5.84	6.48	6.41	5.08	4.75

Table Transformation of percentage to probits(Vincent, 2008)

%	0	1	2	3	4	5	6	7	8	9
0	—	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
—	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

(3) Regression analysis Probit at 120-hpf

log10[phorate], X	0	1	2	3	4
Probit at 120-hpf, Y	5.84	6.48	6.41	5.08	4.75

Regression Statistics	
Multiple R	0.727997243
R Square	0.529979986
Adjusted R Square	0.373306648
Standard Error	0.61553229
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1.28164	1.28164	3.382706926	0.163165516
Residual	3	1.13664	0.37888		
Total	4	2.41828			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	6.428	0.47679	13.48185	0.00088	4.91064	7.94536
log10Concent	-0.358	0.19465	-1.83921	0.16317	-0.97746	0.26146

y = -0.358x + 6.428

(4) Calculation LC_{50} concentration

x = (y – 6.428) / -0.358

for 50% hatching → y = 5.0, so x = 3.99

[EC_{50}] = 10^(x) = 10^(3.99)

= 9,746 ug.L^{-1}