

Using *Daphnia magna* Straus to monitor toxicity of sustainable oil sludge bio-phytoremediation

Nia Rossiana¹, Keukeu Kaniawati Rosada¹, Monica Natasha Almapangesti¹, Yayat Dhahiyat²

¹Department Biology Faculty of Mathematic and Natural Sciences University of Padjadjaran

²Department Fisheries Faculty of Fisheries and Marine Sciences University of Padjadjaran

Abstract. Oil sludge (OS) is the toxic product of various oil refinery processes. Oil sludge biophytoremediation is sustainable remediation efforts were conducted to reduce OS toxicity level and they also need to be monitored through toxicity test. In this study, the toxicants were OS post-biophytoremediation for 24 months (OS₂₄) and 40 months (OS₄₀), and were tested to *Daphnia magna*. The toxicity test consisted of Range Finding Test, acute and chronic toxicity test. The purpose of this research is to obtain the LC₅₀ value of each OS and the chronic effects shown by *D. magna*. The method in this study was experimental with Completely Randomized Design (CRD). The LC_{50-96 hours} values were calculated from 50% of dead individuals while *D. magna* reproduction was calculated from the number of neonates (offspring) produced within three weeks. The LC₅₀ of OS₂₄ and OS₄₀ were 0.042 ppm and 0.366 ppm respectively. Both were categorized as 'extremely toxic' refer to Swan, 1994, observed chronic effects were reproduction delays and a decrease in the number of neonates.

1 INTRODUCTION

Biophytoremediation is Green Sustainable Technology for Industrial Waste Management. Oil sludge is a waste derived from the petroleum industry and includes hazardous and toxic substances that must be biologically managed. One of the efforts to overcome oil sludge waste is by using bioremediation. Oil sludge (OS) is the toxic product of various oil refinery processes that is encountered during crude oil refining, refinery-wastewater treatment and cleaning of oil storage vessels [15]. Oil sludge contains viscous mixture of water, oil, sediments and high concentrations of hydrocarbon. The main composition of oil sludge are benzene, toluene, ethylbenzene, xylene and heavy metal such as lead (Pb).

Taking into consideration, the thick and viscous form of OS can also harm living organisms in the contaminated area. The negative impacts of OS contamination must be observed in order to determine the valid management step. Oil sludge in soils pose a risk of leaching or the separation process of soluble (solute) and insoluble substances (inert) through the hydrological cycle. Solute substances will eventually reside in the aquatic ecosystem and cover water surfaces. As a result, sunlight cannot penetrate the water body which leads to a decrease in the rate of photosynthesis. Furthermore, the chemical compounds of oil sludge can accumulate in aquatic organisms' tissues and the buildup of these hazardous materials can travel through the food chain which will then be harmful for humans to consume any of those aquatic animals. This process is called biomagnification. These consequences will be society's health issue.

According to the Ministry of Environment Decree No. 128/2003, petroleum waste and contaminated soil should be treated biologically as an alternative of waste treatment technology. Bioremediation utilizes bacteria, fungi, macroalgae, microalgae, plants and animals. [10] made a combination of *Pseudomonas aeruginosa* and *Bacillus sphaericus* as bioremediators, along with the mycorrhizal plant *Paraserianthes falcataria* as a phytoremediator. Therefore, the whole process is called biophytoremediation. The mixture is composed of 40% oil sludge, 40% soil and 20% sand which had been composted by bacteria for 2 months. Afterwards, the OS mixture was planted with mycorrhizal plant for 40 months.

Monitoring the toxicity rate of oil sludge post-biophytoremediation is necessary to measure how much time is required for biophytoremediation processes to reduce the toxicity rate to zero or until it becomes non-toxic. The monitoring is carried out biologically so it is called biomonitoring, with a goal to evaluate the water body conditions [4]. Therefore, biomonitoring using post-biophytoremediation of oil sludge is carried out in this research for 24 and 40 months to evaluate the decrease in toxicity rate. The toxicity in this research is determined by LC₅₀ or *Lethal Concentration 50*, i.e. the term used to define the concentration that kills 50% population of test biota at one time exposure for 24, 48, 72, or 96 hours [7]. The research begins with preliminary test or *Range Finding Test* (RFT) to determine the range concentration of oil sludge that would be used for acute and chronic tests. The range was obtained by testing widely-spaced sample dilutions in a logarithmic series to *D. magna*; it is the range between the upper and lower limit that kills 50% of *D. magna*

population during the 24-hour test. For each oil sludge sample of 24 and 40 months, they were diluted into 5 concentrations within the tested range using a logarithmic scale interval series table in accordance to [9]. These series of concentrations were used as the sample concentration for the acute toxicity test using LC₅₀. The test animals were exposed to the diluted oil sludge sample for 96 hours and the number of animals of each test concentration were measured every 24 hours. The chronic test was carried out after the acute test. The parameter was the amount of offsprings produced from each concentration for 14 days.

The toxicity of oil sludge post-biophytoremediation must be tested to various organism levels (a.k.a multilevel organisms) and it will reveal different results between aquatic or terrestrial, and vertebrate or invertebrate animals. This process is called biomonitoring. To monitor the ecosystem's condition, biomonitoring uses organisms in which their viability can represent changes in water body quality. This group of animals is called bioindicator. If there is a correlation between the existence of one group of animals in nature with the ecosystem's condition, they can be considered as bioindicators [13]. In this study, the test animals were *Daphnia magna* which belongs to the class Branchiopoda. This species is often used in the fields of ecotoxicity because of its sensitivity. They show post-exposure effects to toxicants such as a change in heartbeat, immobility, reproduction cycle, and even death [12]. Thus, *D. magna* is the ideal test animal for toxicity test with oil sludge as the toxicant, using the [5, 16] guidelines.

2 MATERIALS AND METHODS

2.1 Materials

The materials used in this work were divided into *D. magna* living media and food, such as ground water, baker's yeast and green water as well as the materials used to mix the toxicants, Tween-80 and *oil sludge* post-biophytoremediation of 24 and 40 months.

2.2 Methods

This study applied the Completely Randomized Design (CRD) method. The test animals were *D. magna* neonates that were less than 24 hours old. The study was first started with preliminary Range Finding Test prior to initiate a static, acute, definitive toxicity test. The result of this test was the range between the upper and lower concentration limit which caused 50% death of test animals. The series of concentrations (within the tested range) in the acute test were determined using the logarithmic scale interval series table [9]. A total of 500 neonates were required to be placed randomly in five test chambers. The treatment given was five different concentrations, each repeated five times; each test chamber contained ten neonates. Neonates were separated from the mother two hours before the exposure. The number of living and dead individuals were noted in a logbook every 24 hour for four days in order to calculate the LC₅₀ value. After 96 hours of

exposure, the remaining living individuals were moved into a new media. The new media (a mixture of ground water, green water and baker's yeast) was prepared to observe the post acute test effect which was shown by the reproduction rate of the surviving animals. The reproduction test was held for 14-21 days, or long enough for the development of at least three broods. New offsprings from each test chamber must be counted and allocated every Monday, Wednesday and Friday.

The mortality rate of *D. magna* during the acute test period was analyzed with probit method using SPSS 26.0 program to calculate the LC₅₀ value. Meanwhile, the reproduction rate was analyzed with ANOVA test of 5% significance level and was continued with the Duncan test. ANOVA and Duncan tests were analyzed with the XLSTAT 2019 program. The LC₅₀ would be compared to the toxicity category of petroleum sludge according to [14].

Table 1. Toxicity Category of Petroleum Sludge (Swan et al., 1994)

Toxicity Category	LC ₅₀ (96 hr) ppm
Extremely toxic	< 1
Highly Toxic	1 to < 100
Moderately toxic	100 to < 1000
Lowly toxic	1000 to < 10000
Slightly toxic	10000 to < 100000
Non toxic	> 100000

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3 RESULTS AND DISCUSSION

In this study, the toxicity of oil sludge post-biophytoremediation for 24 months (OS₂₄) and 40 months (OS₄₀) was tested to *D. magna*. The toxicity test consisted of the Range Finding Test (RFT) or the preliminary test that is required to find the upper and lower limit of oil sludge concentration that killed 50% population of test animals. RFT was carried out for one day and it was discovered that the range for OS₂₄ is between 1 ppm - 0,01 ppm whereas the range for OS₄₀ is between 1 ppm - 0,1 ppm. The upper and lower limit were used to determine the acute toxicity test (LC₅₀) and chronic test (reproduction) using the logarithmic scale interval series table [10]. The series of concentrations used for OS₂₄ were 0.65, 0.21, 0.056, 0.0135 and 0 ppm (control), whereas for OS₄₀, the concentrations were 0.87, 0.56, 0.37, 0.18 and 0 (control).

3.1 Acute Toxicity Test (LC₅₀)

Acute toxicity test was conducted to evaluate the effects inflicted by the test solutions OS₂₄ and OS₄₀

towards the death of 50% population of *D. magna* that were exposed for 96 hours. The results of OS₂₄ and OS₄₀ are represented by the average mortality of *D. magna* in each test solution and can be seen in Table 2 and Table 3.

Table 2. Average Mortality of *D. magna* in OS₂₄ Test Solution

No.	Test Solution Concentration (ppm)	Average Mortality (%)
1	0.65	100
2	0.21	78
3	0.056	72
4	0.0135	16
5	0 (control)	10
LC ₅₀ = 0.042 ppm		

Table 3. Average Mortality of *D. magna* in OS₄₀ Test Solution

No.	Test Solution Concentration (ppm)	Average Mortality (%)
1	0.87	100
2	0.56	84
3	0.37	58
4	0.18	26
5	0 (control)	10
LC ₅₀ = 0.366 ppm		

Table 2 and Table 3 show different ranges of average mortality. Based on the probit analysis, the LC_{50-96 hours} of OS₂₄ is 0.042 ppm (between 0.0135 – 0.65 ppm). According to Swan’s toxicity category table, this value is considered very toxic since it is under 1 ppm. Likewise, the LC_{50-96 hours} of OS₄₀ is 0.366 ppm (between 0.18 – 0.87 ppm). Both of these values are categorized as ‘extremely toxic’, but the LC_{50-96 hours} value of OS₂₄ is lower than OS₄₀. This shows that the test solution of OS₂₄ was more toxic than OS₄₀. Moreover, these results explain that biophytoremediation could reduce toxicity in a period between 24 and 40 months, although the reduction rate is not too significant. A long-term bioremediation process is required to reduce the toxicity level of sludge-type pollutants in large amount. [8] stated that oil sludge is a complex emulsion which is cytotoxic, mutagenic and potentially carcinogenic. It also affects soil fertility. The use of microorganisms can transform pollutants to less-toxic compounds through composting processes. Nevertheless, the degradation of oil sludge will have to go through several complex remediation processes such as physical, chemical, and biological to reduce the toxicity level in a shorter period of time. Based on these facts, the oil sludge in this study must be treated further to decrease its toxicity.

Both OS₂₄ and OS₄₀ were still extremely toxic when they were tested to *D. magna*. The toxicants are categorized as extremely toxic and this is shown by the previous GCMS results. Nevertheless, the GCMS test showed that the compounds contained in OS₄₀ had been splitted into simpler compounds when compared to the compounds that made up OS₂₄. Oil sludge is basically a toxic, complex compound, thus in order to reduce its toxicity, a series of remediation processes are required to break the complex compounds into their constituents. The Environmental Literacy Council (2015) suggested that pollutants should be processed without producing residues and being harmful to the environment, hence bioremediation is highly recommended.

D. magna has been used widely to evaluate water body quality due to its sensitivity towards any toxicants in their living media [11]. [3] tested the toxicity of oil sludge waste before and after phytoremediation to

Daphnia carinata and the results showed that the LC_{50-48 hours} values of OS before and after phytoremediation are 28.62 ppm and 126.55 ppm. Furthermore, [10] carried out a biophytoremediation process of oil sludge for 14.5 months and tested it to *Rattus norvegicus*. The result was categorized as ‘low toxic’ with a LD₅₀ of 9.785,184 mg/kg. *D. magna* use filtration mechanism to acquire food from their environment. Therefore, when they were exposed to harmful compounds, the substances do not only affect their digestive systems but also their entire body surface. Meanwhile, the mixture of oil sludge and rat feed needed more time to be digested in the digestive system of *Rattus norvegicus*. Thus, during the time that the toxicity tests were carried out, the toxic effects of the oil sludge were deadlier for *D. magna*. These comparisons explain that oil sludge has worse impacts towards aquatic organisms than to terrestrials.

3.2 Chronic Toxicity Test (Reproduction)

Chronic toxicity test was carried out to evaluate the effect caused by toxicants after the acute toxicity test. The observed parameters in this test were the decrease in the number of *D. magna* parents and offsprings produced during the test. These procedures were conducted based on the [16] guidelines.

3.2.1. The Reduction of *D. magna* Parents

The reduction in number of *D. magna* parents must be observed to evaluate the parents’ viability after the acute toxicity test. The results of ANOVA analysis for this parameter are shown in Table 4 and Table 5.

Table 4. ANOVA Test Result towards The Reduction of *D. magna* Parents in OS₂₄

Conclusion for D8 (ANOVA):	
	D8
R ²	0.133
F	1.000
Pr > F	0.397

Table 5. ANOVA Test Results towards The Reduction of *D. magna* Parents in OS₄₀

Conclusion for all Y (ANOVA):		
	D11	D13
R ²	0.143	0.143
F	1.000	1.000
Pr > F	0.397	0.397

Annotation: ANOVA table shows significance if (pr>F) value is less than 0,05

Table 4.6 and 4.7 showed that the results were insignificant. This insignificance explained that the number of parents were relatively constant in OS₂₄ and OS₄₀. In other words, the mortality rate was nearly zero. Throughout the chronic test, food was given periodically in accordance to the US EPA guidelines, thus nutrition from the food could sustain the parents’ life. Food was

given two hours before the acute toxicity test and was not given again during the 96-hour test for *D. magna* to signify effects that were purely caused by the oil sludge's toxicity. *D. magna* is known for its sensitivity and is able to show morphological and activity changes when exposed to particular toxicants [2]. During the acute toxicity test, *D. magna* showed their adaptations by reducing their activity in order to survive the food-limited environment [17]. Afterwards, *D. magna* were moved to a food-provided environment to support their growth and development to prepare them for the chronic toxicity test. Each test animal had sufficient nutrition, thus reducing the competition between test individuals. This could also suggest that the exposure in the acute toxicity test was not toxic enough to affect the growth and development of *D. magna* in the chronic toxicity test.

3.2.2 The Reduction of Offsprings Produced by *D. magna*

The number of offsprings produced must be observed to evaluate the fertility of *D. magna* as it was one of the chronic effects shown after the exposure. The results of ANOVA analysis showed significance, hence the analysis was continued to Duncan test. The Duncan test results for the number of offsprings produced by *D. magna* are shown in Table 6 and Table 7

Table 6. Duncan Test Results for The Number of Offsprings Produced by *D. magna* in OS₂₄

	D5	D8	D11	D13
0	1.000 a	69.600 a	44.000 a	26.000 a
0.0135	0.000 a	87.200 a	38.600 a	21.000 ab
0.056	0.000 a	3.200 b	4.800 b	6.000 b
Pr > F(Treatment)	0.397	< 0.0001	0.001	0.041
Significant	No	Yes	Yes	Yes

Table 7. Duncan Test Results for The Number of Offsprings Produced by *D. magna* in OS₄₀

	D5	D8	D11	D13
0	8.0 a	66.2 a	63.0 a	11.4 a
0.18	1.0 a	57.0 a	64.8 a	19.8 a
0.37	1.0 a	22.0 a	28.4 a	24.6 a
Pr > F(Treatment)	0.062	0.003	0.006	0.253
Significant	No	Yes	Yes	No

Annotation: The Duncan table shows significance if (pr>F) value is less than 0,05

According to the Duncan analysis results, the number of offsprings produced by *D. magna* throughout the chronic toxicity test in OS₂₄ and OS₄₀ showed significance starting from day 8 because every parent in each test chamber had produced offsprings. However, the parents were 4 days old when they were moved to the new chamber for the chronic toxicity test and were 12 days old when they all produced the first offsprings. This is due to the fact that *D. magna* generally could produce offsprings in the age of 4 days as they reach maturity quickly [6]. If the optimum condition is provided, *D. magna* could produce as many as 4-22 eggs per day. During the chronic toxicity test, the reproduction rate of *D. magna* is assumed to be delayed due to the toxic effect from the exposure of test solutions (OS₂₄ and OS₄₀) in the previous acute toxicity test. Nonetheless, the test organisms did not produce ephippia or resting eggs. [2] stated that *D. magna* will

produce dormant, sexual eggs when exposed under stressful environment as these eggs can withstand heat, cold, lack of oxygen and nutrition, fluctuations in the population and drought. Ephippia was formed sexually to produce genetic variation and increase the chance of species survival. Therefore, the absence of ephippia in this test signify that *D. magna* had enough nutrition after the exposure, hence the toxic effect did not affect the formation of the asexual eggs. Still, the egg formation was delayed. This fact explains that OS₂₄ and OS₄₀ gave significant effect to the reproduction of *D. magna*.

The data on Table 6 and 7 showed that the lowest increase of offsprings was in the concentration 0.056 of OS₂₄ and 0.37 of OS₄₀. Oil sludge affected the reproduction of *D. magna* chronically, thus the offsprings produced were lesser than in other concentrations. In the test solutions of OS₄₀, the number of offsprings produced in day 13 was not significant. This might be caused by the microorganisms' activities living in the media. They broke down organic matters in the media which resulted in the formation of carbon dioxide. The increase of carbon dioxide caused the water's pH to decrease, thus some compounds (such as ammonia) in the water became toxic. This could lead to a decrease in the number of offsprings produced in the media [1].

The negative effects that occurred in the test organisms of this study indicate that the use of oil sludge is really harmful to the environment, especially heavy metals that are contained in the toxicants. According to the GCMS test, the heavy metals in oil sludge are Pb (lead), Ni (nickel), Cd (cadmium), Cr (chromium), and Hg (hydragyrum/mercury). These heavy metals are able to induce toxic effects that harm the physiological function of the exposed organisms. Other than that, there were originally 9 compounds of hydrocarbons in OS₂₄. After OS₂₄ underwent the biophytoremediation process for 40 months, 4 out of 9 compounds were degraded into 93 simpler compounds. The compounds that were not degraded consisted of hexane, cyclopentane, cyclohexane, decane and cyclohexasiloxane. This proved that biophytoremediation can break down oil sludge into less toxic forms. According to the analysis of both acute and chronic toxicity test data in this study, the process of oil sludge degradation requires a longer period of time in order to fully remove its toxic characteristics, mainly in aquatic environment.

As it was mentioned previously, the toxicity of oil sludge is more harmful to aquatic organisms than it is to terrestrial organisms. Based on this comparison, the toxic effects have to be known widely for many different types of test animals. Therefore, the toxicity of oil sludge must be tested towards multilevel organisms and various oil sludge's impacts will be discovered for various ecosystems.

4. CONCLUSION

Based on the explanation above, the LC₅₀ values for both OS₂₄ and OS₄₀ are 0.0149 ppm and 0.2844 ppm respectively. Both values are considered extremely toxic. The test solutions OS₂₄ and OS₄₀ slowed the reproduction rate of *D. magna*. A concentration of 0.056 ppm OS₂₄ and 0.37 ppm OS₄₀ had the largest effect on

D. magna in which the average number of offsprings produced were less than the ones produced in other treatments.

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