Journal of Environment and Earth Science
ISSN 2224-3216 (Paper) ISSN 2225-0948 (Online)
Vol 2, No.8, 2012

Molecular Exploration of Biomarkers as Early Warning System of Aquatic Pollution

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This research financed by Directorate of Research and Community Services, the Directorate General of Higher Education, Ministry of Education and Culture of the funding provided for Fundamental grants in 2011.

Abstract

LAS exposure to potentially cause pollution in aquatic environments. LAS Content in the waters, harmful to aquatic organisms and humans. Biomarkers required as "early warning" of pollution on aquatic biomonitoring program. Biomarkers can provide a picture of the effect of changes in environmental quality. The response to the stress caused by LAS is used as a biomarker of pollution and is expected to provide an overview effect on humans in the future.

This study analyzes the differences in molecular changes in the hepatocyte Cyprinus carpio L were exposed to various concentrations of LAS. LAS as stressors created by the concentration of 0.01, 0.02; 0.03; 0.04; 0.05 mg / l with a long exposure of 24 hours, 48 hours, 72 hours, 96 hours, and 8 days. Biomarker expression in hepatocyte observed using immunohistochemical methods. The results obtained were analyzed statistically using the 2-way MANOVA.

The results showed a significant level of p = 0.0005 on HSP70 protein expression, iNOS, p38 MAPK, and CYP 1A compared to the control group. Increased concentrations of LAS and the duration of exposure resulted in increased number expressing hepatocyte biomarker with a significant level (p = 0.0005). The conclusion of this study is that the expression of HSP70, iNOS, p38 MAPK, and CYP 1A can be used as a biomarker of pollution in aquatic environments LAS.

Keywords: Aquatic, biomarkers, early warning system, molecular, pollution.

1. Introduction

Synthetic cleaning agents commonly known detergent increased use in the community. One of the pollutants in aquatic environments is Linear Alkylbenzene Sulfonate (LAS), which is the active ingredient in the detergent product. Detergent wastes from domestic sources (households) and non-domestic sources (industries) are entering the water body. It will directly cause environmental pollution and indirectly to humans, because of the limited of water sources and the declining quality of raw water in Indonesia.

Linear Alkylbenzene Sulfonate (LAS) has better characteristics than other types of surfactants, although it can not be said to be environmentally friendly. Linear Alkylbenzene Sulfonate rapidly biodegradable and potentially limited to expected . The study was conducted [1] studying the effects of LAS, Chromium (Cr)-Nickel (Ni) and the combination of LAS-Cr-Ni for 30 days. The results showed that an increase in activity of aspartate amino transferase, alanine amino transferase, acid phosphatase, and gamma glutamyl transpeptidase, decreased superoxide dismutase activity and increased accumulation of Cr and Ni in the network. Biochemical changes and accumulation of heavy metals found in the more complex the Cr-Ni-LAS. It shows that LAS is more toxic when forming complexes with other pollutants, but the waters there are various types of pollutant [2]. Kalimas is one source of raw water for the people of Surabaya. Monitoring results showed that the levels of detergent in Kalimas Surabaya during the dry season of 4.65 mg/l, while the threshold value of detergent in the water according to PP 82/2001 at 0.2 mg/l. To avoid further damage to the ecosystem is needed as a biological marker of early warning system of water pollution.

At this time the Ecological Risk Assessment (ERA) or Biological Monitoring (Biomonitoring) is widely used in the analysis of water standards. Biomonitoring process is based on the biological response to the presence of pollutants, to monitor ecological changes in the ecosystem. Aquatic organisms response to stressful conditions due to the presence of pollutants in the environment.

The response to stress is a mechanism to protect themselves from changes in the environment, can be adapted during a stressful environment or changes in the system of biological organisms, if the stress in the environment has reached advanced level [3]. Response of the organism to changes in environmental quality can be indicative of the presence of certain pollutants, which are called "markers" or markers. The term "bio" is used as "markers" are expressed by the organism. Biomarkers obtained through toxicological testing of certain pollutants in the environment, using species that represent the place of pollution [4]. (Biomarkers can be found in all parts of organisms, one of which is liver. The liver is a vital organ in the metabolism and excretion of xenobiotics materials, so the chemical concentration can be higher in the liver than other organs [5]. Appropriate biomarkers for environmental monitoring programs, such as: stress proteins (eg heat shock protein, metallothionein), physiological parameters, morphological, and histological [6].

This study aims to explore the response of living beings to changing environmental conditions due to pollution of the waters of LAS. The research was conducted by researchers recognize the vital role water for all living beings, so the quality must be maintained. The results of this study are expected to be detecting the presence of contaminants that later became "early warning", so that the heavier ecosystem damage can be prevented. Biomarkers were observed in this study is the expression of HSP-70 iNOS, p38 MAPK, and CYP1A in hepatocyte Cyprinus carpio L.

3. Material and Methods

2.1 Material

The organisms used in this study is the fish Cyprinus carpio L. (Common carp) obtained from Fish Seed (BBI) in Punten, Batu, Malang, Indonesia. Preparation begins with preparing the same parent then bred to obtain Cyprinus carpio L. to be used as a sample, this is done to reduce the bias. Maintenance seeds for 3 months for reasons already sexually mature fish (mature sexuality) and changes in body weight during the study was relatively small. Cyprinus carpio L. who used male sex of each fish with a length of 15.6 ± 0.74 cm and weight 44.72 ± 7.07 g. Maintenance cultivated in the same conditions, eg food and drink together, the maintenance of the same place, the type and species of the same origin, age Cyprinus carpio L. but still get the same difference in terms of weight and length. Cyprinus carpio L. as many as 135 fish taken from hatcheries to then acclimatized before the sample used for the study. The process of acclimatization lasts for 7 days, then take 120 fish were divided into 6 groups aquarium, which is 5 group variation LAS exposure concentration (0.01 mg / l, 0.02 mg / l, 0.03 mg / l, 0, 04 mg / l and 0.05 mg / l) and 1 control group (not given exposure to LAS).

2.2 Methods

Examination of hepatocyte expressing HSP70, iNOS, p38 MAPK, and CYP1A performed used immunohistochemical methods, with HE staining. The principle of the method is complex immunohistochemical HSP70-binding protein HSP70 antibody bound by the secondary antibody (biotin-labeled affinity purified antibody to mouse IgG (H + L), which carries the label (DAB). Addition of enzyme and substrate will cause the substrate in the form of visualization hazel , indicating hepatocyte expressing HSP70 in the network. observations on the expression of HSP70 performed on tissue slices under a microscope at a magnification of 1000X on 20 field of view (1 viewing area = 0.015 m^2). The same examination performed to detect the presence of iNOS, p38 MAPK, and CYP1A.

Calculations performed using the NO production by Griess enzymatic colorimetric principles. This method is based on the enzymatic conversion of nitrate to nitrite by nitrate reductase as azo dyes Griess reaction. Griess reaction by a two-step reaction diazosiasi, where NO2 - which are in the acidic producing agent that reacts with sulfanilic acid diazonium ion produced. Ion is then coupled with N-(1-naphthyl) ethylenediamine to form the chromophoric azo derivative that absorbs light with a wavelength of 540 nm. The color change is then measured used Nitric Oxide (NO2-/NO3-) Assay Kit. Production of NO is the number of nitrite-nitrate, expressed in µmol / L.

3. Results and Discussion

3.1 Heat Shock Protein-70

Immunohistochemical techniques using specific antibodies against HSP70, suggesting that HSP70 strongly expressed in the cytoplasm Cyprinus carpio L. the treatment of LAS at various concentrations and duration of exposure (Fig.1).

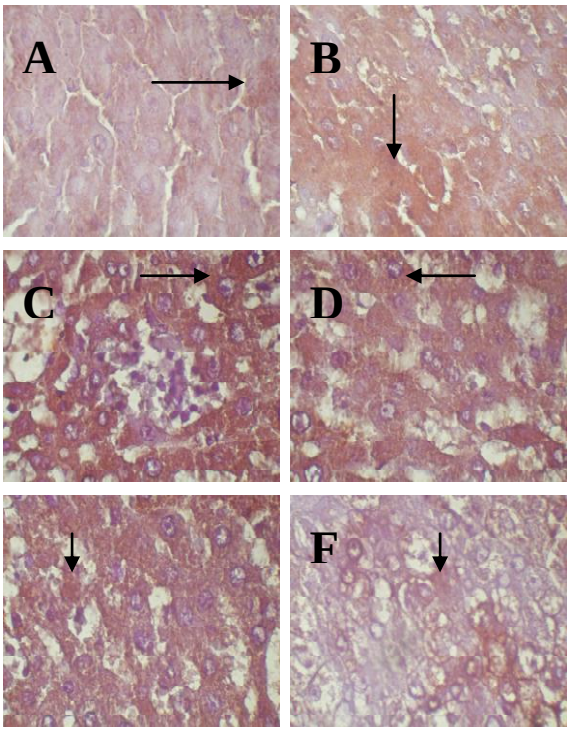

Figure 1. HSP70 expression in hepatocyte Cyprinus carpio L

MANOVA test on the General Linear Model showed that the concentration of LAS exposure significantly influence the expression of HSP70 ($p = 0.0005$). Linear regression on the Curve Fit, strengthen significantly the level obtained in the General Linear Model (Fig.2), which found a strong positive correlation ($R = 0.97$) with HSP70 equation $x + 11.679 = 322.857$, and $p = 0.0005$. The equation shows the amount of hepatocyte expressing HSP70 for each concentration of the multiplication of the large dose 322.857 11.679 coupled with a confidence level of 97%. This indicates that increasing the dose increased hepatocyte expressing HSP70.

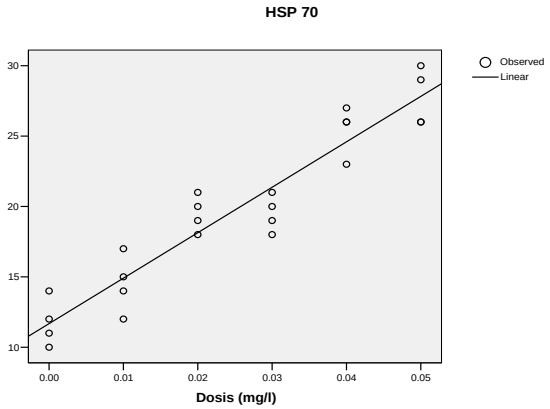

Figure 2. Linear regression of LAS concentration on HSP70

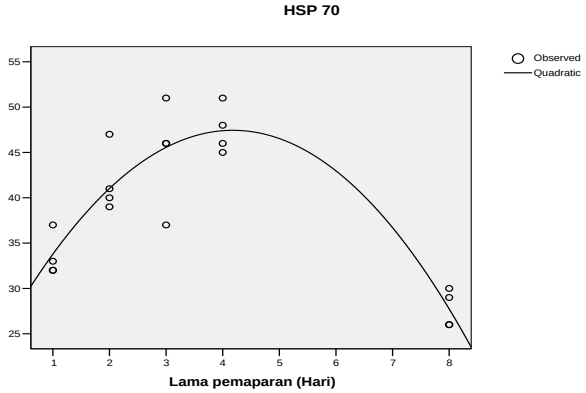

Figure 3. Linear regression of length of exposure on HSP70

Added time of exposure also showed a significant effect on the expression of stress proteins in the hepatocyte ($p = 0.0005$). HSP70 expression pattern can be observed in the Post Hoc Test showed that the expression of HSP70 on the first day to the fourth day there was an increase, but a decrease from 5-8 days. In Figure 3 shows that in the 24, 48 and 72 hours the amount of hepatocyte expressing HSP70 continued to increase, but at the 96th hour though slight decline. Significantly decrease seen in the exposure time of 8 days. Quadratic regression on the Curve Fit (Figure 3) shows a positive correlation ($R = 0.921$) with HSP70 equation $x2 = -1.35 +11.29 +23.843$ x and $p = 0.0005$. The equation shows that the number of hepatocyte expressing HSP70 at each observation time is the sum of -1.35 (day squared) to 11.29 (day) plus 23.84, with a significant level of 92.1%.

3.2 inducible Nitric Oxide Synthase (iNOS)

Immunohistochemical techniques using specific antibodies against iNOS, suggesting that iNOS terekpresi strong in the cytoplasm by treatment with LAS at various doses and longer exposure time. Expression of iNOS to LAS exposure concentration indicated by specific colors maroon and black arrows in Fig 4.

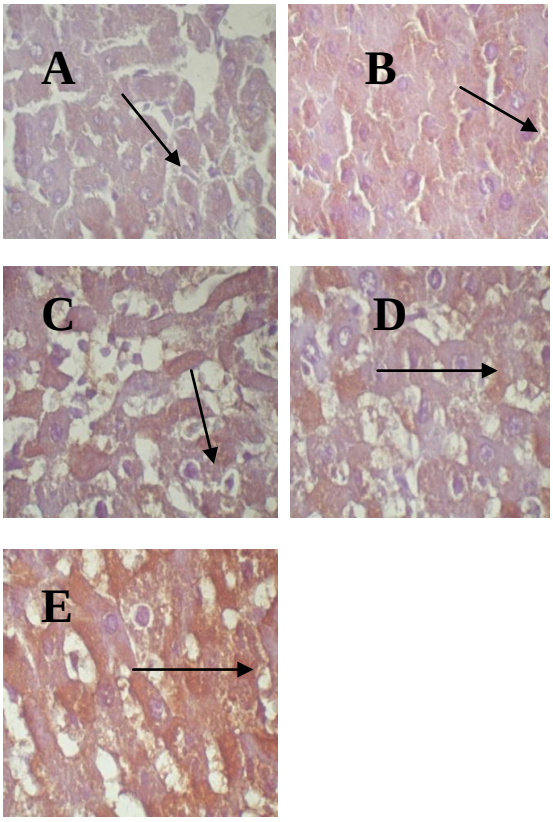

Figure 4. iNOS expression in hepatocyte Cyprinus carpio L

The results of immunohistochemical analysis showed that the control (Fig. 4a) looks clean so no tan at all. On the other hand, in Figure B, C, D, and E are the LAS concentration 0.01 mg / l (Fig 4b) and 0.02 mg / l (Fig 4c); slowly emerging brown color, which is the longer intensifying. In the LAS concentration 0.03 mg / l (Fig. 4d), 0.04 mg / l (Fig. 4e) and 0.05 mg / l (Fig. 4f) iNOS expression was evident in the cytoplasm began. Expression of iNOS are starting to look at the concentration of 0.01 mg / l and did not appear on the control indicates that the nature of the inducible iNOS protein or only appear as a result of a certain stimuli.

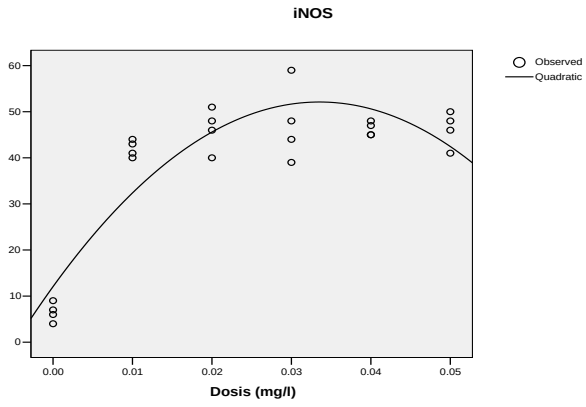

Figure 5. Linear regression of LAS concentration on iNOS

MANOVA test on Between-Subject Effects indicate that the duration of exposure and dose of LAS exposure showed a significant level of p = 0.0005 for iNOS expression. Increasing the dose and duration of exposure that resulted in an increase in the number of hepatocyte iNOS expression was also shown in Fig 5.

In the figure 6 shows that the increase in concentration, which is characterized by color changes indicate an increase in the number of hepatocyte iNOS expression, while increasing the exposure time is shown per group 4 replication. MANOVA test the correlation table, obtained significant value p = 0.0005 and the value of the Pearson correlation R = 0.9 showed a very strong correlation between the dose by iNOS expression. Lines quadratically on Curve Fit shows that the number expressing hepatocyte iNOS = -35625 x2 + x +12.054 2389.107, p = 0.0005. The equation shows that the number of iNOS expressing hepatocyte for each dose of the sum of -35 625 (quadratic dose) plus the multiplication result of the large doses of LAS 2389.107 plus 12.054, with 90% confidence level.

Figure 6 shows a brownish red color more specifically expressed in hepatocyte seiiring with the length of time of exposure. MANOVA test the correlation table, obtained significant value p = 0.0005 and the value of the Pearson correlation R = 0.456 for duration of exposure to the expression of iNOS. Lines quadratically on Curve Fit showed that long exposure, resulting in the number of iNOS expressing hepatocyte x2 = -0.913 + 8.8883 x +33.28; with p = 0.0005.The equation shows that the number of iNOS expressing hepatocyte for each day of the sum of -0.913 (days squared) plus the theoretical 33.28 plus 8.8883 of the day, with a 45.6% confidence level. Smaller R value due to that the regression calculations for the day is done the entire LAS exposure concentration. If the linear regression performed on each exposure concentration, it will get a high R value.

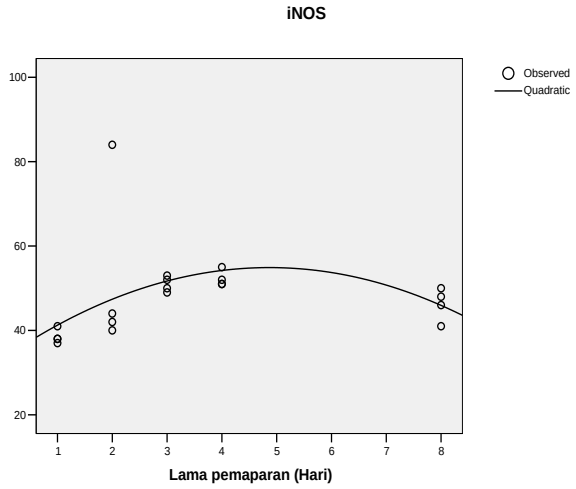

Figure 6. Linear regression of length of exposure on iNOS

3.3 Mitogen Activated Protein Kinase-38 (MAPK P38)

Immunohistochemical techniques using specific antibodies on MAPK-p38, indicating that p38 MAPK-expressed strongly on hepatocyte nuclei by treatment with LAS various doses and duration of exposure (Fig.7).

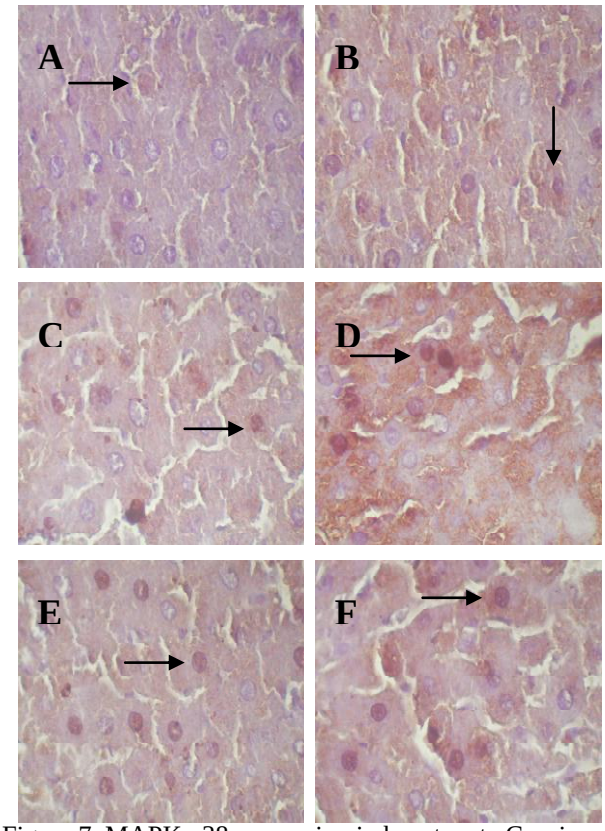

Figure 7. MAPK p38 expression in hepatocyte Cyprinus carpio L

P38 MAPK expression patterns can be seen on the Post Hoc Test (Tukey HSD test). Duration of exposure and dose of exposure also interacted significantly p = 0.0005 on p38 MAPK expression, it can be seen in the table test between-subject effect.

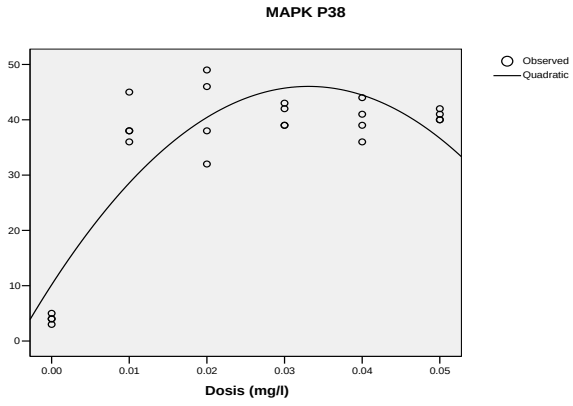

Figure 8. Linear regression of LAS concentration on MAPK p38

Increased duration of exposure per group also resulted in an increase in the number of hepatocyte expressing p38 MAPK. 24,48,72,96 Increased exposure time hours to the day-to-8, there are increased hepatocyte expressing p38 MAPK (Figure 8) to a maximum of 10% for each exposure time interval.

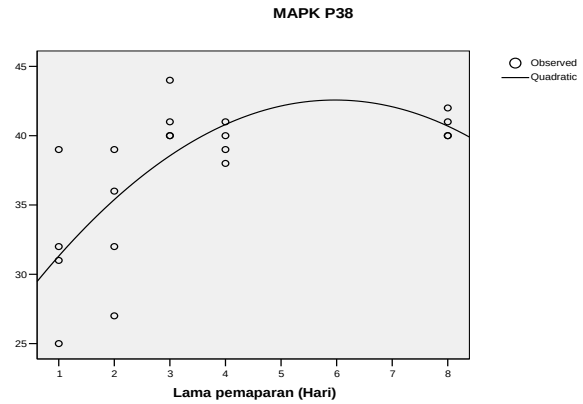

Figure 9. Linear regression of length exposure on MAPK p38

Equation of a line in the Curve Fit LAS showed a long exposure has a positive correlation on value of the expression of p38 MAPK, where the number of hepatocyte expressing p38 MAPK x2 = -0.456 + 5.443 x + 26.36. This is indicated by the value of the Pearson correlation R = 0.714 and significant value of p = 0.0005 (Figure 9). The equation shows that the number of hepatocyte expressing p38 MAPK for each duration of exposure for the sum of -0.456 (squares a day) plus the result of multiplying 5.443 (day) and 26.36, the 71.4% confidence level.

3.4 Cytochrome P450 (CYP1A)

Immunohistochemical techniques using specific antibodies on CYP1A, indicating that CYP1A expressed strongly on hepatocyte induced by LAS exposure with various doses.

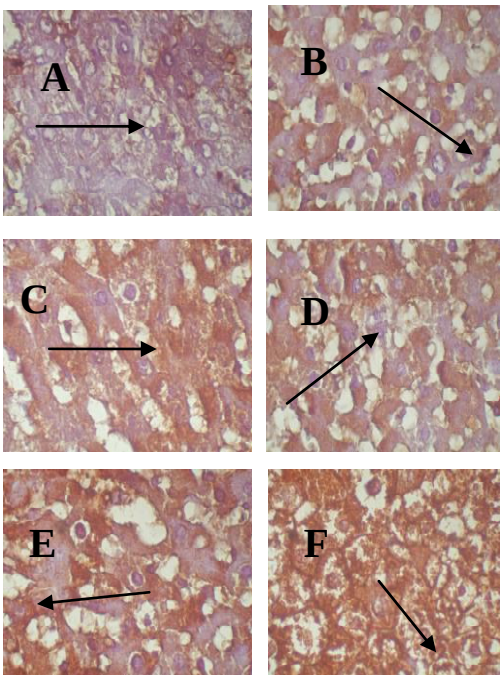

Figure 10. CYP1A expression in hepatocyte Cyprinus carpio L

Role of CYP1A can be seen also on the test table MANOVA test between-subject effect and the General Linear Model (Multivariate Tests) showed that the duration of exposure and dose of exposure had a significant level of p = 0.005 on CYP1A

expression. Equation lines show the CYP1A = 1256944.44 x3-111 011, 905x2 +3229.067 x +6.399, with a confidence level of 98.6%.

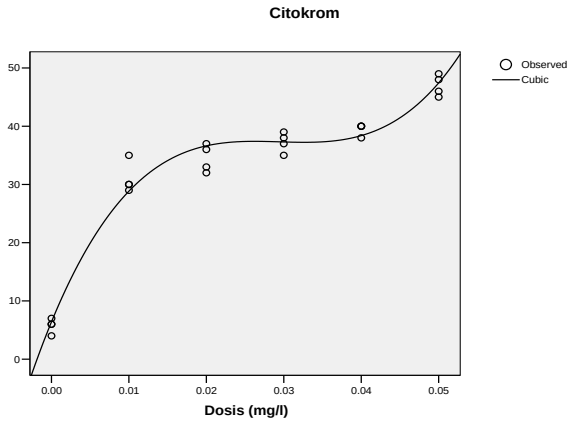

Figure 11. Linear regression of LAS concentration on CYP1A

Curve Fit linear regression showed a longer exposure LAS positive correlation value on the expression of CYP1A, where the number of hepatocyte expressing CYP1A = 1.853 x + 32.38. This is indicated by the value of the Pearson correlation R = 0.877 and significant value of p = 0.0005 (Figure 12). The equation shows that the number of hepatocyte CYP1A expression for the sum of 32.38 with 1.853 and the theoretical duration of exposure, with a 87.7% confidence level.

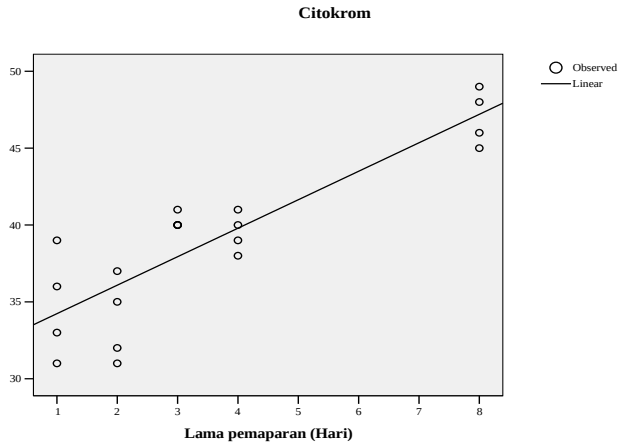

Figure 12. Linear regression of length exposure on CYP1A

These results complement previous studies, that turned out to LAS as a detergent component triggers the expression of HSP70, iNOS, p38 MAPK, and CYP1A overall. Expression of HSP70, iNOS, p38 MAPK, and CYP1A increased with increasing exposure concentration and duration of exposure, which describes the molecular changes induced by LAS. Expression of HSP70, iNOS, p38 MAPK, and CYP1A qualified definition and criteria to be used as a biomarker, but its existence did not provide specific information that refers to one of the causes of pollution. This makes the presence of HSP70, iNOS, p38 MAPK, and CYP1A only be used as a biomarker of pollution in marine environment.

Biomarkers generated in this study, when applied in a biomonitoring program makes HSP70 a priority. This is because HSP70 is able to describe the condition of the marine environment through the description of the stress experienced by aquatic organisms. On the implementation of biomonitoring in the field, observation of HSP70 and apoptosis adequately describe the condition of the waters. In this study, apoptosis was not observed parameters. The results of this study indicate that the three other biomarkers when observed separately, namely iNOS, p38 MAPK, and CYP1A not been able to ascertain the occurrence of environmental pollution. In this study the expression of iNOS, p38 MAPK, and CYP1A gives an overview of the effects caused by LAS and defense mechanisms against pollution LAS aquatic organisms.

4. Conclusion

Conclusions resulting from the study are as follows : HSP70, iNOS, P38 MAPK and CYP1A can be used as a biomarker of pollution LAS in aquatic environments. LAS exposure triggers hepatocyte for expressing HSP70, iNOS, P38 MAPK and CYP1A where growing numbers with increasing exposure concentration and duration of exposure to LAS.

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