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Are simple biomarkers sensitive to detect toxicity of low concentration of metal?

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Abstract. Biomarkers are considered as appropriate and sensitive tools to detect contaminants which effects are recorded on a sentinel organism. Research on simple biomarkers has been conducted in the laboratory to detect the toxicity of low concentrations of Arsenic by using green mussels, *Perna Viridis*, as sentinel organisms. The simple biomarkers used are biogenesis, Stress On Stress (SOS), and the condition index. Green mussels of 2.5-3.5 cm were exposed to concentrations of 0, 0.008, 0.038 and 0.19 mg/L of arsenic for 14 days. The results revealed that biogenesis was not sensitive to arsenic toxicity in green mussels. The air survival test or Stress on Stress was tested in terms of LT_{50} of Kaplan-Meier test. The results showed that LT_{50} for treatments of 0, 0.008, 0.038 and 0.19 mg/L were 3.15, 2.76, 2,63 and 3.03 days respectively. It demonstrated that the air survival of mussels in control was longer than those were exposed to a series of arsenic concentrations descriptively, but statistically, they were not significantly different. However, the condition index of the green mussel revealed a significant difference statistically between control and 0.19 mg/L of Arsenic. This suggested that the condition index can detect the toxicity of Arsenic to the green mussel.

1. Introduction

A biological approach has been used as a complement or counterpart of the classical approach in monitoring and studying the negative effects of marine pollution on many international monitoring programs [1–4]. Chemical analysis solely is considered inadequate to interpret the impact of pollution in the marine ecosystem, since it does not directly depict the destructive effects on the organism and its environment[5,6] and the presence of biotransformed chemicals within the organism's body [7]. In a lot of cases, biotransformation may increase the level of toxicity of pollutants in organisms through the production of more toxic and reactive metabolite compounds than their parent compounds [8]. Furthermore, the classic chemical approach is expensive, requiring sophisticated tools of high value such as AAS (Atomic Absorption Spectrophotometer), GC-MS (Gas Chromatography-Mass Spectrometry), produces little data that has biological benefits and consequently simplifies or reduces the complexity of the monitored ecosystem [9].

Biomarkers are regarded as relevant and sensitive devices for detecting either the contaminant in the form of exposure or effects that appear on a sentinel organism[10–13]. This is because biomarkers can record and provide biological information that is relevant and comprehensive concerning the health status of the organism [14–16]. In the case of pollutants that have low stability in the

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environment, such as organophosphates and carbamates, biomarkers are good tools for estimating their impact on biota even after the pollutants no longer exist in waters [17]. This is due to biomarkers that can detect a persistent response or effect from the pollutant recorded throughout the range of a biological organization of a biota that is targeted for study or monitoring. [18]. Therefore, biomarkers become a trend that is used as a tool of biomonitoring around the world to estimate the risk of pollutants in aquatic environments[16,19–23].

Over time, the worldwide development and use of biomarkers tend toward studying and using biomarkers that require expensive tools [24]. The basic idea is that biomarkers are used in biomonitoring as well as ecotoxicological tests in the laboratory as a substitute for expensive chemical analysis approaches [1,2,24–26]. This tendency makes it difficult for scientists and environmental practitioners, especially those in developing countries, to use biomarkers [24]. The consequence is that a lot of valuable evidence in the field of ecotoxicology is missed without any research [24]. Therefore, studies are needed to establish simple biomarkers using sentinel organisms such as the green mussel, *Perna Viridis*.

The bivalves of the Mytilidae family, including the blue mussel *Mytilus edulis*, which are closely related to green mussels, have long been applied as sentinel organisms in biomonitoring [19,27–30]. As sedentary animals and filter feeders, mussels cannot escape from the polluted waters in which they live and accumulate contaminants that exceed the level of contaminants in the water [31,32]. This behavior makes mussels valuable sentinel organisms that can realistically record pollutant concentrations that can biologically impair aquatic organisms (bioavailability). The bioavailability of contaminants in the mussel's body is also strengthened by the fact that mussels have ineffective enzymes in detoxifying the contaminants so that only a small amount of pollutants can be expelled from the body [7]. Hence, mussels are suitable as an eco-sentinel organism [32]. Blue mussels *Mytilus edulis* have been extensively applied as eco-sentinel organisms in temperate regions. Currently, studies of biomarker and green mussels as sentinel organisms have been widely performed in the tropical region, though not as extensively. In Indonesia, the use of biomarkers as an integral part of monitoring, especially by using green mussels, is still very rare. Therefore, studies underlying simple biomarker applications that use green mussels as eco-sentinel organisms are indispensable. The studies will improve the effectiveness and efficiency of ecotoxicological tests in laboratory and biomonitoring in the field based on the effects of pollutants in Indonesian marine waters.

A simple biomarker that can be extracted from the green mussels is a biomarker-based on morphometric body shells formulated in the form of condition index [24] and growth index [33]. In addition, the physiological condition of the shellfish can also be used as a simple biomarker to detect contaminants such as Stress On Stress [24] and byssus production [34–36]

This paper will describe and discuss the results of laboratory research using simple biomarkers to detect the effects of arsenic contaminants. We propose that the simple biomarkers of green mussels can detect the effects of the low concentration of Arsenic.

2. Materials and Methods

2.1. Study area and sampling

Three hundred green mussels with a size of 2.5-3.5 cm were collected from green mussels collectors and ropes of seaweed cultivation in Mandalle waters, Pangkejene Kepulauan (PANGKEP) district, South Sulawesi, Indonesia. Once collected, the green mussel samples were put into a cool electric box and brought to the laboratory. At the laboratory, samples were acclimatized in aquariums for 21 days in seawater, which was filtered with salinity 30 o/oo. During acclimation, the green mussels were fed with *Spirulina sp* algae with a density of 10 x 406 cells / 1 [37] after seawater was changed daily.

2.2. Chemical

The chemical used in the study was arsenic acid (H_3AsO_4 in HNO_3 0.5 mol/l) ordered from Merck, Germany.

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2.3. Exposure experiment
The study used a complete randomized design. Twelve aquaria containing four liters of seawater were used, and ten green mussels measuring approximately 2.5-3.5 cm were inserted into each aquarium. Three green mussels were put into a plastic bottle for the measurement of byssus production. After that the medium was contaminated with arsenate (AsV) of Na_3AsO4 (0, 0.008, 0.038 and 0.19 mg/L for 14 days). Three replicate aquaria were used for each concentration. During the exposure experiment, the aqueous media was aerated to maintain dissolved oxygen concentrations in order to keep the ideal oxygen conditions for the animals. The green mussels were fed *Spirulina* sp with a density of 10 x 406 cells/L [37] one hour before the water was changed. Temperature, oxygen, pH and salinity were measured daily before feeding time.

2.4. Parameters
Biomarker parameters consist of three types, namely biogenesis, Stress On Stress (SOS), and condition index. Byssogenesis was determined by counting plaques attached on the plastic bottle. We measured Stress On Stress (SOS) by putting green mussels from all treatment in chambers at 25^{o} C for aerial exposure. Dead animals were recorded daily. The death symptom was determined to be an open valve and no reaction.

The condition index was measured using the formula suggested by Lucas & Beninger [38], which is the dry tisssue weight/dry shell weight ratio. This index is widely used, more universal and proposes valuable information on the physiological condition of an animal since it easily standardized, describes the response of a biological state under environmental changes and eliminates the bias because of water content fluctuation of entire tissue [38]. Hence, it is a good indicator of environmental Stress [38].

2.5. Data analysis
Biomarker parameter data were examined for normality and homogeneity before ANOVA was performed. If the data were not normally distributed and homogeneous, they were transformed. If the ANOVA revealed differences, the data were tested using a Bonferroni test to determine the difference between treatments. If, after transformation, the data remained abnormal and not homogeneous, then a non-parametric test (Kruskal-Wallis test) was used to determine the difference between treatments. If the Kruskal-Wallis test indicated such differences, we proceeded by using Dunn's Multiple Comparison to quantify the differences between exposure treatments. The correlation between the values of the condition index on each treatment with the nominal arsenic concentrations was determined using the Pearson test.

3. Results and Discussion
3.1. Results
3.1.1. Byssogenesis. From the statistical test to the byssus production parameter, it is known that there is no difference in the production of byssus of the green mussels in the control treatment with those exposed to series of arsenic concentrations. This suggested that the production of byssus is not sensitive to arsenic toxicity in term of the concentration range being treated (Figure 1 and 2).

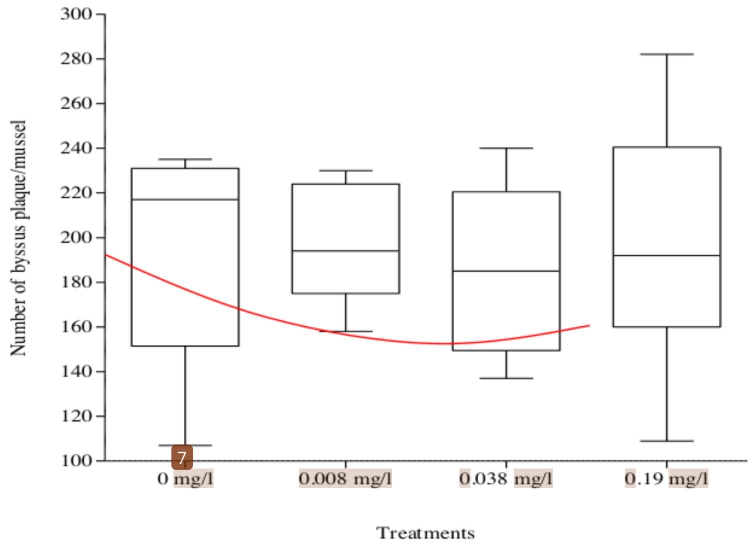

Figure 1. Byssogenesis of green mussels which treated by series concentration of Arsenic for 14 days (N= 3 muessels per treatment). Red curve describes the phenomenon of hormetic like-effect.

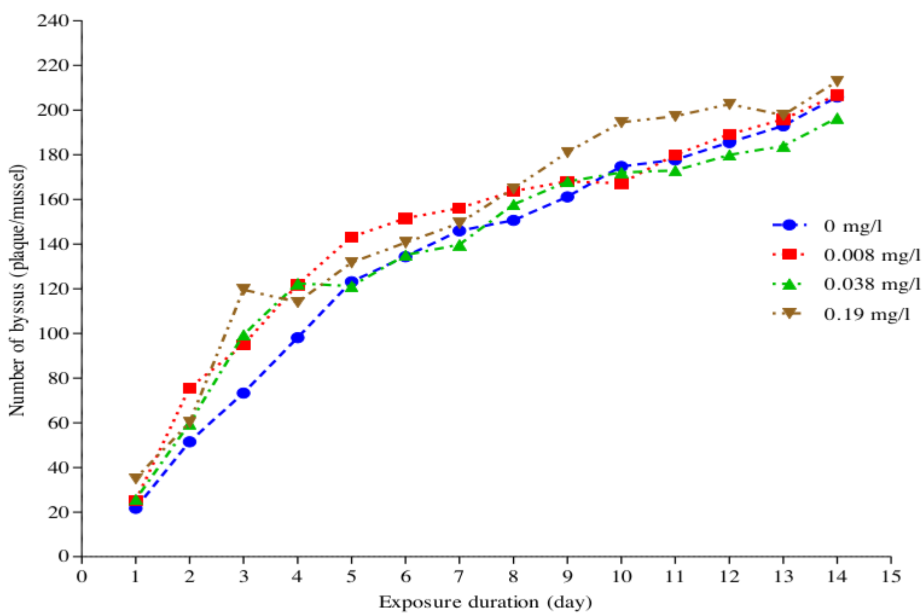

Figure 2. Average daily byssus production which exposed to Arsenic for 14 days.

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3.1.2. Stress On Stress. Stress On Stress is one of the parameters in ecotoxicology to measure the ability of mussels to live in the air after being exposed to a certain contaminant. Stress On Stress is analyzed with LT_{50} concept. The LT_{50} is measured by the Kaplan-Meier test, which measures 50% of the population, which died for a certain period of time. LT_{50} measurements showed that LT_{50} for treatment 0, 0.008, 0.038 and 0.19 mg/L were 3.15, 2.76, 2.63 and 3.03 days, respectively (Figure 3). This Kaplan-Meier curve, when tested with statistical tests, showed no significant difference. However, if the LT_{50} value plotted in the histogram will show the phenomenon of hormetic-like effect (Figure 4).

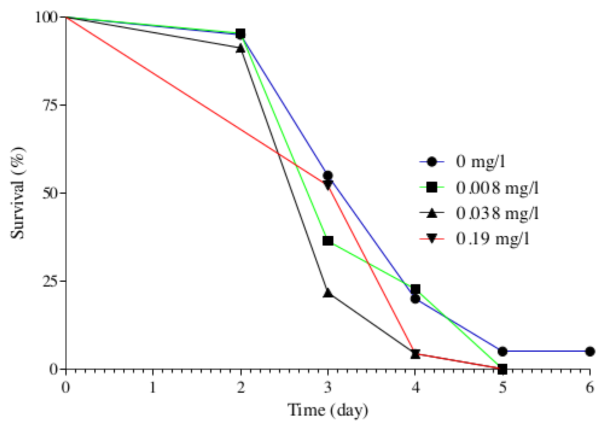

Figure 3. LT_{50} in term of Kaplan-Meier test.

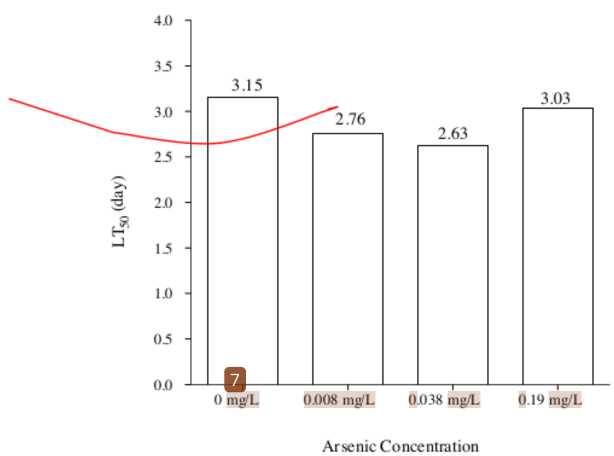

Figure 4. LT_{50} of green mussel, *P. Viridis* exposed to Arsenic. The red color curve shows the phenomenon of hormetic like-effect.

3.1.3. Condition Index. Green mussel condition index is a parameter that describes the health condition of mussels. Theoretically, the higher the index value, the higher the level of health [38]. The results showed that there was a significant difference in the condition index between the controls and the concentration of 0.19 mg/L. The condition index of mussels under control conditions was significantly higher compared to those kept at 0.19 mg/L. However, the controls did not show statistically significant differences ($p < 0.05$) compared to those exposed to lower concentrations of 0.008 and 0.038 mg/L (Figure 5).

The correlation analysis between the values of the condition index in each treatment with the tested sarsen level showed a statistically significant correlation ($p < 0.05$) with the value of R is -0.9885. The condition index value is inversely proportional to the concentration of arsenic exposure. The data shows that the higher the Arsenic, the lower the conditions index (Figure 6).

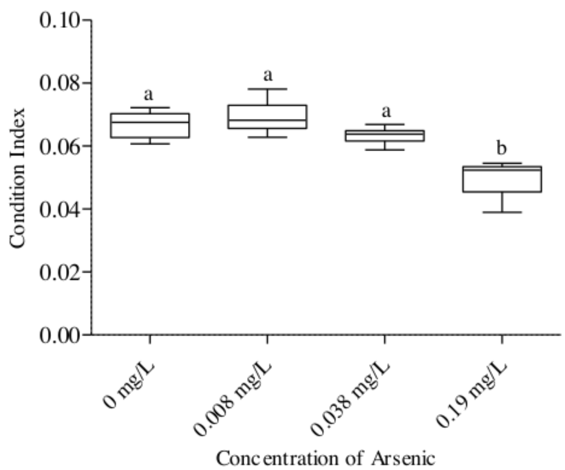

Figure 5. Index of conditions of green mussels, *P. viridis* exposed to Arsenic. Different letters show significant differences ($p < 0.05$). The bar is the median with the highest quartile is 90% and the lowest quartile10%.

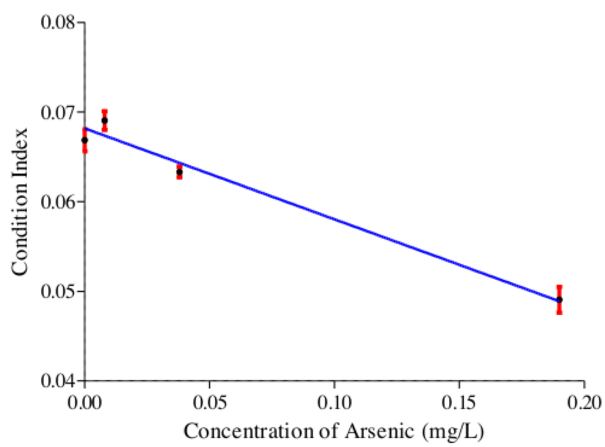

Figure 6. The correlation between green mussel condition index, *P. viridis* with the nominal arsenic concentration, i.e 0, 0.008, 0.038 and 0.19 mg/l was used in the study. Statistically, the correlation is significant ($p < 0.05$) with the value of R is -0.9885. Point is average, bar is the standard deviation.

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3.2. Discussion

Arsenic is a marine biogeochemical group which naturally or anthropogenically present in the marine ecosystem which has important toxicity implications for marine biota and who consume it including human [39–41]. The average of total arsenic concentration in the oceans is about 1.7 mg/l [39]. The dominant form of Arsenic in the ocean and the brackish water is arsenate (As V). While the most toxic arsenic form of arsenite (As III) rarely exceeds 20% of the total Arsenic present in the ocean [39].

In determine the toxicity of Arsenic to marine organisms, green mussels are rarely or even almost never used as sentinel organisms by utilizing simple biomarkers. Current research attempts to use simple biomarkers from green mussels to detect arsenic toxicity with relatively low concentrations. Biomarkers used are byssogenesis or byssus production, Stress On Stress, and the condition index.

The production of byssus is a way by which the mussel remains stick on a substrate so that it is not disturbed by currents or waves. Biological responses in the form of byssus production have been used as biomarkers to detect the toxicity of pollutants in the laboratory[36] and environmental monitoring [42]. The two studies have successfully detected the toxicity of pollutants by using byssus production.

Yaqin and co-worker (2014) demonstrated that byssogenesis of green mussel was able to detect the toxicity of Pb and Cd mixtures at concentrations of 0.2 mg/L [36]. To examine the sensitivity of *Mytilus trossulus* byssogenesis in the field, Moles and Hale (2003) compared the byssogenesis from the mussels living in polluted and uncontaminated areas [42]. The results indicate the mussels that live in uncontaminated waters produce more byssus than those in the polluted area. In the present study, the byssus production of green mussels between the control treatment and the series of arsenic concentrations did not show significant differences statistically. This indicates that the series of arsenic concentrations are not toxic to inhibit the byssus production of green mussels. This may be because byssus on low arsenic pollution is used by green mussels as a metal excretion [43], so the green mussels still use the energy to produce byssus. In fact, exposure to low-concentration cadmium of 0.05 or 0.1 mg/l to green mussel can strengthen the ability of green mussels' byssus in accumulating metals by producing various proteins known as binding metals such as cysteine, tyrosine, and histidine [44].

However, if we look at the graph descriptively, there appears to be a phenomenon of hormetic like-effect. Hormetic like-effect is defined as a response to a biphasic dose-response where the low-dose biological effects of pressure show the opposite effect of high-dose biological effects of a similar type of pressure [45]. The type of hormesis observed in the byssus production of green mussels exposed to Arsenic is J-shape hormesis, where at low doses, there is a decrease in byssus production when compared to controls until a condition in which increased arsenic concentrations actually causes increasing the byssus production.

A J-shape hormesis also appears on the Stress On Stress biomarker. Measurement of Stress On Stress or the ability of mussels to live in the air will give us an idea of exogenous and endogenous pressures on mussels. Mussels that live in the intertidal region will experience hypoxia and anoxia associated with tides and other oceanographic events. To overcome the conditions, the mussels decrease the rate of metabolism so that it can store energy. Prolonged exposure in the air gradually leads to anoxic conditions in the body and metabolic processes will change from aerobic pathways to anaerobic pathways. When under conditions of environmental Stress, the mechanism of adaptation or detoxification will increase the energy requirement. Therefore, the influence of biotic or abiotic factors on the ability of the shell to survive in the air makes the parameter sensitive as a tool to evaluate the environmental stress conditions. In the present study, Stress On Stress (SOS) biomarkers showed no significant difference between exposure treatments using arsenic concentrations. However, if the LT_{50} value is plotted against the arsenic concentration series it will appear J Shape hormesis.

The results of the present study differ from those of some bivalve-related SOS studies, which show that there is an effect of pollutants on SOS [24,46–48]. This is probably because in the present study, the concentrations of arsenic metals used were so low that they have not shown any effect on SOS. LC_{50} of Arsenic for biota of the mollusk group ranges from 0.350-0.750 ppm [49].

In contrast to the two biomarkers described above, the condition index showed a significant difference between control and arsenic treatment of 0.19 mg/L. This shows that the condition index can detect arsenic toxicity at concentrations of 0.19 mg/L. Some green mussels researchers also pointed out that the conditions index of green mussels are sensitive in detecting metal pollutants [50–55].

In the present study, the condition index used is a condition index with a formula that involves measuring the weight of mussel tissue, which is a reflection of the growth of the mussel. The condition index value of treatment 0.19 mg/L, which is lower than the control, is mostly due to the decrease of mussel tissue weight. This is because the exposed mussels will more often close their shells to avoid metal exposures [51]. Closed mussel shells result in a reduction in the filtration rate of mussels, which is resulting in a decrease in the amount of food supply in mussels. The reduced food supply will result in a decrease in the growth rate of tissue weight. This condition causes the low value of condition index at treatment 0.19 mg/L compared to the condition index of the control. A low value for this index exhibits that a major biological effort has been expended, either as maintenance energy under poor environmental conditions or disease or in the production and release of gametes. Thus, as an indicator of Stress, or sexual activity, this index gives significant information about the physiological state of the animal [38].

4. Conclusion

This study shows that not all simple biomarkers using green mussel as sentinel organisms can detect arsenic toxicity. Byssogensis and Stress On Stress were not statistically able to detect arsenic toxicity in the range of used concentration. However, the biomarkers show a phenomenon of a hormetic-like effect. This phenomenon is interesting because it suggests that if research is conducted using a wider and higher range of concentration series, it is likely that the two biomarkers can detect arsenic toxicity. In contrast to the two biomarkers, the condition index can detect arsenic toxicity at a concentration of 0.19 mg / L. This evidence gives us a new perspective and hope that something simple in terms of biomarkers is not necessarily insensitive. Therefore, it is necessary to explore more deeply the potential of simple biomarkers as monitoring tools in field and laboratory studies.

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