100 *Current Nutrition & Food Science*, 2014, *10*, 100-106

The Use of Byssogenesis of Green Mussel, *Perna Viridis*, as a Biomarker in Laboratory Study

Khusnul Yaqin[1,*], Joeharnani Tresnati[1], Rohani Ambo Rape[2] and Muhammad Aslam[1]

[1]*Department of Fisheries, Faculty of Marine Science and Fisheries, Hasanuddin University, Jalan Perintis Kemerdekaan Km 10, Makassar 90245, Indonesia;* [2]*Department of Marine Science, Faculty of Marine Science and Fisheries, Hasanuddin University, Jalan Perintis Kemerdekaan Km 10, Makassar 90245, Indonesia*

Abstract: Marine pollution monitoring is important for food bio-safety as well as the conservation of the environment. The green mussel, *Perna viridis* has previously been used as an eco-sentinel organism in marine pollution monitoring. In this study the byssogenesis of *P. viridis* was used as a biomarker during an *in vivo* study. Fifteen *P. viridis* were exposed for 14 days in filtered seawater to metal mixtures of lead (Pb) and cadmium (Cd) containing 0.008, 0.04, 0.2, 1, 5 mg/l of each metal for 14 days. The results showed that Pb and Cd residues in the mussel tissue were proportional to the metal concentration in water. Kruskal-Wallis and Dunn's Multiple Comparison tests were used to assess the effects of metal exposure on the production of byssus. The test results showed that the byssus production in 0.2 and 1 mg/l treatments was significantly different from controls ($p < 0.05$). Backward elimination regression was used to discern the role of Pb and Cd in the byssus productions. The regression demonstrated that Pb played a more important role than Cd in terms of byssogenesis. The study suggested that the byssogenesis production of *P. viridis* has potential to be used in biomarker studies.

Keywords: Byssogenesis, green mussel, biomarker, metals.

INTRODUCTION

As sedentary animals which live in the intertidal zone mussels must withstand the fluctuating conditions generated by biological, chemical and physical factors in order to survive. To avoid displacement by current and wave action mussels attached to solid substrata by means of byssus. The cost for making and maintenance of the byssus is about 8% and 12% respectively of the total metabolic energy of a mussel [1, 2]. The byssus is composed by five proteins and collagens that are produced by the foot of the mussel and divided into three parts; 1) the root which is located in base region of the foot and links the whole musculature of byssus retractor, 2) the stem which stretches out from the root and supports each byssal thread, and (3) the byssal threads that produced along the groove of the foot and stretch out from the stem [3, 4]. The byssal thread consists of three parts which are distal, proximal parts and the attachment disc, plaque [3-5].

Mussels produce the byssal thread to resist hydrodynamic factors such as tide and wave action in fluctuating ecosystems. The byssal thread is one of the mussel organs which has a capacity to absorb and accumulate chemical compounds [6-8]. The distribution of metals such as Zn, Cu, Pb and Cd in different part of byssus and soft body of the green mussel, *Perna viridis*, has been studied [9]. Yap and coworkers [9] found that the byssal thread, particularly the plaque, has the highest uptake and accumulation rates of metals. Although metal residues in byssus are lower than those in shell of green mussel [10], the phenomenon still inspired the use of the byssal thread of *P. viridis* as a target organ for biomonitoring of marine pollution in Malaysian waters [11-13].

Mussels have been extensively used as eco-sentinel organisms which provide biomarkers from molecular [14-20], cellular [20-23] to behavior levels [24, 25] in laboratory and field studies. However, byssogenesis or byssus production in the context of mussel behavior as a potential and meaningful biomarker has not been studied extensively using green mussel, *P. viridis*. Since Holmes [26] studied the reduction of byssus production of bivalves caused by chlorine, this chemical compound has been used as an antifouling agent in sea water cooling systems. Roberts [27] used the byssal thread production of *Mytilus edulis* to detect the effects of the pesticide, endosulfan. He showed that the byssus production was inhibited by 50% after exposure to endosulfan for 24 h at 0.45 mg/l. Hence he suggested that the byssogenesis is a suitable biomarker that provides a rapid and convenient method for routine testing of aquatic xenobiotics. Since the byssal thread is "an external organ" of mussel that can be measured manually, the use of byssogenesis as a biomarker is potentially a non-destructive, simple, and cost-effective biomarker.

For those reasons the use of byssogenesis of green mussel *P. viridis* as a biomarker for laboratory and field biomonitoring is intriguing and in need of further study. The current study investigated the use of green mussel byssogenesis production in response to metal exposure in the laboratory. Finding from this study may be expanded to field

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*Address correspondence to this author at the Department of Fisheries, Faculty of Marine Science and Fisheries, Hasanuddin University, Jalan Perintis Kemerdekaan Km 10, Makassar 90245, Indonesia;
Tel/Fax: +62411588828; E-mail: khusnul@gmail.com

2212-3881/14 $58.00+.00

Table 1. The residue of Pb and Cd in tissue of *P. viridis* after exposure to Pb and Cd for 14 days.

Exposure Dose (mg/l)	**0**	**0.008**	**0.04**	**0.2**	**1**
Cd (µg/g dry weight)					
1	13.06	84.52	67.94	225.51	1046.03
2	9.09	23.4	40.9	221.78	1048.5
3	15.05	21.23	55.93	200.8	795.6
Mean	12.40	43.050	54.923	216.03	963.377
Pb (µg/g dry weight)					
1	1.14	6.41	2.8094	20.65	36.53
2	0.707	1.13	3.22	7.49	58.14
3	0.55	1.55	3.211	13.85	63.78
Mean	0.799	3.030	3.080	13.997	52.817

investigations of marine pollution biomonitoring for food biosafety and ecosystem conservation.

MATERIALS AND METHODS

P. viridis (length 4-5 cm) were hand-collected from a seaweed culture area in Pangkajene Kepaulauan regency. The mussels were transferred directly using a humid cool box to the laboratory of Marine Science and Fisheries Faculty, Hasanuddin University. Before acclimating the mussels in filtered sea water for at least seven days, fouling organisms on the mussels shell were removed.

Each group of 15 mussels was exposed to nominal mixtures of Pb and Cd at concentration of 0, 0.008, 0.04, 0.2, 1.0, 5 mg/l in 4 l of filtered sea water for 14 days. Their replicates were used for each dose resulting in a total of 18 aquariums each containing 4l of seawater and 15 mussels. Three mussels from each group were kept in plastic petri dishes for counting the byssal thread. The mussels were fed by *Spirulina sp.* one hour before replacing the filtered sea water every 24 h. The production of the byssal threads was counted each day commencing from day 4. After fourteen days of exposure to metals the experiment was terminated and mussels were dissected out. The metals residues were analyzed from mussel soft tissue using Atomic Absorption Spectrophotometry (ASS).

STATISTICAL ANALYSIS

Kruskall-Walis with Dunnett's post hoc test were used to distinguish the differences in the byssal thread production among the treatments at a significance level of $p < 0.05$. Backward elimination regression was used to determine the metal residues that influence the byssus production. Statistical analyses were conducted using GraphPad Prism trial version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. The association between metal concentrations and the byssogenesis was analyzed by simple and multiple regressions.

RESULTS

Metal

Metal body burden of an animal is potentially an important sign of water contamination. However, metal burden does not suggest that harm has been caused to organisms and to get precise toxicity information of metals it is necessary to study responses of organisms to such contamination [28]. (Table **1**) describes the metal body burden of Pb and Cd in tissue of *P. viridis* after exposure to mixtures of Pb and Cd for 14 days. Organisms exposed to 5 mg/l Pb and Cd were not used as they did not survive the experiment beyond 4 days.

(Figs. **1** and **2**) shows that Pb and Cd loading in green mussel tissue was dose-dependent. Statistical analysis showed that the correlation coefficients of Pb and Cd residues with serial dilution of the metal exposures were 0.956 ($p<0.05$) and 0.988 ($p<0.05$) respectively, indicating strong associations between the serial dilution of the mixture metals and the metal loads in mussel tissue. Notwithstanding, the accumulation levels of the two metals in the mussel tissue were different. Cadmium (Cd) was accumulated by green

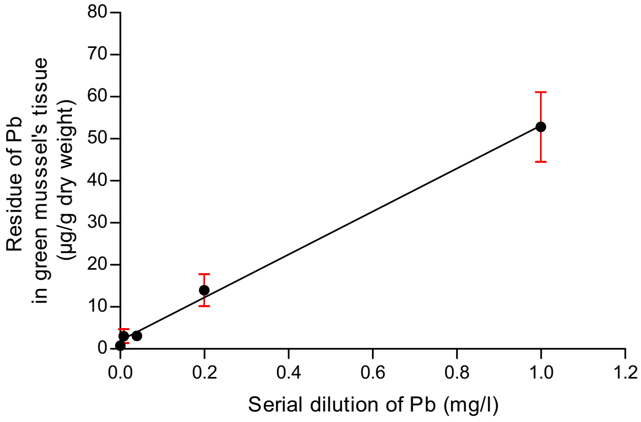

Fig. (1). Relationship between residue of Pb in green mussel's tissue and serial dilutions of Pb exposure for 14 days.

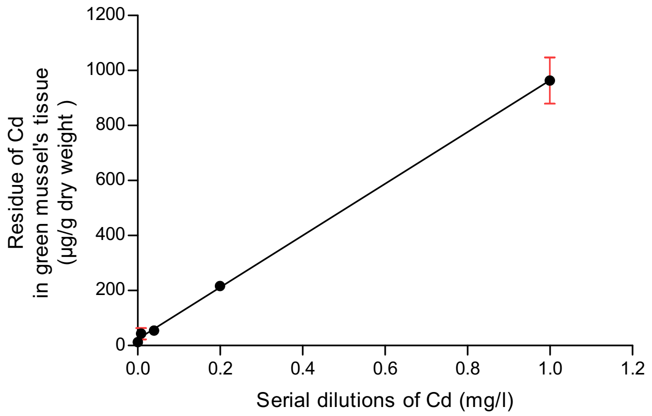

Fig. (2). Relationship between residue of Cd in green mussel's tissue and serial dilutions of Cd exposure for 14 days.

mussel at a much higher rate compared to lead (Pb). By employing a multicolinearity test, VIF (Variance Inflation Factor) values of Pb and Cd in relation to the production of byssus were determined. The VIP of Pb and Cd were 6.804.

BYSSUS PRODUCTION

After exposing the mussel to the serial dilution for four days the production of byssus was measured. Later on, the measurement of byssus production was performed daily for ten days, from day 4 to day 14 of exposure. The results highlight that the byssus production trend was a dose-dependent response. The byssus productions decreased with the increasing metal concentrations. Statistical analyses did not show different byssus production for treatments of 0.008 and 0.04 mg/l compared to the controls and the median of the treatments suggested a decreasing trend. Statistical analysis using Kruskal-Wallis method depicted that the byssus productions of green mussels exposed to concentrations of 0.2 and 1 mg/l Cd and Pb combined differed from those of the control ($p<0.05$) (Fig. **3**).

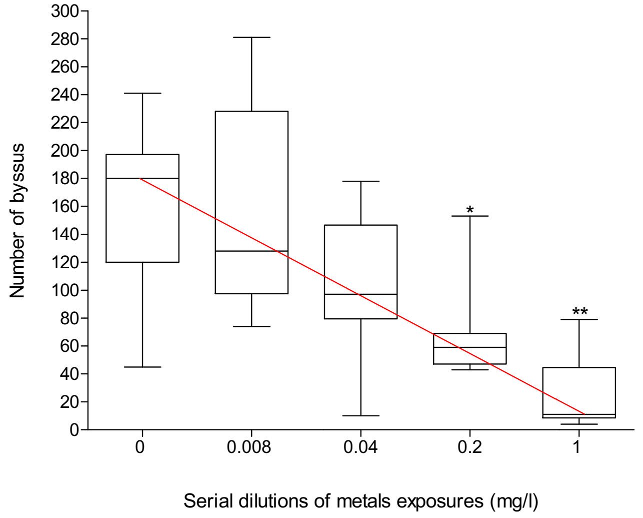

Fig. (3). Byssus production of green mussel after exposure to Cd and Pbfor 14 days. * indicated significant difference from control ($p< 0.05$). ** Indicated significant difference from control ($p < 0.001$).

(Fig. **4**) shows that on the fourth and fifth day of exposure the byssus production was not influenced by metal concentrations. However, after six days of exposure the treatment of 0.2 mg/l had significantly increased byssus production ($p<0.001$). In contrast, the byssus production of green mussels exposed to 1 mg/l decreased significantly ($p<0.05$). The byssus production in metal exposures of 1 mg/l was consistently lower than controls over time. However, on the ninth day of exposure byssus production was somewhat higher than at other time periods. In concentration of 0.2 mg/l the byssus production was higher than that of 1 mg/l treatment, but it was significantly lower than that of the control animals. The byssus production of the green mussel exposed to 0.008 and 0.04 mg/l concentrations increased with

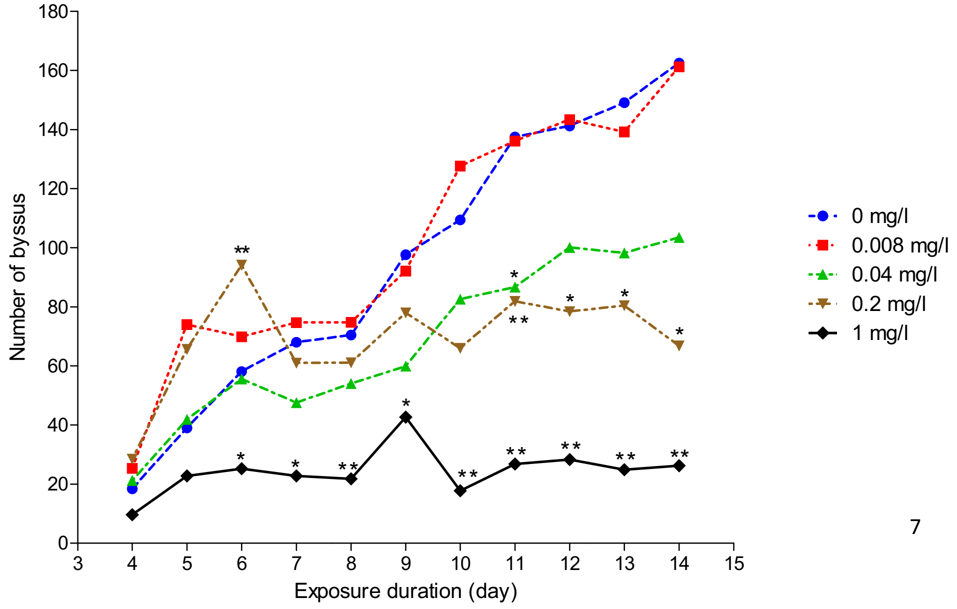

Fig. (4). Byssus productions under mixture metals exposures for 14 days. * indicated significant differencefrom control ($p < 0.05$). ** indicated significant difference from control ($p<0.001$).

the duration of exposure and not significantly different to that of the control animals ($p<0.05$). Notwithstanding, on the eleventh day the byssus production in exposure concentrations of 0.04 mg/l was significantly lower than that of the control ($p<0.05$).

DISCUSSION

Information on metal concentrations alone in aquatic ecosystem as a result of monitoring programs is not adequate as an indicator of aquatic ecosystem health. Metal toxicity is influenced by some factors such as metal speciation, route of metal to body of living organism which can produce disruption to the organism, and the ability of the living organism to detoxify metals [29, 30]. Hence, the understanding of processes that control metal speciation in aquatic ecosystem, metal uptake by living organism and organisms response to metals plays important roles in developing criteria for acceptable levels of metals that not only considers the environmental chemistry aspects but also the responses by living organism [31-33].

The bioavailability of metal residues in green mussel body was considered as the main factor in data analysis along with the serial dilution of contaminants that were used in laboratory study. The advantages of this method compared to the classical method that uses metal concentration of water as the main factor are providing direct and undoubting measurements which integrate the effects of some chemical compounds and biological factors that influence the availability of pollutants to raise effects on living organism [34].

There were differences in the results of studies of metal residues of Pb and Cd in tissues of green mussels that have been studied in some Asian countries. In some field studies on the concentration of several metals including Cd and Pb, researchers found that the residue of Pb was greater than Cd in the tissue of green mussels that live in the Malaysian [35, 36] and Hong Kong waters [37]. However, Sasikumar and co-workers [38] found that the residue of Cd was greater than the residue of Pb in green mussels that live in the South West Coast, Karnataka India. Laboratory study was conducted by Yap and co-workers [39] on the accumulation of Cd in mussel tissues. The study showed that the rate of shellfish bioaccumulate Cd was 9 µg/g/day. Meanwhile, Yap and co-workers [40] examined the rate of Pb accumulation in the tissues of green mussels and found that the rate of bioaccumulation of Pb was 60 µg/g/day. Our results showed that the residue of Cd was greater than the residue of Pb (Table **1**). Research that was conducted by Yap and co-workers [39, 40] reaffirmed *in situ* study conducted by some authors [35-37]. Those authors found that the residual concentration of Pb was greater than Cd, while our results affirmed the results of research conducted by Sasikumar and co-workers [38].

Indeed, it is very difficult to compare research results on residues of certain metals such as Pb and Cd in the body of mussels because the comparison between a single study with other studies related to metal concentration requires the information of the metal complexity in the media and how the metal is absorbed by the body of mussels and the consequences of this. For example, *in situ* studies conducted by Yap and co-workers [35] and Kamaruzzaman and co-workers [36] in Malaysia, Fang and co-workers [37] in Hong Kong and Sasikumar and co-workers [38] in India did not include data on the speciation of Pb and Cd concentrations in the water column or sediment, turbidity levels and density of phytoplankton. Whereas the presence and bioavailability of Pb and Cd are strongly influenced by those conditions [28]. Fang and co-workers [37] in their *in vivo* studies found that Cd uptake of mussels was three times larger in *P. viridis* through water than *via* the medium of phytoplankton. In contrast, Pb tends to be bound by suspended particles [41] and then enters the body of mussels. At high levels of turbidity caused by both organic and non-organic particles, mussels tend to flocculate the particles that enter their body. By using mucus, mussels coagulate the particles as pseudo-feces that are excreted through the exhalant canal [42]. In this manner, the accumulation of Pb in the body of mussels is dependent on particle adsorption and desorption mechanisms as well as solubility. Moreover, the digestive organ of mussel is less effective in absorbing Pb [43]. Therefore, the presence of suspended particles and the ineffectiveness of the digestive organ of mussels affect the rate of accumulation of Pb in the body of mussels. This point may explain why the concentration of Cd in the tissue of green mussels was greater than that of Pb in tissue of the animals that were found in this study.

Speciation of metals such as Cd and Pb will affect the metals accumulated in the body of mussels. Cd^{2+}ions will be more easily absorbed by mussels than in inorganic or organic forms [44]. While the Pb in the form of alkil plumbum is more easily absorbed by organisms [41]. This was what might be the basis of rationality in the studies that were conducted by Yap and co-workers [35], Kamaruzzaman and co-workers [36] in Malaysia and Fang and co-workers [37] in Hong Kong, so they found that the content of Pb was greater than Cd in the body of green mussels. Perhaps the waters in which the researchers conducted the studies contained Pb in alkil plumbum form and Cd in complex organic and inorganic forms. In addition, the environment in which the mussels are alive greatly affects the absorption of the metal by mussels [45].

Our *in vivo* study revealed different results from those conducted by Yap and co-worker [9, 40]. The two last studies were conducted with metal doses of Cd or Pb and in contrast to our study which was performed by exposing the mussels to mixture of metal serial dilutions of Pb and Cd for 14 days with filtered sea water as the media. Interaction between Pb and Cd was expected to be the reason why our research found residues of Pb were lower when compared to the residues of Cd in the body of mussels. Multi-colinearity test of Pb and Cd in relation to the production of byssus demonstrated that VIF (Variance Inflation Factor) value of Pb and Cd exceeded 5 (VIFPb=6.804; Cd=6.804). This value indicated the existence of multi-colinearity between Pb and Cd in which two of these metals interact to affect the metabolism of green mussels. The high concentration of Cd in the green mussels compared to the concentration of Pb might be due to Cd being more easily absorbed by the green mussels compared to Pb. This is because the Cd^{2+} ions might mimic Ca^{2+} ions in terms of the radius and the electric potential [44]. Therefore, it is more readily absorbed by biological systems through the mechanism of Ca transport. Cadmium used in this study was $CdCl_2$ which easily splitted into ions

Cd^{2+} in seawater media [41]. Therefore the Cd accumulation in the cells blocking the accumulation of Pb in body of mussels, especially the entry of Pb into the body of the green mussel, is a process that involves organic particles and phytoplankton rather than direct binding of the organ that occurred with Cd.

The concentrations of Cd and Pb residues differed in the body of mussels but the relationship between Cd and Pb residues and serial dilution of the metals showed a linear graph (Figs. **1** and **2**). The concentration of the metals in green mussel is not only due to the number of concentration of the metals exposure, but also because the duration of exposure of the metals to green mussels, and elimination ability of green mussels to the metals. Yap and co-workers [9] showed that after depuration treatment of Cd-contaminated mussels, Cd levels in soft tissues of *P. viridis* were 10-30 times higher than that before exposure. It indicated that the depuration process of the green mussels was low and still stored the metals although they had been transferred into a clean medium.

Green mussels were still alive while their body contained a high concentration of metals. This finding suggests that green mussels can control the whole-body metals residue by managing uptake or depuration or by storage in granules or methallothionein proteins thus making them biologically inactive so that they do not have harmful effects on the mussels [44].

High accumulation and low depuration ability of the mussels to metals and the ability of the mussels to maintain the metals in their body are part of *raison de'etre* for using green mussel as an eco-sentinel organism in marine pollution biomonitoring for food biosafety. By understanding the metal tissue concentration of green mussels and its relation to such biological response of eco-sentinel organism such as byssus production, the magnitude of harmful effect of pollutant on individual, population, community and ecosystem can be better understood.

Byssus

Yap and co-workers [11] divide morphologically the byssus organ into roots hidden in the foot, stalks, and the proximal and distal plaque. The byssus production is influenced by several oceanographic factors such as salinity, temperature, water velocity [46] and predators [47]. In environmental biomonitoring campaigns, the byssus has been used as a target organ [11]. The authors found that the plaque was part of byssus mussels which accumulated the highest of Zn, Cu, Pb and Cd when compared with the other parts of byssus. In the plaque, concentrations of Zn, Cu, Pb and Cd were 73.94, 17.48, 11.92, and 2.22 µg/g respectively. When compared to the green mussel tissue, the concentration of metals in the plaque was also much higher. This suggested that the plaque was an important part of the mussels that can be used as a target organ in biomonitoring of pollutant effects.

Besides the byssus can be used as a target organ in pollution monitoring program, specially in terms of byssus production it can also be used as a biomarker. In laboratory studies, pesticides endosulfan inhibited the byssus production of blue mussel (*Mytilus edulis*) by 50% in the period of acute exposure, 24 hours [27]. The author concluded that the production of byssus was a good biomarker for monitoring the environment and also an end point in studies *in vivo*. Ayad and co-workers [48] also found that the production of byssus was greatly influenced by the pesticide Cypermethrin.

The byssus production of the green mussel, *Perna viridis* has so far not been investigated as a biomarker. In the present study, byssus production was used as a biomarker to recognize the response of mussel to the metal, Pb and Cd. Byssus production of the green mussel showed an interesting response after being exposed to a series of mixture metal concentrations of Pb and Cd. Treatment of 1 mg/l was the treatment that had the greatest influence on the byssus production. Even in the early exposure durations, the production of byssus was very low in this treatment compared to the control. Furthermore, treatment of 0.2 mg/l caused a hormetic-like effect on the byssus production. At the beginning of exposure the byssus production tended to increase until the day sixth of exposure and immediately dropped to form a flat curve on the seventh day of exposure until the end of exposure (14 days). The increasing byssus production at the beginning of the exposure period was not a sign that the health status of the green mussel was improving, but it demonstrated the phenomenon that the green mussel was trying to counter the presence of Pb and Cd which entered its body. This increasing byssus production might be an anticipation of the possibility of uprooting byssus plaque from the substrate due to the onset of the metals, Pb and Cd. On the seventh day of exposure, part of the byssus plaques uprooted and the green mussel was no longer able to produce more byssus until the end of the study.

The treatments of 0.04 and 0.008 mg/l resulted in a similar byssus production of the green mussel compared to the control. The byssus production formed a linear curve over time. On the eleventh day, the byssus production of 0.04 mg/l showed a noticeable difference compared to the control. However, it seemed that the mussels were able to adapt to the metal exposure until the end of the study, the mussels in the treatment of 0.04 mg/l were capable of consistent production of the byssus.

The phenomenon of byssus production of the mussels described in this study has highlighted that the byssus production or byssogenesis was quite reliable for use as a biomarker, however concentrations at which a response was measured were somewhat high suggesting that this is not a highly sensitive tool. In addition, the use of byssus production as a biomarker did not need to sacrifice the mussels which reduce the depletion of the target organism due to sampling activity. The usefulness of byssogenesis of the green mussel as a biomarker is also supported by the fact that besides byssogenesis's sensitivity to Pb and Cd, it is also a biomarker that is simple, cost effective and easy to measure. It is because the application of byssogenesis as a biomarker needs only simple tools such as a transparent plastic bottle or petri dish as substrate of byssus plaque. Afterwards, the number of byssus can be counted based on the plaque that was attached on the transparent plastic bottle or petri dish (Fig. **5**).

The result of correlation test demonstrated that Pb has a major contribution towards byssus production when compared to Cd. Yap and co-workers [9] found that concentra-

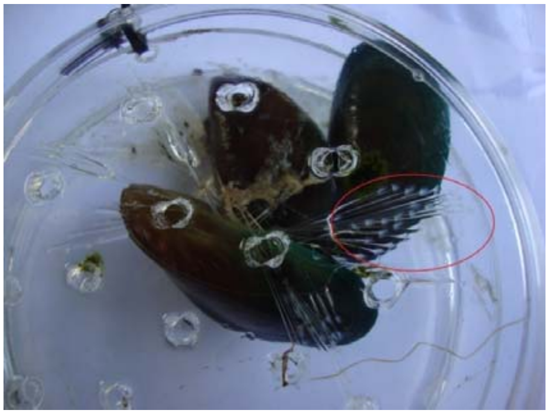

Fig. (5). Byssus of green mussel, *P. viridis* attached to the plastic petri dish. Byssus plaques are inside red circle.

tions of Pb in the foot, where the mussels produce byssus, were greater than the concentration of Cd.

This laboratory study conclusively revealed the potential use of byssogenesis to detect the effect of mixture compound of Pb and Cd on physiological level which can be reflected on ecological level. Reduction of byssus production will cause decreased capacity of mussel to attach to the substrate which in turn eventually results in the damage of mussel population as it may be swept away by the wave or current action [46] or ravaged by predators [47]. Ecologically, the damage of the mussel population will eliminate the role of mussel to absorb harmful pollutants such as Pb and Cd or atmospheric CO_2 which can induce acidification of marine habitat. It is recognized that mussel is not only a beneficial filter feeder animal, but also one of the good carbon sink marine animals [49] by flocculating marine organic particles that enter their body and excrete them as pseudo feces in marine sediment. Economically, the depletion of the mussel population will decrease income of artisanal fishermen livelihood of which depends on the population of marine organisms such as green mussel. Hence, the study should be elaborated from the laboratory level to field condition or *in situ* to validate these findings and enhance the development of the biomarker as a tool in marine pollution biomonitoring for food biosafety.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

We thank The Institute for Research and Community Services, Hasanuddin University, Indonesia for supporting the study. We would also like to thank Dr. Amanda Reichelt-Brushett Senior Lecturer from School of Environment, Science and Engineering, Southern Cross University for editing and stimulating discussion and her advices.

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Received: December 30, 2012 Revised: June 11, 2013 Accepted: July 15, 2013