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Short Communication

Retention and Survival Optimization of Juvenile Green Mussel (*Perna viridis*) by Using Substrate from Different Seaweed Extracts

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Abstract

The low retention of juvenile of green mussels (*Perna viridis*) in the aquaculture holding system has become a constraint for its production. The declining number of juvenile mussel on the collector rope might be caused due to both limited spawning season and their secondary settlement behaviour. Therefore, providing suitable substrate which able to improve green mussel seed retention is required. One of the solutions is by applying inducer mediating settlement as substrate enrichment in order to optimize the retention of juvenile *P. viridis*. The potential substrates thought to have these inductive activities is seaweed. Seaweed bioactive compound which may improve juvenile mussel retention is terpenoid. Six seaweed extracts used in the current study and the terpenoid of these six macroalgae species were tested. Qualitatively all six seaweed showed a positive result on the terpenoid compound. The retention and survival of juvenile green mussel were observed by using 20 conical tanks with a complete randomized design experiment. Each of the seaweed species tested separately comparing with three other experimental treatments under 24 h observation time, A (rope), B (rope + Phytagel™), C (rope + Phytagel™ l+ solvent), and D (rope + Phytagel™ + seaweed extract), made four experimental treatments with 5 replications. The result indicated a variation pattern on the retention of juvenile mussels according to the experimental substrate. The juvenile mussels were preferably settled on enriched substrate of *G. latifolium* and *S. polycystum* extracts ($p<0.05$). Adding seaweed extracts on the substrate did not affect the mussels survival ($p>0.05$).

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1. Introduction

Increasing the production of the aquaculture sector is one of the priorities for national food security for strengthening the national economic market. One of the cultivation sectors that becomes a concern to the government which increased to 32% in 2019 is green mussels (*P. viridis*) (Kementrian Kelautan Perikanan, 2015). Green mussels are a commodity with high economic value (Fahrudin and Susanto, 2019) because it is easy to cultivate and the seeds are available all year round. Apart from its rules as a source of protein (Affandi and Tang, 2002), green mussels can be used as animal feed and decoration to strengthen the economy of coastal communities (Fahrudin and Susanto, 2019; Kementrian Kelautan Perikanan, 2015). Green mussels production in Indonesia in 2015 increased by 50% from the previous years with a total production of up to 233,700 tons and is predicted to continue to increase until 2019 (Kementrian Kelautan Perikanan, 2015). The existence of green mussels seeds is available throughout the year, however, the mussel farmers still rely on natural catches (Fahrudin and Susanto, 2019) with the peak of seed acquisition in April - July (WWF, 2015). The less optimal attachment of green mussel seeds to the collector substrate can be a constraint to increase the production of green mussels. The attachment enhancement of juvenile green mussel on the substrate can be increased by applying inducer mediating settlement to enrich the substrate from chemical or natural substances (Puglisi *et al*., 2019; Soares *et al*., 2008).

Juvenile mussels are known to attach to various substrates such as the surface of macroalgae (seaweed) (Alfaro, 2005; Eyster and Pechenik, 1988; Soares *et al.*, 2008). *Mytilus californianus* is found attached to the surface of seaweeds, especially filaments, rocks, and mussel beds. Filamentous macroalgae are known to play an important role in the attachment and settlement processes of juvenile shellfish from various species (Soares *et al.*, 2008). Moreover, juvenile mussels prefer filamentous macroalgae as a substrate for attachment, this type of macroalgae can also be a refuge from predators (Davis and Moreno, 1995) and from hydrodynamic stress in the waters (Alfaro and Jeffs, 2003; Alfaro, 2005; Sanjayasari and Jeffs, 2019).

One of the predicted causes of juvenile mussels attached to macroalgae is due to the presence of substances or compounds that can act as attractants for the settlement of shellfish seeds. The results of previous studies showed that secondary metabolites from the extract of the seaweed *Scytothamnus australis* and *Melanthalia abscissa* could increase the adhesion of juvenile *P. canaliculus* (Alfaro, 2006b). In Indonesia, *P. viridis* seeds were obtained by relying on substrate collector (WWF, 2015), however, the use of attractants to improve the adhesion and juvenile settlement of *P. viridis* using inducer media from seaweed extract has not been widely studied and applied. Therefore, a study on the potential of secondary metabolite compounds in seaweed as an inducer of mediating settlement for juvenile *P. viridis* is necessary to support government achievements in increasing the production of green mussels in the future.

2. Materials and Methods

2.1 Materials

The tools used in this research include; cool box, ziplock bag, 20L container, camera, Erlenmeyer tube, glass beaker, coffee grinder (Kris), analytical scale (Metler-Tuledo), rotary evaporator (RE-2010, Lanphan), measuring cup, binocular compound microscope (Omax), petri dish, tweezers, magnifying glass, coconut fiber coire, aerator, and conical tank.

Six species of seaweed used in the study namely *Ulva lactuca*, *Kapaphycus alvarezii*, *Kappaphycus striatum*, *Sargassum polycystum*, *Gelidium latifolium*, and *Eucheuma denticulatum.* One species of microalgae used to feed the juvenile mussel, *Nannochloropsis oculata*, Media F2 (Guillard), and several solvent for the extraction and bioassay on terpenoid test, namely sulfic acid, glacial acetic acid, methanol 95% p.a, and dichloromethane.

2.2 Methods

2.2.1 Sample collections and seaweed extractions

Seaweed was obtained and washed using fresh running water to clean salt and biota that stick to it. After washing, the seaweeds were then drained and dried, let it air dry protected from the sun for approximately 2-5 days resulting the moisture content ~ 12%. Dried seaweed was grinded into dust by using a coffee grinder and ready to be extracted.

The extraction process used a multilevel maceration method using Methanol 95% p.a solvent (polar and dichloromethane (semi-polar) (1:1) (Alfaro *et al.*, 2006b). The use of different solvents aims to attract secondary metabolites which have different polarity properties. The extract obtained was concentrated using a rotary evaporator. The crude extract obtained was then used as an inducer mediating settlement to enrich the substrate medium for juvenile *P. viridis*.

2.2.2 Juvenile mussel preparation

Juvenile *P. viridis* was obtained from green mussel aquaculture farmers in Grinting, Bulakamba, Brebes. The extraction process and experimentation

set up were carried out at the Laboratory of Fisheries and Marine Sciences Faculty, Jenderal Soedirman University. Juvenile mussels collected from the field were then cleaned and size graded. The size used in this research is 1-4 mm shell length with the mean of initial size at 3.25±0.85 mm shell length (n = 400 individual) of juvenile mussel. These juvenile mussels were then acclimated in 20 L containers which were aerated and fed with *N. oculata* as much as 2 x 10^5 cells of mussel^{-1} day^{-1} (Gui *et al*., 2016b; Sanjayasari and Jeffs, 2019).

2.2.3 Screening of terpenoid compound on seaweed extracts

The analysis of the bioactive compounds of seaweed extract was a phytochemical test for terpenoid compounds. The terpenoid test was carried out based on the method by Sangi *et al*., (2008) in which 50-100 mg of seaweed extract samples were placed on a drop plate and 98% anhydrous acetic acid was added until the samples were completely submerged. The sample was left for about 15 minutes, then six drops of the solution were transferred into the test tube and added 2-3 drops of concentrated sulfuric acid 98%. The presence of terpenoids was indicated by the occurrence of red-orange or purple.

2.2.4 Experimental design Seaweed extracts as inducer mediating settlement for juvenile P. viridis

The research designed were One-Way ANOVA with the main factor of four different treatments using 5 replications. Each seaweed species was subjected to six separated of experiment set up. Each of the experimental set up per macroalgae (seaweed) species was held on 20 conical tanks. The seaweed extract obtained was dissolved into a diluent medium (methanol and Phytagel™). The four treatments in the study, were treatment A (rope), B (rope + Phytagel™), C (rope + Phytagel™ + solvent), and D (rope + Phytagel™ + seaweed extract). To facilitate the juvenile mussel to settle on the seaweed extract, the rope substrate (coire from Rosella fibers) were enriched by soaking them in 10 ml of Phytagel™ mixed with crude extract of seaweed. The Rosella ropes were soaked in Phytagel™ for 13 min to harden the gel. The rope which enriched with extracts were placed in an experimental conical tank that had been filled with filtered seawater (1000 ml).

After all the experimental set up were ready, the juvenile *P. viridis* were put into each experimental container as many as 20 juvenile mussel per tank. Observations were made for 24 hours. The variables observed were the number of juvenile attached to the rope (retention) and the survival of juvenile mussels. Juvenile mussel retention and survival were measured based on Sanjayasari and Jeffs (2019).

2.3 Data Analysis

The qualitative data from seaweed extracts on their terpenoid compound assay were presented in Table and descriptively discussed. The data of retention and survival which obtained in the form of percent were arcsine transformed prior the data analysis. All data were tested for normality and homogeneity before the statistical analysis process was carried out. The statistical analysis was performed by using Sigmaplot version 14.0. and Minitab 19.

3. Results and Discussion

3.1. Qualitative assay of terpenoid compound

The test results for qualitative assay of terpenoid revealed that two of the three species of seaweed used in this study contained terpenoids (Table 1).

Qualitatively, the extract of seaweed sample showed three specific colors; orange, red, and purple when they are positive containing terpenoid. These three colors appeared after the extract sample was immersed using glacial acetic acid and was dripped with 2 - 3 drops of sulfuric acid. It was observed that all of six macroalgae species used in the study were positively contained terpenoid (Table 1).

Green seaweed (*U. lactuca*) produces various bioactive compounds (Nurjanah *et al*., 2018). Meanwhile, according to Mayore *et al*. (2018) the bioactive compounds detected in Kappaphycus seaweed are alkaloids, flavonoids, saponins, terpenoids, tannins, and phenols. *Kappaphycus alvarezii* has more composition on terpenoid compounds compared to other red seaweeds such as *Hypnea musciformis*. Terpen *K. alvarezii* has the main component of β amyrin. This tarpaulin was detected at retention time intervals ranging from 23.30 to 44.9 minutes (Sumayya and Murugan, 2018). However, in current experiment, specific characteristic of terpenoid compound was not observed. Further investigation is required to characterize the terpenoid compound on *G. latifolium, S. polycystum,* and *K. striatum*.

3.2. Retention of juvenile P. viridis

The response of juvenile mussels which remain retain to the enriched substrate with seaweed extracts varied. The result on enriched substrate with *G. latifolium*, and *S. polycystum* extracts significantly influenced the retention of juvenile *P. viridis* (p<0.05). Whereas, substrate enriched with *U. lactuca*, *E. denticulatum K. striatum* and *K. alvarezii* extracts did not influence the mean retention of juvenile *P. viridis* (p>0.05) (Table 2). Although, there was no significant effect of *K. striatum* extract to juvenile mussels retention, but it clearly showed that the extract of this species of microalgae has

the potential to make juvenile mussel remain retain on the rope (Figure 1).

Higher retention of juvenile mussel displayed on the enriched substrate with *G. latifolium* extract (treatment D) at 5±0.03%, this number had lower retention than on the rope substrate (treatment A) at 16±0.08%, however, the retention response was similar to enriched substrate C at 11±0.07%. The lowest retention of juvenile *P. viridis* showed on the B treatment (Tukey test, $p<0.05$).

In the same way, the treatment group of the *S. polycistum* extract contributed at 5.38 ± 6.3% to the retention of juvenile mussel, the same response was also shown in the rope substrate (A) with the number of mussel's retention at 4.48 ± 2.66%. Higher mean of retention found on the substrate B at 11.9 ± 7.39% and the lowest retention found on treatment C with no juvenile mussel attached to the substrate (Tukey's test, $p < 0.05$).

Favorable type of seaweed which was used as settlement substrate for juvenile shellfish was red seaweed (Yang *et al*., 2007). One of the red seaweeds which is widely used as settlement substrate for juvenile clams is *Gelidium sp* (Beckley, 1979). Juvenile mussels prefer to settle on filamentous substrate, debris, hydroid filamentous seaweeds (Jeffs *et al*., 1999). In addition, mussel juvenile can also be influenced by chemical cues produced by seaweed (Soares *et al*., 2008) to select the substrate. Previous studies reported that secondary metabolites were able to attract young shellfish such as juvenile *P. perna* (Soares *et al*., 2008) and juvenile *Mytillus galloprovincialis* (Yang, *et al*., 2007). It has been identified that one of the chemical cues that can attract adolescent roles is terpenoids (epitaondiol) (Soares *et al*., 2008) which is categorized as polycyclic meroditerpenoids (Sánchez-Ferrando and San-Martin, 1995). Terpenoids are compounds that have many variations in biochemical structures and have a variety of different functions (Jiang *et al*., 2016). Not all types of terpenoids can be a chemical signal for the attachment process, such as the type of Sesquiterpene (+) - elatol which is responsible for the association between *L. dendroidea* seaweed and sea rabbit *A. brasiliana* (Nocchi *et al*., 2017), while the polygodial Sesquiterpene becomes chemical compounds inhibiting the attachment of blue shellfish *Mytilus edulis galloprovincialis* (Fahrudin and Susanto, 2019). In this study, the qualitative results showed that all macroalgae species were positive for terpenoid compounds, but this experiment has not characterized certain terpenoid compounds (Table 1). Other chemical compound which may possibly improve settlement and retention for juvenile mussel is el-Dopa (Alfaro *et al*., 2006b). However, studies on other secondary metabolites compounds aside from terpenoid which able to attract their retention were still limited.

Current laboratory experiment on introducing seaweed extract (treatment D) as substrate enrichment for 24 h indicated lower overall retention (< 13%) compared to when mussel provided with rope substrate (Treatment A) at >18%. This finding indicated that pretreatment on seaweed species as induction cues for juvenile mussel settlement may potentially lower their inductive activities. Similar response was observed on post larvae *Mytilus galloprovincialis* which lowered their settlement and metamorphosis when exposed to extract *Chlorodesmis fastigiata* and *Ceramiun tenerrimum* which was extracted with ethanol solvent compared to non-solvent (water) (Yang *et.al*, 2007). Longer observation time (>24 h) may potentially result different response on retention (Sanjayasari and Jeffs, 2019).

The largest proportion of the juvenile mussel in current experiment for all three seaweeds preferred to settle on the bottom of tank at ≈75%. This might be due to the mean of initial size of juvenile mussel used in the study had larger (>3 mm) initial shell length than the post settlement juvenile size (<1 mm) in shell length (Gui *et al*., 2016a; Sanjayasari and Jeffs, 2019). Despite of the initial size, juvenile mussel used in current study was probably too heavy to drift through byssus-pelagic drifting, other factor such as water motion may influence the retention (Hayden and Woods, 2011; Sanjayasari and Jeffs, 2019). High water motion may stimulate byssal thread productions in juvenile mussels (Nishida *et al*., 2003; Young, 1985) and enhance their attachment (Alfaro, 2006a).

G. latifolium, *S. polycystum*, and *K. striatum* have the potential as an inducer of mediating settlement in juvenile *P. viridis* (t test, $p < 0.05$) compared to *K. alvarezii*, *U. lactuca*, and *E. denticulatum* (Figure 1). Therefore, further research is necessary to determine the types of terpenoid compounds in *G. latifolium, S. polycustum*, and *K. striatum* to determine the optimum concentration of this macroalgae extract to enrich the collector substrate so that it can increase juvenile deposition in the collector rope. This study proved that juvenile *P. viridis* were found attached to substrates enriched by the extracts of *G. latifolium*, *S. polycustum,* and *K. striatum*.

3.3. Survival of juvenile Perna viridis

After 24 hours, there was no difference in the average survival rate of juvenile shellfish as a result of different treatments on five seaweed extracts ($p > 0.05$), except for the substrate enriched with *G. latifolium* extract as substrate enrichment showing mean differences on *P. viridis* juvenile survival ($p < 0.05$) (Table 3).

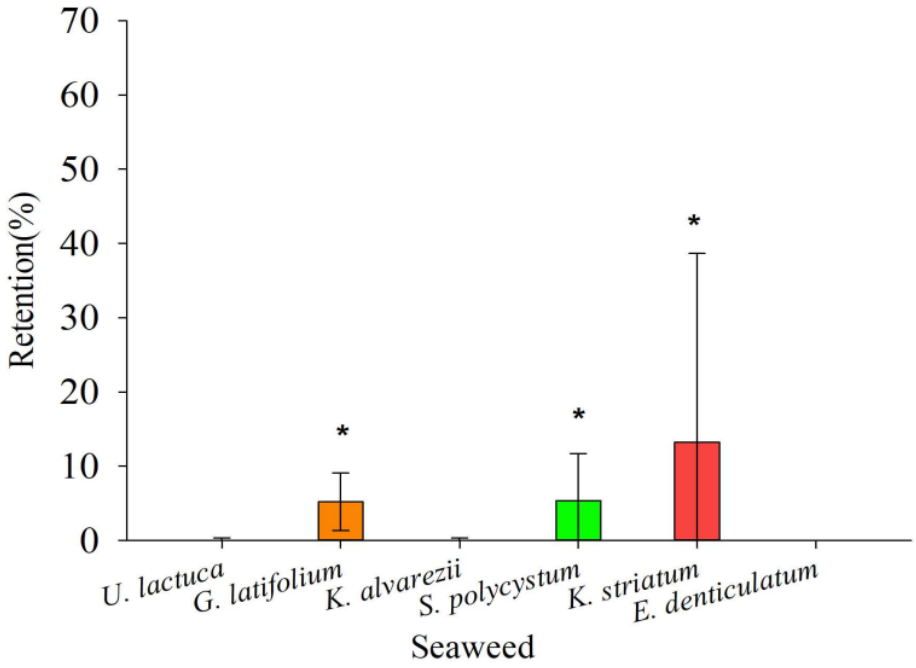

Figure 1. Juvenile mussel retention (%) on three different enriched extract.
(*) indicated significant result (t-test, $p < 0.05$).

Table 1. Qualitative test results of three seaweed extracts on terpenoid compound.

Seaweed species	Terpenoid
Ulva lactuca	Orange
Gelidium latifolium	Redish-orange
Sargassum polycystum	Orange
Kappaphycus striatum	Orange
Kappaphycus alvarezii	Orange
Eucema denticulatum	Orange

Tabel 2. Juvenile mussel retention profile (%) on different enriched substrate from three macroalgae species. Treatment A (rope), B (rope + phytagel), C, (rope + phytagel + solvent), and D (rope + phytagel + seaweed extract).

Treatments	Seaweed species					
	U. lactuca	***G. latifolium***	***K. alvarezii***	***S. polycystum***	***K. striatum***	***E. denticulatum***
A	16±0.13	16±0.08^{b}	22.03±0.39	4.48±2.66^{ab}	18.36±26.13	8.38±5.83
B	8±0.09	4±0.04^{a}	0.00±0.00	11.90±7.39^{b}	7.22±10.32	13.0±14.83
C	12±0.15	11±0.07^{ab}	9.17±0.15	0.00±0.00^{a}	7.50±10.27	2.00±4.47
D	0.0±0.00	5±0.03^{ab}	0.00±0.00	5.38±6.30^{ab}	13.21±25.45	0.00±0.00

Different letter following the retention value within column showed significance (Tukey test, $p<0.05$).

Table 3. Mean survival of the juvenile *P. viridis* when exposed to different erinched substrate. Treatment A (rope), B (rope + phytagel), C, (rope + phytagel + solvent), and D (rope + phytagel + seaweed extract).

Treatments	*U. lactuca*	*G. latifolium*	*K. alvarezii*	*K. striatum*	*S. polycystum*	*E. denticulatum*
A	96±2.23	96±4.13^{ab}	93±4.47	95±7.07	93±13.04	96 ± 4.18
B	93±2.73	100±0.00^{b}	92±8.36	98±2.74	88±10.37	100±0.00
C	95±3.53	94±4.18^{a}	89±7.41	93±5.70	89±12.45	99±2.24
D	94±4.18	97±2.73^{ab}	97±2.73	89±10.84	82±13.04	98±2.74

Different letter following the retention value within column showed significance (Tukey test, $p<0.05$).

There are several factors influencing survival of juvenile mussel such as various environmental conditions (Hayden and Woods, 2011; Sanjayasari and Jeffs, 2019), desiccation (Carton *et al*., 2007), and nutritional condition (Sim-Smith and Jeffs, 2011; Supono *et al*., 2020). Current study indicated that the rope substrate enriched with solvent and Phytagel™ has lowered the mean of survival at 94% on *G. latifolium* experimental set up, however, this phenomenon was not detected on the other five species of seaweed experimental set up. The decrease on inductive activities of pre-treatment *C. fasgiata* and *C. tenerrimum* which extracted with ethanol solvent causes ~ 80% lower survival on post larvae *M. galloprovinciallis* compared to the extract without solvent (Yang *et al*., 2007).

4. Conclusion

Six macroalgae species qualitatively contained terpenoid. Seaweed extracts of *G. latifolium, S. polycystum*, and *K. striatum* have the potential to induce retention of juvenile *P. viridis* compared to the three other seaweed extracts. The addition of seaweed extract did not significantly affect the survival rate of juvenile *P. viridis*. Early treatment in handling seaweed samples has the potential to reduce the inductive activity of seaweed and the viability of mussels. Further research is required to determine the specifications and concentration of terpenoid compounds in *G. latifolium, S. polycystum*, and *K. striatum* to be applied to collector ropes of *P. viridis*.

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Authors' Contributions

All authors have contributed to the final manuscript. The contribution of each author as follows, DS; collected the data, drafted the manuscript and designed the figures. DS, MD, and TW; devised the main conceptual ideas and critical revision of the article. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

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