KORESPONDENSI PAPER

1. C 4.

JUDUL : Identification of agents causing vibriosis in *Litopenaeus vannamei* shrimp culture in Kendal, Central Java, Indonesia and application of microalgae *Dunaliella salina* and *Tetraselmis chui* as bio-control agents against vibriosis.

JURNAL : AACL - Bioflux.

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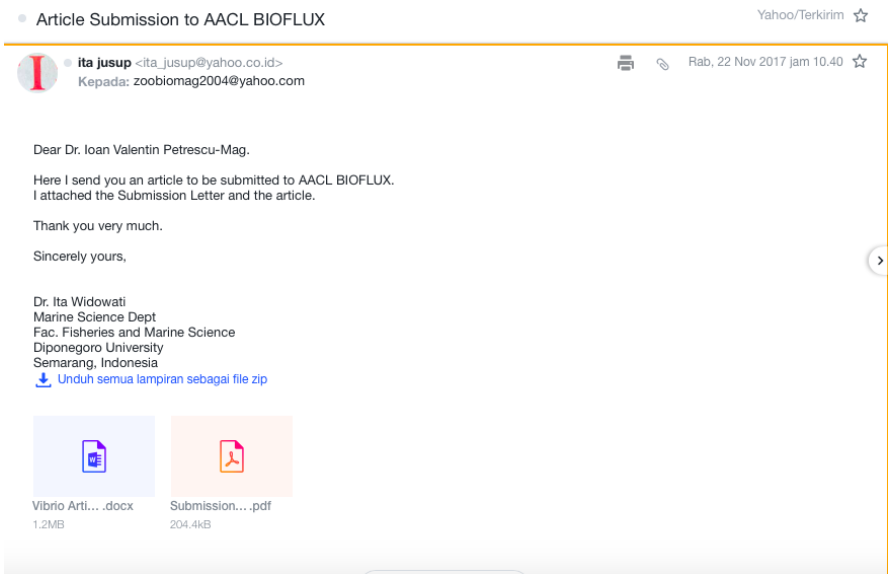
1. Submission Artikel (22 November 2017)



Article Submission to AACL BIOFLUX

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Rab, 22 Nov 2017 jam 10.40

Dear Dr. Ioan Valentin Petrescu-Mag.

Here I send you an article to be submitted to AACL BIOFLUX.
I attached the Submission Letter and the article.

Thank you very much.

Sincerely yours,

Dr. Ita Widowati
Marine Science Dept
Fac. Fisheries and Marine Science
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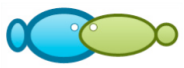
Submission Letter (1b)



Submission letter

Article title:
Identification of Agents causing Vibriosis in *Litopenaeus vannamei* Shrimps Culture in Kendal, Central Java and application of Microalgae *Dunaliella salina* and *Tetraselmis chuii* as Bio control Agents against Vibriosis.

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Hereby I would like to submit the manuscript entitled **"Identification of Agents causing Vibriosis in *Litopenaeus vannamei* Shrimps Culture in Kendal, Central Java and application of Microalgae *Dunaliella salina* and *Tetraselmis chuii* as Bio control Agents against Vibriosis."** to Aquaculture, Aquarium, Conservation & Legislation - International Journal of the Bioflux Society.

This manuscript was not submitted or published to any other journal. The authors declare that the manuscript is an original paper and contain no plagiarised text. All authors declare that they are not currently affiliated or sponsored by any organization with a direct economic interest in subject of the article. My co-authors have all contributed to this manuscript and approve of this submission.

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Date: 22 November 2017.

Initial Manuscript (1c)

INITIAL MANUSCRIPT
(22 November 2017)

Identification of Agents causing Vibriosis in *Litopenaeus vannamei* Shrimps Culture in Kendal, Central Java and application of Microalgae *Dunaliella salina* and *Tetraselmis chuii* as Bio control Agents against Vibriosis.

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Abstract. Aquaculture of Vannamei shrimp at Kendal, Central Java, has been using an intensive system, which could provoke the degradation of water quality in the pond. One of the emerging effects due to such degradation becoming threat for shrimp aquaculture is the loss caused by vibriosis disease. This study aimed to identify the presence of vibriosis-causing bacteria in shrimp pond at Kaliwungu, Kendal, Central Java and use the microalgae *Dunaliella salina* and *Tetraselmis chuii* as bio control agents against Vibriosis. The Vibriosis-causing bacteria were isolated from the hepatopancreas and tails of ten shrimps showing clinical signs of vibriosis infection, cultured on the Thiosulfate Citrate Bile Salt Sucrose media. Out of 20 isolates, three isolates were selected for further gene sequence identification of 16rRNA using the *Reprective Sequence-based Polymerase Chain Reaction* (rep-PCR) and were analysed with BLAST. Universal primer 27F (5'AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'TACGGTTAACCTTGTTACGACTT-3') were selected as the primers to identify the isolated bacteria. Results demonstrated that the three isolated bacteria were positive as vibriosis-causing agent in shrimp, which were U1H1 AND U4H1, identified as *Vibrio alginolyticus,* and U1E1 as *V. harveyi.* The use of microalgae as biocontrols was performed. Forty-five shrimps infected by vibrio reared during 21 days and feed with *D. salina* and *T. chuii* showed a decreased of bacteria amount, which were counted by Total Plate Count method. The result indicated that the microalgae was capable to produce an antibacterial compounds against vibrio.

Keywords: bio control, *Dunaliella salina*, *Litopenaeus vannamei, Tetraselmis chuii*, Vibriosis.

Introduction

Community trend towards the need of fisheries products is constantly increasing, thus, the aquaculture activities are highly developed, and one of them is the Vanamei shrimp. This shrimp is a fresh water commodity. The production of Vanamei in Central Java reached 411.729 tons in 2014. From 2010 to 2014, the production of this commodity increased to 20.49% (LAKIP-Directory General of Aquaculture Fisheries, 2014). The augmentation of Vanamei production at Central Java has been generated by the application of intensive systems characterized by spreading high quantity of breed stocks and the usage of 100% of artificial feeds. The application of intensive systems decreases the water quality in ponds making the Vanamei shrimp susceptible to diseases caused by parasites, virus and bacteria. The mortality of shrimp due to vibriosis infection might reach 80 to 100% in the larval, post larval, juvenile, and adult stages within 1 to 3 days. Case of mass mortality in shrimp aquaculture occurring across every region in Indonesia in the 1900s, including Central Java, had made great loss for most of the company.

Vibrio has been widely reported to be one of the main pathogenic bacteria causing high mortality rate in shrimp aquaculture (Haldar *et al.*, 2011; Heenatigala and Fernando, 2016; Solidum *et al.*, 2016). The presence of this pathogenic bacteria itself is a part of natural microflora contributing to 60% of total bacteria population as mentioned by Heenatigala and Fernando (2016). As opportunistic organism, the infection of *Vibrios* to the cultivated organisms occurs when the environmental condition degrades affecting the health condition of the cultivated organism (Kumaran and Citarasu, 2016). Bacterial disease outbreak provoked by *Vibrios* has become constraint on the sustainable production of shrimp (Manilal *et al.*, 2010). In order to suppress the outbreak, marine secondary metabolites have been mentioned as promising resources to further develop, especially from microalgae. It has been reported that the extracts of microalgae could prevent the bacterial infections (Cadiz *et al.*, 2016; Dash *et al.*, 2017).

Despite of the intensive shrimp farming activities in order to fulfill markets demand, infectious disease caused by bacteria remains the main problem that still happens present days. As a consequence, it is crucial to carry out a study that evaluates and identifies the vibriosis-causing bacteria as an update and also serves as basic information prior to further treatment. It is meant to apply the suitable and appropriate treatment to overcome the infection. The objectives of this study were to identify the agents causing vibriosis in the cultivated *Litopenaeus vannamei* and to evaluate the efficacy of microalgae extracts from *Dunaliella salina* and *Tetraselmis chuii* in suppressing the vibriosis infection.

Material and method

Collection of infected Vanamei shrimp

The sampling location of Vanamei shrimp, infected with vibriosis, was collected from the Panggangayom village, Kaliwungu district, Kendal, Central Java.

The tenth (10) Vanamei shrimp (*Litopenaeus vanamei*) had an average weight of 4.30 g and length of 8 cm. Samples chosen wich having the morphological signs of infected shrimps (Prayitno and Lachtford, 1995). The research was conducted on October 2016 – March 2017. Sample was analyzed at Integrated Laboratory, Diponegoro University.

Isolation of vibriosis-causing bacteria

Tryptone Soya Agar (TSA) as universal agar media and *Thiosulfate Citrate Bile Salt* (TCBS) (Merck, Jerman) as vibrios-specific agar media for bacterial culture were selected. Bacteria were isolated from the infected shrimp using streack method. (Haldar *et al*., 2001).

DNA extraction, sequencing and identification of isolated bacteria

DNA extraction was conducted by using chelex method from Walsh (2013) as performed in Susilowati (2015). Selected colonies were inoculated in 50-100 µl ddH2O and 1 ml of 0,5% saponin in PBS 1x (saved overnight). The mixture was centrifuged (12000 RPM, 10 min). Supernatant was discard. Then 100 µl ddH2O and 50 µl of 20% chelex 100 (shake up chelex solution and ensure that some of the crystals make it into sample) were added to a final solution and the solution was boiled for 10 min and vortex once after 5 min. The mixture was sentrifuged (12000 RPM, 10 min) and stored at -20oC. The DNA concentrations were quantified and qualified by using NanoDrop 2000 spectrophotometer (Thermo Scientific). The concentration of 1 µl DNA sample was determined by using the NanoDrop 2000 spectrophotometer (Thermo Scientific). The 260/280 and 260/230 nm ratios was calculated by the NanoDrop spectrophotometer and used to evaluate the DNA purity and also the concentration of DNA.

DNA extracts for 16S rRNA genes sequences were amplified by PCR using universal primers 27F (5'AGAGTTTGATCMTGGCTCAG-3') and 1492 R (5'TACGGTTAACCTTGTTACGACTT-3'). The PCR mixture consisted GoTaq®Green Master Mix Promega (25 µl), primer 27F (0,5-5 µl), primer 1492R (0,5-5 µl), DNA extract (1-5 µl), and Nuclease-Free Water (50 µl). The PCR reaction was performed in a MJ Mini Personal Thermal Cycler (BIO RAD) using cycling conditions consisting of an initial denaturation at 95 °C for 3 min followed by 30 cycles of denaturation at 95 oC for 1 min, annealing at 55 °C for 1 min, and extension at 72 oC for 1 min. A final extension was performed at 72 °C for 7 min (Lee *et al.*, 2006). The PCR products were analyzed by agarose 1 % gel electrophoresis and the result showed by using UVIDoc HD5 (UVITEC cambridge).

The isolated bacteria were identified by means of homology at https://blast.ncbi.nlm.nih.gov/Blast.cgi. It is an analysis using BLAST based on the database from the GenBank of the National Center for Biotechnology Information (NCBI).

Extraction of microalgae

Dry biomass of microalgae *T. chuii* (10 g) and *D. salina* (9 g) were dissolved in 100 mL methanol and was left for 5 x 24 h. Methanolic extract of *T. chuii* was passed in ultrasound for 15 min and was evaporated to dryness.

Preparation of feeds

Dry extracts of *T. chuii* and *D. salina* (2 g) were blended with 10 g of commercial pellet and were left to dry in the drying machine for 2 x 24 h. Commercial pellet without microalgae extracts served as negative control. The commercial pellets used in this study contain 30% of protein.

The use of microalgae as bio control (in vivo assay)

Forty-five Vanamei shrimp taken from Vibriosis detected on the pond based on the previous biomolecular test were used for the *in vivo assay*. Shrimp were reared in aerated aquarium with the density of five shrimps in 5 L of water. The water quality was maintained at 26-28 ^{0}C and salinity 25-300 ppt. Shrimp were then acclimatized for three days before the *in vivo* assay.

Shrimps were placed in three different tanks. Each tank represented different treatments related to the type of feeds given. Treated shrimps were fed three times a day, *i.e.* at 08.00, 12.00 and 17.00 WIB (local time). All experiments were performed in three replicates.

Treatment I: commercial pellet mixed with *Tetraselmis chuii* extract of 200 ppm against Vaname shrimp infected with vibriosis.
Treatment II: commercial pellet mixed with *Dunaliella salina* extract of 200 ppm against Vaname shrimp infected with vibriosis.
Treatment II: commercial pellet without the addition of microalgae extract to Vaname shrimp infected with vibriosis.

Microbial enumeration from the hepatopancreas of shrimp

The observed parameters were amount of bacteria in the hepatopancreas of treated and control shrimps counted using the Total Plate Count (TPC) method (Benson, 2001). Hepatopancreas of treated and control shrimp were dissolved in 10 mL sterile seawater. The solutions were stirred for 15 min and were diluted three times (10^{-1}, 10^{-2} and 10^{-3}. The treatment was carried out for 21 days and the bacteria amount was counted at the 7^{th}, 14^{th}, and 21^{st} days.

Results

Morphological signs of the infected shrimp

The clinical signs of the infected shrimps were 'luminescent pleopoda', melanocyst on the shrimp's body, reddish tail, and brownish-red hepatopancreas.

Bacterial isolats

Based on the isolation of vibriosis-causing bacteria, there were 13 isolats that was morphologically characterized based on their colonial edge form, characteristic and colour. Out of 13 bacterial isolats, three potential bacteria were selected and results are presented in Table 1.

Table 1. Morphological characteristic and colour of 13 bacterial isolats.

No	Colonial edge form	Colonial characteristic	Colonial color	Total	Isolates code
1	Jagged	Convex	Green	1	U1H1
2	Smooth	Convex	Yellow	7	U1H2,U1E1,U2H1, U2H1, U3H2, U4H1, U5H1,
3	Smooth	Convex	Green	2	U1E2, U3H1
4	Smooth	Cembung	White milk	1	U7E1
5	Jagged	Convex	White milk	1	U8E1
6	Jagged	Convex	Yellow	1	U8E2

Bacterial identification

Based on the biomolecular analysis, there were two bacterial isolats that were classified as same species. This analysis managed to identify the presence of *V. alginolyticus* and *V. harveyi* as two bacteria strain being responsible for the vibriosis infection on the treated shrimps. Biomolecular analysis is presented in Table 2.

Table 2. Biochemical analysis of three selected bacterial isolates from Vanamei shrimp

No	Code	Universal Primer		Species	Homology
		27F	1429R		
1	U1H1	1.27F	1.1429R	*V.alginolyticus* strain ATCC 17749	97%

2	U1E1	2.27F	2.1429R	*V.harveyi* strain NCIMB 1280	96%
3	U4H1	3.27F	3.1429R	*V.alginolyticus* strain ATCC 17749	97%

In vivo Assay

Forty-five shrimps infected by vibrio reared during 21 days and feed with *D. salina* and *T. chuii* showed a decreased of bacteria amount (Figure 1).

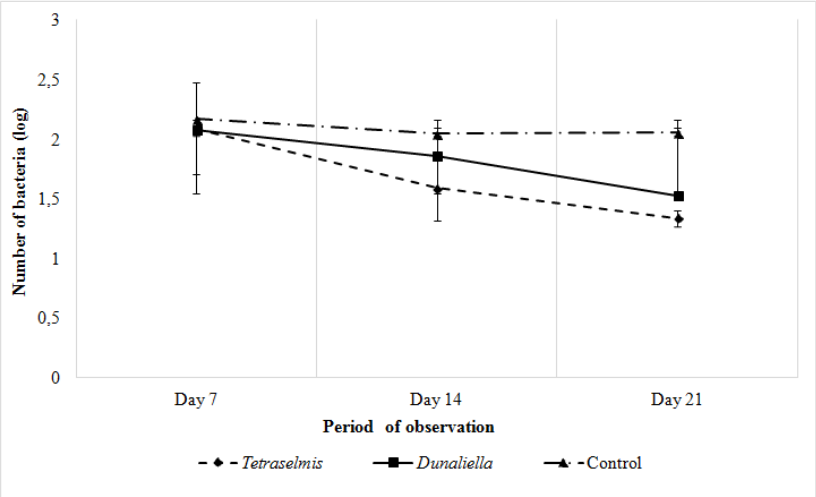

Figure 1. Number of bacteria observed from infected Vannamei shrimp during 21 days experiment.

Discussion

Clinical signs observed on the infected Vanamei shrimp collected from Kaliwungu, Kendal were in accordance as infection signs in Vannamei caused by vibrio's as reported by Lina *et al*. (2001) and Huang *et al.* (2013). Another features remarked correlates to the behavior of shrimp are passive movement, slow response to feed and fluorescent swimming legs. Prayitno and Lachtford (1995) stated that *V. harveyi* is one of causative agent that also known as luminescent bacteria. Those clinical signs became the reference in isolating the vibriosis-causing bacteria from the shrimp.

Bacterial isolates demonstrated that there were 13 isolates with diverse morphology. The selected isolate, which was U1H1, had a jagged colonial edge, convex with green color. Meanwhile, the U1E1 AND U4H1 were characterized with smooth colonial edge, convex with yellow color. These three isolates showed similar characteristic as *Vibrio* bacteria. Sarjito *et al*. (2015) revealed that morphological features of bacteria with smooth colonial edge, convex with yellow color tested biochemically were identical with those of *V. harveyi.*

DNA amplification results from the rep-PCR exhibited three (3) isolates with similar band profiles as seen on marker 800 bp. Rep-PCR method has been proven as an efficient method to identify bacterial species and the genetic relationship (Lina *et al*. 2001). DNA sequencing of isolate U1H1 and U4H1 had identical nucleotide with *V. alginolyticus* strain ATCC 17749 with homological

level 97%. Isolat U1E1 showed to have similar nucleotide with *V. harveyi* strain NCIMB 1280 with homological level 96%.

Presence and abundance of *V.alginolyticus* and *V. Harveyi* are highly correlated with environmental conditions. Asplund (2013) stated that the abundance of bacteria *Vibrio sp.* usually relates to temperature and salinity and increases in stable environmental condition. The abundance of *V.alginolyticus* and *V. Harveyi* counted was 21 (isolat U1H1) and 4 (isolat U4H1) colony/plate and 11 (isolat U1E1) colony/plate. Environmental condition in the pond when the sampling was performed in October-November was fluctuated due to the rainy season. In this season, the temperature and salinity in the pond tended to change in short period; thus, it affected the abundance of bacterial colony, either in the pond or on the body of Vanamei shrimp.

The result on *in vivo assay* shown that the infected Vibrio shrimp feed with mixed *Tetraselmis salina* extract and *Dunaliella salina* reared at 21 days shown a decreased a number of bacterial infection as shown on Table 3 and 4 compared with those non feed (control) shown on Table 5 with these two micoalgae. Widowati *et.al* (2017) observed that *Dunalliela sp., Tetraselmis chuii* showed an antioxidants potentials which could be considered for future applications in aquaculture. The result of this study indicated that the microalgae capable produce an antibacterial compounds against vibrio.

Acknowledgement

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2. Submission Acknowledgement (22 November 2017) (2)

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Rab, 22 Nov 2017 jam 22.06

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3.Respon to Submission Acknowledgement (3)

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Kepada: Miklos Botha

Rab, 29 Nov 2017 jam 11.41

Dear Dr. Miklos Botha
Editor AACL Bioflux,

Thank you for the information that my paper submission is under evaluation and about the publication fee.

I will follow your assistance concerning the submission of the manuscript.

Thank you.
Sincerely yours,

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Fac. Fisheries and Marine Science
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4. Revision Required #1 22 januari 2018 (4)

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Kepada: ita jusup

Sen, 22 Jan 2018 jam 19.11

Dear Dr. Ita Widowati,

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4a. Manuscript Revision required (22 Januari 2018) (4a)

Identification of Agents causing Vibriosis in *Litopenaeus vannamei* Shrimps Culture in Kendal, Central Java and application of Microalgae *Dunaliella salina* and *Tetraselmis chuii* as Bio control Agents against Vibriosis

Ita Widowati[1*)], Muhammad Zainuri[1], Hermien Pancasakti Kusumaningrum[2], Yusri Maesaroh[3], Yann Hardivillier[5], Vincent Leignel[6], Nathalie Bourgougnon[7], Jean-Luc Mouget[8]

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Abstract. Aquaculture of Vannamei shrimp at Kendal, Central Java, has been using an intensive system, which could provoke the degradation of water quality in the pond. One of the emerging effects due to such degradation becoming threat for shrimp aquaculture is the loss caused by vibriosis disease. This study aimed to identify the presence of vibriosis-causing bacteria in shrimp pond at Kaliwungu, Kendal, Central Java and use the microalgae *Dunaliella salina* and *Tetraselmis chuii* as bio control agents against Vibriosis. The Vibriosis-causing bacteria were isolated from the hepatopancreas and tails of ten shrimps showing clinical signs of vibriosis infection, cultured on the Thiosulfate Citrate Bile Salt Sucrose media. Out of 20 isolates, three isolates were selected for further gene sequence identification of 16rRNA using the Reprective Sequence-based Polymerase Chain Reaction (rep-PCR) and were analyzed with BLAST. Universal primer 27F (5'AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'TACGGTTAACCTTGTTACGACTT-3') were selected as the primers to identify the isolated bacteria. Results demonstrated that the three isolated bacteria were positive as vibriosis-causing agent in shrimp, which were U1H1 AND U4H1, identified as *Vibrio alginolyticus,* and U1E1 as *V. harveyi.* The use of microalgae as biocontrols was performed. Forty-five shrimps infected by vibrio reared during 21 days and feed with *D. salina* and *T. chuii* showed a decreased of bacteria amount, which were counted by Total Plate Count method. The result indicated that the microalgae was capable to produce an antibacterial compounds against vibrio.
Key Words: bio control, *Dunaliella salina*, *Litopenaeus vannamei, Tetraselmis chuii*, Vibriosis.

Introduction. Community trend towards the need of fisheries products is constantly increasing, thus, the aquaculture activities are highly developed, and one of them is the Vanamei shrimp. This shrimp is a fresh water commodity. The production of Vanamei in Central Java reached 411.729 tons in 2014. From 2010 to 2014, the production of this commodity increased to 20.49% (LAKIP-Directory General of Aquaculture Fisheries, 2014). The augmentation of Vanamei production at Central Java has been generated by the application of intensive systems characterized by spreading high quantity of breed stocks and the usage of 100% of artificial feeds. The application of intensive systems decreases the water quality in ponds making the Vanamei shrimp susceptible to diseases caused by parasites,

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virus and bacteria. The mortality of shrimp due to vibriosis infection might reach 80 to 100% in the larval, post larval, juvenile, and adult stages within 1 to 3 days. Case of mass mortality in shrimp aquaculture occurring across every region in Indonesia in the 1900s, including Central Java, had made great loss for most of the company.

Vibrio sp. has been widely reported to be one of the main pathogenic bacteria causing high mortality rate in shrimp aquaculture (Haldar et al 2011; Heenatigala & Fernando 2016; Solidum et al 2016). The presence of this pathogenic bacteria itself is a part of natural microflora contributing to 60% of total bacteria population as mentioned by Heenatigala & Fernando (2016). As opportunistic organism, the infection of *Vibrios* to the cultivated organisms occurs when the environmental condition degrades affecting the health condition of the cultivated organism (Kumaran & Citarasu 2016). Bacterial disease outbreak provoked by *Vibrios* has become constraint on the sustainable production of shrimp (Manilal et al 2010). In order to suppress the outbreak, marine secondary metabolites have been mentioned as promising resources to further develop, especially from microalgae. It has been reported that the extracts of microalgae could prevent the bacterial infections (Cadiz et al 2016; Dash et al 2017).

Despite of the intensive shrimp farming activities in order to fulfill markets demand, infectious disease caused by bacteria remains the main problem that still happens present days. As a consequence, it is crucial to carry out a study that evaluates and identifies the vibriosis-causing bacteria as an update and also serves as basic information prior to further treatment. It is meant to apply the suitable and appropriate treatment to overcome the infection. The objectives of this study were to identify the agents causing vibriosis in the cultivated *Litopenaeus vannamei* and to evaluate the efficacy of microalgae extracts from *Dunaliella salina* and *Tetraselmis chuii* in suppressing the vibriosis infection.

Material and Method

Collection of infected Vanamei shrimp. The sampling location of Vanamei shrimp, infected with vibriosis, was collected from the Panggangayom village, Kaliwungu district, Kendal, Central Java.

The tenth (10) Vanamei shrimp (*Litopenaeus vanamei*) had an average weight of 4.30 g and length of 8 cm. Samples was chosen having morphological signs of vibriosis infection (Prayitno & Lachtford 1995). The research was conducted in October 2016 – March 2017. Sample was analyzed at Integrated Laboratory, Diponegoro University.

Isolation of vibriosis-causing bacteria. Tryptone Soya Agar (TSA) as universal agar media and Thiosulfate Citrate Bile Salt (TCBS) (Merck, Jerman) as vibrios-specific agar media for bacterial culture were selected. Bacteria were isolated from the infected shrimp using streack method (Haldar et al 2001).

DNA extraction, sequencing and identification of isolated bacteria. DNA extraction was conducted by using chelex method from Walsh (2013) as performed in Susilowati (2015). Selected colonies were inoculated in 50-100 µL ddH_2O and 1 mL of 0.5% saponin in PBS 1x (saved overnight). The mixture was centrifuged (12,000 RPM, 10 min). Supernatant was discarded. Then 100 µL ddH_2O and 50 µL of 20% chelex 100 (shake up chelex solution and ensure that some of the crystals make it into sample) were added to a final solution and the solution was boiled for 10 min and vortex once after 5 min. The mixture was centrifuged (12,000 RPM, 10 min) and stored at -20°C. The DNA concentrations were quantified and qualified by using NanoDrop 2000 spectrophotometer (Thermo Scientific). The concentration of 1 µL DNA sample was determined by using the NanoDrop 2000 spectrophotometer (Thermo Scientific). The 260/280 and 260/230 nm ratios was calculated by the NanoDrop spectrophotometer and used to evaluate the DNA purity and also the concentration of DNA.

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DNA extracts for 16S rRNA genes sequences were amplified by PCR using universal primers 27F (5'AGAGTTTGATCMTGGCTCAG-3') and 1492 R (5'TACGGTTAACCTTGTTACGACTT-3'). The PCR mixture consisted GoTaq®Green Master Mix Promega (25 µL), primer 27F (0.5-5 µL), primer 1492R (0.5-5 µL), DNA extract (1-5 µL), and Nuclease-Free Water (50 µL). The PCR reaction was performed in a MJ Mini Personal Thermal Cycler (BIO RAD) using cycling conditions consisting of an initial denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. A final extension was performed at 72°C for 7 min (Lee et al 2006). The PCR products were analyzed by agarose 1% gel electrophoresis and the result was showed by using UVIDoc HD5 (UVITEC Cambridge).

The isolated bacteria were identified by means of homology at https://blast.ncbi.nlm.nih.gov/Blast.cgi. It is an analysis using BLAST based on the database from the GenBank of the National Center for Biotechnology Information (NCBI).

Extraction of microalgae. Dry biomass of microalgae *T. chuii* (10 g) and *D. salina* (9 g) were dissolved in 100 mL methanol and was left for 5 x 24 h. Methanolic extract of *T. chuii* was passed in ultrasound for 15 min and was evaporated to dryness.

Preparation of feeds. Dry extracts of *T. chuii* and *D. salina* (2 g) were blended with 10 g of commercial pellet and were left to dry in the drying machine for 2 x 24 h. Commercial pellet without microalgae extracts served as negative control. The commercial pellets used in this study contain 30% of protein.

The use of microalgae as bio control (in vivo assay). Forty-five Vanamei shrimp taken from Vibriosis detected on the pond based on the previous biomolecular test were used for the *in vivo assay*. Shrimp were reared in aerated aquarium with a density of five shrimps in 5 L of water. The water quality was maintained at 26-28°C and salinity 25-300 ppt. Shrimp were then acclimatized for three days before the in vivo assay.

Shrimps were placed in three different tanks. Each tank represented different treatments related to the type of feeds given. Treated shrimps were fed three times a day, i.e. at 08:00, 12:00 and 17:00 WIB (local time). All experiments were performed in three replicates.

Treatment I: commercial pellet mixed with *Tetraselmis chuii* extract of 200 ppm against Vaname shrimp infected with vibriosis.
Treatment II: commercial pellet mixed with *Dunaliella salina* extract of 200 ppm against Vaname shrimp infected with vibriosis.
Treatment II: commercial pellet without addition of microalgae extract to Vaname shrimp infected with vibriosis.

Microbial enumeration from the hepatopancreas of shrimp. The observed parameters were amount of bacteria in the hepatopancreas of treated and control shrimps counted using the Total Plate Count (TPC) method (Benson 2001). Hepatopancreas of treated and control shrimp were dissolved in 10 mL sterile seawater. The solutions were stirred for 15 min and were diluted three times (10^{-1}, 10^{-2} and 10^{-3}). The treatment was carried out for 21 days and the bacteria amount was counted at the 7^{th}, 14^{th}, and 21^{st} days.

Results

Morphological signs of the infected shrimp. The clinical signs of the infected shrimps were 'luminescent pleopoda', melanocyst on the shrimp's body, reddish tail, and brownish-red hepatopancreas.

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Bacterial isolats. Based on the isolation of vibriosis-causing bacteria, there were 13 isolates that was morphologically characterized based on their colonial edge form, characteristic and color. Out of 13 bacterial isolates, three potential bacteria were selected and results are presented in Table 1.

Table 1

Morphological characteristic sand color of 13 bacterial isolates

No	Colonial edge form	Colonial characteristic	Colonial color	Total	Isolates code
1	Jagged	Convex	Green	1	U1H1
2	Smooth	Convex	Yellow	7	U1H2,U1E1,U2H1,U2H1, U3H2, U4H1, U5H1, U1E2, U3H1
3	Smooth	Convex	Green	2	
4	Smooth	Cembung	Milk white	1	U7E1
5	Jagged	Convex	Milk white	1	U8E1
6	Jagged	Convex	Yellow	1	U8E2

Bacterial identification. Based on the biomolecular analysis, there were two bacterial isolates that were classified as same species. This analysis managed to identify the presence of *V. alginolyticus* and *V. harveyi* as two bacteria strain being responsible for the vibriosis infection on the treated shrimps. Biomolecular analysis is presented in Table 2.

Table 2

Biochemical analysis of three selected bacterial isolates from Vanamei shrimp

No	Code	Universal Primer		Species	Homology
		27F	1429R		
1	U1H1	1.27F	1.1429R	*V. alginolyticus* strain ATCC 17749	97%
2	U1E1	2.27F	2.1429R	*V. harveyi* strain NCIMB 1280	96%
3	U4H1	3.27F	3.1429R	*V. alginolyticus* strain ATCC 17749	97%

In vivo Assay

Forty-five shrimps infected by vibrio reared during 21 days and feed with *D. salina* and *T. chuii* showed a decreased of bacteria amount (Figure 1).

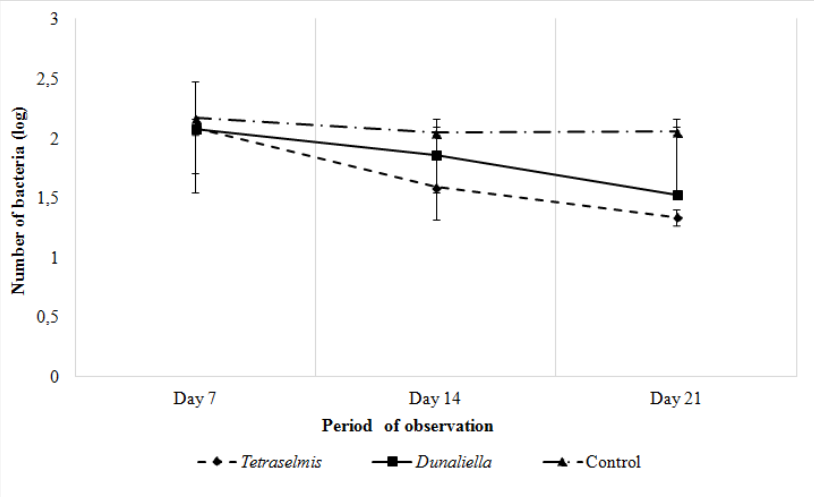

Figure 1. Number of bacteria observed from infected Vannamei shrimp during the 21 days experiment.

Discussion. Clinical signs observed on the infected Vanamei shrimp collected from Kaliwungu, Kendal were in accordance as infection signs in Vannamei caused by vibrio's as reported by Lina et al (2001) and Huang et al (2013). Another features remarked correlates to the behavior of shrimp are passive movement, slow response to feed and fluorescent swimming legs. Prayitno & Lachtford (1995) stated that *V. harveyi* is one of causative agent that also known as luminescent bacteria. Those clinical signs became the reference in isolating the vibriosis-causing bacteria from the shrimp.

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Bacterial isolates demonstrated that there were 13 isolates with diverse morphology. The selected isolate, which was U1H1, had a jagged colonial edge, convex with green color. Meanwhile, the U1E1 AND U4H1 were characterized with smooth colonial edge, convex with yellow color. These three isolates showed similar characteristic as *Vibrio* bacteria. Sarjito et al (2015) revealed that morphological features of bacteria with smooth colonial edge, convex with yellow color tested biochemically were identical with those of *V. harveyi.*

DNA amplification results from the rep-PCR exhibited three isolates with similar band profiles as seen on marker 800 bp. Rep-PCR method has been proven as an efficient method to identify bacterial species and the genetic relationship (Lina et al 2001). DNA sequencing of isolate U1H1 and U4H1 had identical nucleotide with *V. alginolyticus* strain ATCC 17749 with homological level 97%. Isolate U1E1 showed to have similar nucleotide with *V. harveyi* strain NCIMB 1280 with homological level 96%.

Presence and abundance of *V. alginolyticus* and *V. harveyi* are highly correlated with environmental conditions. Asplund (2013) stated that the abundance of bacteria *Vibrio sp.* usually relates to temperature and salinity and increases in stable environmental condition. The abundance of *V. alginolyticus* and *V. harveyi* counted was 21 (isolate U1H1) and 4 (isolate U4H1) colony/plate and 11 (isolate U1E1) colony/plate. Environmental condition in the pond when the sampling was performed in October-November was fluctuated due to the rainy season. In this season, the temperature and salinity in the pond tended to change in short period; thus, it affected the abundance of bacterial colony, either in the pond or on the body of Vanamei shrimp.

The result of the in vivo assay shown that the vibrio infected shrimps feed with mixed *Tetraselmis salina* extract and *Dunaliella salina* reared for 21 days shown a decreased of the number of bacterial infection as shown on Table 3 and 4 compared with control shown in Table 5. Widowati et al (2017) observed that *Dunalliela sp., Tetraselmis chuii* showed antioxidant potential which could be considered for future applications in aquaculture. The result of this study indicated that the microalgae are capable to produce antibacterial compounds against vibrio.

Conslusions.

Acknowledgements. The authors wish to thank the Directorate of Research and Community Service, Directorate General of Research Strengthening and Development, Indonesian Ministry of Research Technology and Higher Education, for funding this research through the Implementation Agreement Assignment of Research Collaboration and International Publication 2017.

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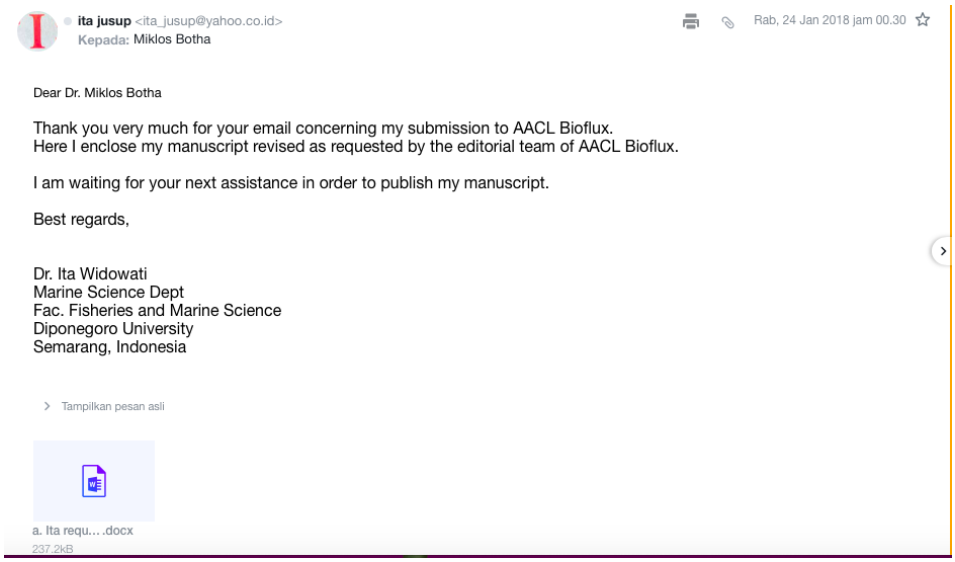
5. Revision #1 submission (24 Januari 2018) (5)



ita jusup <ita_jusup@yahoo.co.id>
Kepada: Miklos Botha

Rab, 24 Jan 2018 jam 00.30

Dear Dr. Miklos Botha

Thank you very much for your email concerning my submission to AACL Bioflux.
Here I enclose my manuscript revised as requested by the editorial team of AACL Bioflux.

I am waiting for your next assistance in order to publish my manuscript.

Best regards,

Dr. Ita Widowati
Marine Science Dept
Fac. Fisheries and Marine Science
Diponegoro University
Semarang, Indonesia

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5.a. Manuscript Revision #1 Submission (24 January 2018) (5a).

MANUSCRIPT REVISION #1
(24 Januari 2018)

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Collection of infected Vanamei shrimp. The sampling location of Vanamei shrimp, infected with vibriosis, was collected from the Panggang Ayom village, Kaliwungu district, Kendal, Central Java, Indonesia.

The tenth (10) Vannamei shrimp (*Litopenaeus vannamei*) samples had an average weight of 4.30 g and length of 8 cm. Samples was chosen having morphological signs of vibriosis infection (Prayitno & Lachtford 1995). The research was conducted in October 2016 – March 2017. Sample was analyzed at Integrated Laboratory, Diponegoro University, Indonesia.

Isolation of vibriosis-causing bacteria. Tryptone Soya Agar (TSA) as universal agar media and Thiosulfate Citrate Bile Salt (TCBS) (Merck, Jerman) as vibrios-specific agar media for bacterial culture were selected. Bacteria were isolated from the infected shrimp using streack method (Haldar et al 2011).

DNA extraction, sequencing and identification of isolated bacteria. DNA extraction was conducted by using chelex method from Walsh et al (2013) as performed in Susilowati et al (2015). Selected colonies were inoculated in 50-100 µL ddH_2O and 1 mL of 0.5% saponin in PBS 1x (saved overnight). The mixture was centrifuged (12,000 RPM, 10 min). Supernatant was discarded. Then 100 µL ddH_2O and 50 µL of 20% chelex 100 (shake up chelex solution and ensure that some of the crystals make it into sample) were added to a final solution and the solution was boiled for 10 min and vortex once after 5 min. The mixture was centrifuged (12,000 RPM, 10 min) and stored at -20°C. The DNA concentrations were quantified and

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qualified by using NanoDrop 2000 spectrophotometer (Thermo Scientific). The concentration of 1 µL DNA sample was determined by using the NanoDrop 2000 spectrophotometer (Thermo Scientific). The 260/280 and 260/230 nm ratios was calculated by the NanoDrop spectrophotometer and used to evaluate the DNA purity and also the concentration of DNA.

DNA extracts for 16S rRNA genes sequences were amplified by PCR using universal primers 27F (5'AGAGTTTGATCMTGGCTCAG-3') and 1492 R (5'TACGGTTAACCTTGTTACGACTT-3'). The PCR mixture consisted GoTaq®Green Master Mix Promega (25 µL), primer 27F (0.5-5 µL), primer 1492R (0.5-5 µL), DNA extract (1-5 µL), and Nuclease-Free Water (50 µL). The PCR reaction was performed in a MJ Mini Personal Thermal Cycler (BIO RAD) using cycling conditions consisting of an initial denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. A final extension was performed at 72°C for 7 min (Lee et al 2006). The PCR products were analyzed by agarose 1% gel electrophoresis and the result was showed by using UVIDoc HD5 (UVITEC Cambridge).

The isolated bacteria were identified by means of homology at https://blast.ncbi.nlm.nih.gov/Blast.cgi. It is an analysis using BLAST based on the database from the GenBank of the National Center for Biotechnology Information (NCBI).

Extraction of microalgae. Dry biomass of microalgae *T. chuii* (10 g) and *D. salina* (9 g) were dissolved in 100 mL methanol and was left for 5 x 24 h. Methanolic extract of *T. chuii* was passed in ultrasound for 15 min and was evaporated to dryness.

Preparation of feeds. Dry extracts of *T. chuii* and *D. salina* (2 g) were blended with 10 g of commercial pellet and were left to dry in the drying machine for 2 x 24 h. Commercial pellet without microalgae extracts served as negative control. The commercial pellets used in this study contain 30% of protein, lipid of 5%, fiber of 4% and 12 % of water.

The use of microalgae as bio control (in vivo assay). Forty-five *L. vannamei* taken from Vibriosis detected on the pond based on the previous biomolecular test were used for the *in vivo assay*. Shrimp were reared in aerated aquarium with a density of five individuals in 5 L of water. The water quality was maintained at 26-28°C and salinity 25-30 ppt. Shrimp were then acclimatized for three days before the in vivo assay.

Shrimps were placed in three different tanks. Each tank represented different treatments related to the type of feeds given. Treated shrimps were fed three times a day, i.e. at 08:00, 12:00 and 17:00 WIB (local time). All experiments were performed in three replicates.

Treatment I: commercial pellet mixed with *T. chuii* extract of 200 ppm against *L. vannamei* infected with vibriosis.
Treatment II: commercial pellet mixed with *D. salina* extract of 200 ppm against *L. vannamei* infected with vibriosis.
Treatment II: commercial pellet without addition of microalgae extract to Vannamei shrimp infected with vibriosis.

Microbial enumeration from the hepatopancreas of shrimp. The observed parameters were amount of bacteria in the hepatopancreas of treated and control shrimps counted using the Total Plate Count (TPC) method (Benson 2001). Hepatopancreas of treated and control shrimp were dissolved in 10 mL sterile seawater. The solutions were stirred for 15 min and were diluted three times (10^{-1}, 10^{-2} and 10^{-3}). The treatment was carried out for 21 days and the bacteria amount was counted at the 7^{th}, 14^{th}, and 21^{st} days.

Results

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Morphological signs of the infected shrimp. The clinical signs of the infected shrimps were 'luminescent pleopoda', melanocyst on the shrimp's body, reddish tail, and brownish-red hepatopancreas.

Bacterial isolats. Based on the isolation of vibriosis-causing bacteria, there were 13 isolates that was morphologically characterized based on their colonial edge form, characteristic and color. Out of 13 bacterial isolates, three potential bacteria were selected and results are presented in Table 1.

Table 1

Morphological characteristic sand color of 13 bacterial isolates

No	Colonial edge form	Colonial characteristic	Colonial color	Total	Isolates code
1	Jagged	Convex	Green	1	U1H1
2	Smooth	Convex	Yellow	7	U1H2,U1E1,U2H1,U2H1, U3H2, U4H1, U5H1, U1E2, U3H1
3	Smooth	Convex	Green	2	
4	Smooth	Cembung	Milk white	1	U7E1
5	Jagged	Convex	Milk white	1	U8E1
6	Jagged	Convex	Yellow	1	U8E2

Bacterial identification. Based on the biomolecular analysis, there were two bacterial isolates that were classified as same species. This analysis managed to identify the presence of *V. alginolyticus* and *V. harveyi* as two bacteria strain being responsible for the vibriosis infection on the treated shrimps. Biomolecular analysis is presented in Table 2.

Table 2

Biochemical analysis of three selected bacterial isolates from Vannamei shrimp

No	Code	Universal Primer		Species	Homology
		27F	1429R		
1	U1H1	1.27F	1.1429R	*V. alginolyticus* strain ATCC 17749	97%
2	U1E1	2.27F	2.1429R	*V. harveyi* strain NCIMB 1280	96%
3	U4H1	3.27F	3.1429R	*V. alginolyticus* strain ATCC 17749	97%

In vivo Assay

Forty-five shrimps infected by vibrio reared during 21 days and feed with *D. salina* and *T. chuii* showed a decreased of bacteria amount (Figure 1).

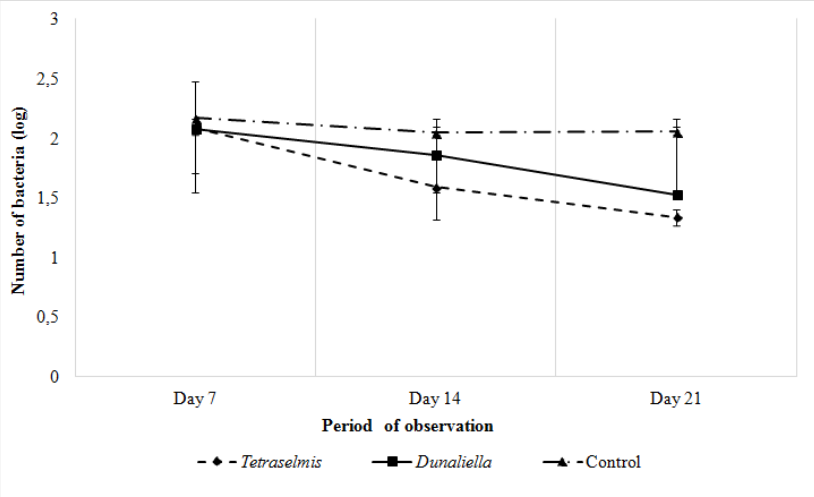

Figure 1. Number of bacteria observed from infected Vannamei shrimp during the 21 days experiment.

Discussion. Clinical signs observed on the infected Vannamei shrimp collected from Kaliwungu, Kendal were in accordance as infection signs in Vannamei caused by vibrio's as reported by Lina et al (2007) and Huang et al (2013). Another features remarked correlates to the behavior of shrimp are passive movement, slow response to feed and fluorescent swimming legs. Prayitno & Lachtford (1995) stated that *V. harveyi* is one of causative agent that also known as luminescent bacteria. Those clinical signs became the reference in isolating the vibriosis-causing bacteria from the shrimp.

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Bacterial isolates demonstrated that there were 13 isolates with diverse morphology. The selected isolate, which was U1H1, had a jagged colonial edge, convex with green color. Meanwhile, the U1E1 AND U4H1 were characterized with smooth colonial edge, convex with yellow color. These three isolates showed similar characteristic as *Vibrio* bacteria. Sarjito et al (2015) revealed that morphological features of bacteria with smooth colonial edge, convex with yellow color tested biochemically were identical with those of *V. harveyi.*

DNA amplification results from the rep-PCR exhibited three isolates with similar band profiles as seen on marker 800 bp. Rep-PCR method has been proven as an efficient method to identify bacterial species and the genetic relationship (Lina, 2011). DNA sequencing of isolate U1H1 and U4H1 had identical nucleotide with *V. alginolyticus* strain ATCC 17749 with homological level 97%. Isolate U1E1 showed to have similar nucleotide with *V. harveyi* strain NCIMB 1280 with homological level 96%.

Presence and abundance of *V. alginolyticus* and *V. harveyi* are highly correlated with environmental conditions. Asplund (2013) stated that the abundance of bacteria *Vibrio sp.* usually relates to temperature and salinity and increases in stable environmental condition. The abundance of *V. alginolyticus* and *V. harveyi* counted was 21 (isolate U1H1) and 4 (isolate U4H1) colony/plate and 11 (isolate U1E1) colony/plate. Environmental condition in the pond when the sampling was performed in October-November was fluctuated due to the rainy season. In this season, the temperature and salinity in the pond tended to change in short period; thus, it affected the abundance of bacterial colony, either in the pond or on the body of Vannamei shrimp.

The result of the in vivo assay shown that the vibrio infected shrimps feed with mixed *T. chuii* extract and *D. salina* reared for 21 days shown a decreased of the number of bacterial infection as shown on Figure 1. Widowati et al (2017) observed that *Dunalliela sp., T. chuii* showed antioxidant potential which could be considered for future applications in aquaculture. The result of this study indicated that the microalgae are capable to produce antibacterial compounds against vibrio.

Conclusions. The present study showed that vibriosis-causing bacteria isolated from *L. vannamei,* which showed clinical signs of vibriosis infection; was identified as *V. alginolyticus,* and as *V. harveyi.* The shrimps infected by vibrio and feed with *D. salina* and *T. chuii* reared during 21 days showed a decreased of bacteria amount. The result indicated that the microalgae was capable to produce an antibacterial compounds against vibrio. Based on the present findings, it could be inferred that the secondary metabolites of *T. chuii and D. salina* may be an excellent source for developing potent formulations for sustainable shrimp farming.

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Name of the authors:
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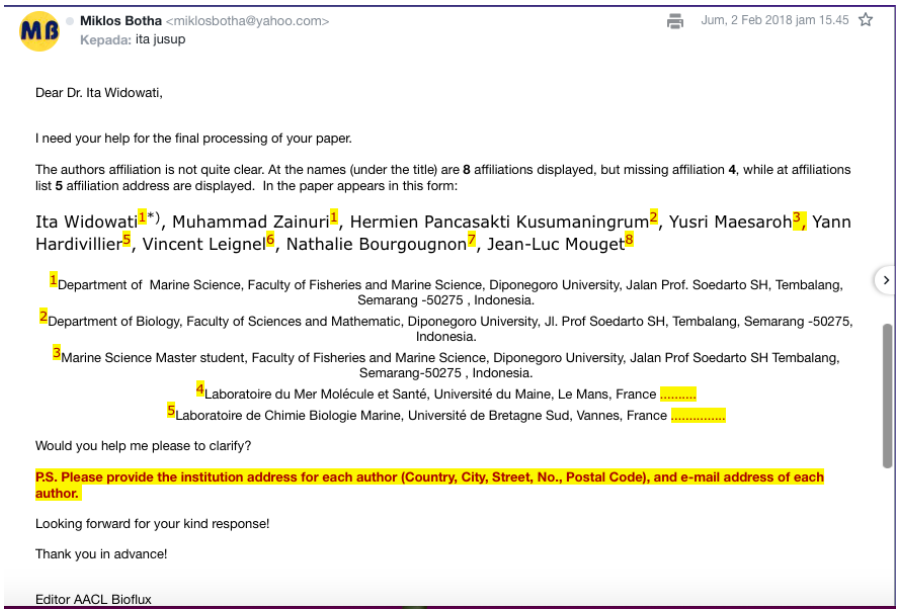

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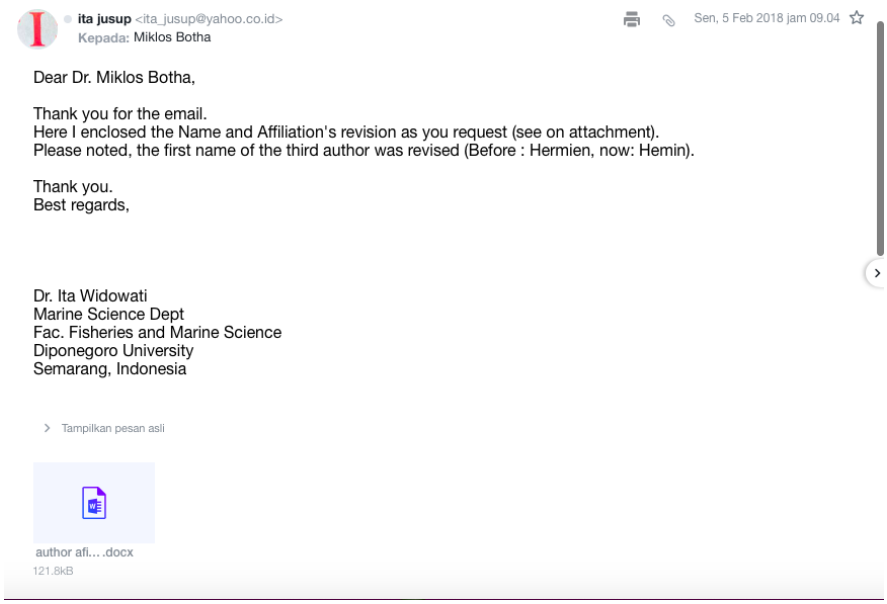

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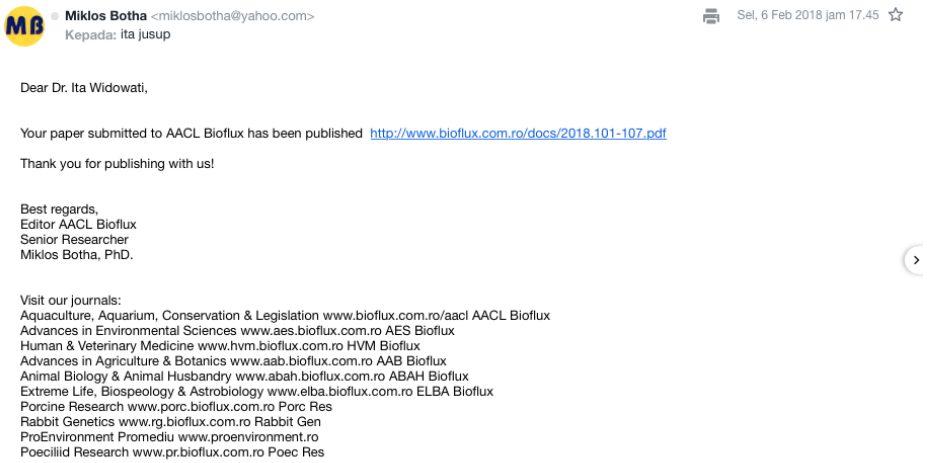

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10. Remerciement (9 Februari 2018). (10)

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Kepada: Miklos Botha

Jum, 9 Feb 2018 jam 14.22

Dear Dr. Miklos Botha,

Thank you very much for publishing my manuscript in AACL Bioflux journal .

Best regards,

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