ABAH BIOFLUX
Animal Biology & Animal Husbandry
International Journal of the Bioflux Society

Ulvan extract from *Enteromorpha intestinalis* enhances immune responses in *Litopenaeus vannamei* and *Penaeus monodon* juveniles

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Abstract. Disease outbreaks are constraints to aquaculture production and immunostimulants from seaweeds have gained increasing attention as a measure towards disease prevention. This study aimed to evaluate the effect of ulvan on the immune responses of *Litopenaeus vannamei* and *Penaeus monodon*, specifically on total haemocyte count, respiratory burst activity, and phenoloxidase activity. Shrimps (3-5 g) were fed with diets containing various levels of ulvan (0.00%, control; 0.05%; 0.10%; and 0.15% kg^{-1} diet) for 21 days. Hemolymph was extracted and used to measure total haemocyte count, respiratory burst activity and phenoloxidase activity. Immune response indices were significantly enhanced in shrimps fed ulvan-incorporated diets than did those fed with the control diet. Estimated optimum dietary ulvan level by the quadratic model was 0.21% for *L. vannamei* and 0.15% for *P. monodon*. Results of the present study indicated that ulvan was an effective immunostimulant for L. *vannamei* and *P. monodon*.
Key Words: Optimum dietary ulvan, *Entermorpha intestinalis*, immunostimulant, black tiger shrimp, Pacific white shrimp.

Introduction. Disease occurrence in shrimp farms have increased as a result of high culture densities, increasing extension of aquaculture farms (Vasquez et al 2009) and ecological disturbances (Walker & Mohan 2009). In recent years, shrimp diseases are responsible for the collapse of aquaculture in many countries (Flegel 2006). Aquaculture shrimps are displaced from their natural environments, provided artificial feeds, stocked in high density, exposed to stress through degraded water quality and by transporting; all these factors have provided opportunities for increased pathogenicity of existing infections, exposure to new pathogens and rapid transmission and trans-boundary spread of disease (Walker & Mohan 2009; Walker & Winton 2010). The wide and frequent use of antibiotics in aquaculture has resulted in the development and spread of antibiotic resistance (Defoirdt et al 2011). Thus, preventive measures need to be developed to achieve a healthy status of the cultured organism. One such method that is gaining importance is the use of immunostimulants from various sources (Sahu & Sivakumar 2010).

Immunostimulants, also called immunomodulators, adjuvants or biological modifiers, stimulate the immune system (Ganguly et al 2010) by increasing the host's resistance against diseases that in most circumstances are caused by pathogens (Bricknell & Dalmo 2005). These can be used as dietary supplements that improve the innate defense of animals providing resistance to pathogens during periods of high stress. The defense mechanism of crustaceans is less developed than that of fish and other vertebrates specifically due to the lack of adaptive memory. Invertebrates do not show high degree of specificity and memory in their immune strategies (Rowley & Powell 2007).

Sources of immunostimulants are varied and include seaweeds that contain sulfated polysaccharides and are known to be rich sources of structurally diverse bioactive compounds that have antibacterial and antiviral activities (Shanmugam & Mody 2000; Persson et al 2011). Yet marine algae, specifically the green algae, have remained largely under-studied (Alves et al 2013). Ulvan is the major water-soluble polysaccharide found in green seaweed of the order Ulvales, e.g. *Ulva* and *Enteromorpha* spp. (Jiao et al (2011). These sulphated polysaccharides play a critical role in major physiological functions in animals (Menard et al 2004).

Enteromorpha intestinalis, which contains considerable amount of ulvan, is considered a fouling organism and has been used as indicator of eutrophication (Blomster et al 1998) and accumulation of these species results in "green tides" (Blomster et al 2002) and has become a global problem. Since seaweeds represent a potential source of antimicrobial substances, discovering other potentials of extracts from *E. intestinalis* is necessary such as its contents of ulvan that could potentially act as an immunostimulant to aquatic invertebrates. As far as we are concern, an evaluation of ulvan as an immunostimulant in penaeid shrimps has not been studied yet. For the first time, the present study aimed to evaluate the effects of ulvan from *E. intestinalis* on the immune responses of *Litopenaeus vannamei* and *Penaeus monodon*.

Material and Method

The experiments were performed between February and May 2014.

Experimental animals. *L. vannamei* and *P. monodon* juveniles (3-5 g) were obtained from Multi-Species Hatchery, UPV, Miagao, Iloilo and SEAFDEC-AQD, Tigbauan, Iloilo, respectively. They were maintained and reared indoor in a 1-tonner concrete tank provided with continuous aeration until the start of the experiments. Shrimps were acclimated to formulated diet and to the experimental condition for one week.

Rearing condition. Culture water was prepared by chlorinating with 100 ppm of sodium hypochlorite for a day and dechlorinated by vigorous aeration for 3 days before use. Water quality parameters such as salinity, temperature, dissolved oxygen, pH, ammonia and nitrite were monitored and maintained at constant levels during the entire experimental period. Each experimental unit in a static system was provided with adequate aeration.

Experimental set-up and diet. Following acclimation, shrimps were randomly divided into 8 tanks (2 tanks per treatment per species) of 300-L capacity with 30 shrimps in each tank. Shrimps were pooled per treatment and were used for the immune assays.

Ingredients in the basal and experimental diets followed the formulation of (Li et al 2007) and were purchased from the Southeast Asian Fisheries Development Center - Aquaculture Department (SEAFDEC-AQD), Tigbauan Iloilo and formulated feeds were prepared at the UP Visayas Nutrition Laboratory in Miagao, Iloilo. Basal diet for the two penaeid shrimps were incorporated with commercially prepared ulvan (Elicityl, France) at inclusion levels of 0%, 0.05%, 0.10% and 0.15% kg^{-1} of feed (Table 1). The prepared diets were subjected to proximate analysis. Each diet was fed to shrimps of each treatment at 10% body weight 3 times daily at 8:00 AM, 12:00 PM, and 4:00 PM for three weeks.

Diet was prepared by mixing all dry ingredients with fish oil (Table 1) followed by the addition of purified ulvan (Elicityl, France) dissolved in hot water by sonication (5 g L^{-1}). Inclusion of ulvan in feed was made by adjusting the amount of cellulose. Cooked starch was added and mixed resulting in moist dough. The resulting dough was passed in the pelletizer twice to ensure proper mixing and was steamed for 5 min and oven-dried at 60°C for 18-24 h. The pellets were cut into appropriate sizes and stored at -4°C until use.

Table 1

Composition of experimental diets fed to *Litopenaeus vannamei* and *Penaeus monodon*

Ingredients	*Dietary ulvan inclusion level*			
	0%	*0.05%*	*0.1%*	*0.15%*
Shrimp meal	34.00	34.00	34.00	34.00
Soybean meal	21.00	21.00	21.00	21.00
CMC	3.48	3.43	3.38	3.33
Vitamin[a]	1.00	1.00	1.00	1.00
Mineral[a]	1.00	1.00	1.00	1.00
BHT	0.02	0.02	0.02	0.02
Lecithin	0.50	0.50	0.50	0.50
Cod liver oil	4.00	4.00	4.00	4.00
Starch	15.00	15.00	15.00	15.00
Ulvan	0.00	0.05	0.10	0.15
Total	100.00	100.00	100.00	100.00

[a]Vitamin-mineral composition (g. 100 g-1 diet): 4,000,000 IU Vitamin A; 300,000 IU Vitamin D2; 1,000 IU Vitamin E; 0.04 Vitamin B1; 0.12 Vitamin B2; 0.12 Vitamin B3; 2.50 Vitamin C; 0.06 Folic Acid; 0.60 Niacin; 1.00 Calcium Pantothenate; 2.00 Biotin; 1.00 Choline Chloride; 1.20 Iron; 0.12 Copper; 0.04 Iodine; 0.50 Manganese; 0.60 Zinc; 1.2 Cobalt; 0.002 Selenium; carrier q.s. ad to make 100 g.

Immune assays

Haemolymph extraction and total haemolymph count. Anticoagulant for hemolymph extraction was prepared by adding 10 nM ethylenediaminetetraacetic acid (EDTA) to a salt solution (450 mM NaCl, 10 nM HEPES, pH 7.3, 850 mOsm kg^{-1} (Hernandez-Lopez et al 1996). Hemolymph was collected from the pleopod at the first abdominal segment near the genital pore, using a 1-mL syringe with 26 gauge hypodermic needle rinsed thoroughly with pre-cooled anticoagulant. Hemolymph at 300 µL $shrimp^{-1}$ was collected from 6 randomly selected shrimps in each treatment for total hemocyte count (THC), superoxide anion assay and phenoloxidase assay.

A drop of anticoagulant-hemolymph mixture was placed on a Neubaeur's hemocytometer for the total hemocyte count expressed as count mL^{-1} hemolymph.

Superoxide anion (NBT reduction) assay. Respiratory burst activity of haemocytes was quantified using reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion production (Muñoz et al (2000).

Haemocyte sample (100 µL) was placed in each well of a microtitre plate and was incubated at ambient temperature for 2 h. The supernatant was discarded and was replaced with 50 µL Modified Hank's Balanced Salt Solution (MHBSS) medium. Nitroblue tetrazolium-phorbol myristate acetate (NBT-PMA) solution (100 µL) was added to each well and was incubated further for 30 min. Supernatants were removed and haemocytes fixed by adding 200 µL absolute methanol for 10 min. The wells were washed twice with 70% methanol and air dried. The formazan deposits were solubilized by adding to each well 120 µL of 2 M KOH and 140 µL of dimethyl sufoxide (DMSO). Intensity of the turquoise blue colour was measured at 620 nm by a microplate reader and the activity expressed as optical density 100 μL^{-1} haemolymph. Blank control reaction was also performed using 120 µL of KOH and 140 µL of DMSO.

Phenoloxidase activity assay. Phenoloxidase (PO) activity was assayed spectrophotometrically by the formation of dopachrome from *L*-dihydroxyphenylalanine (*L*-DOPA) as described by Hernandez-Lopez et al (1996). Anticoagulant-free hemolymph was placed in sterile 1.5 mL microcentrifuge tubes and subjected to a freeze-thaw cycle 5 times to induce cell lysis and degranulation. Samples were vortexed, centrifuged at 13,000 x g for 15 min at 4°C and haemocyte supernatant collected. The reaction mixture consisted of 25 µL haemocyte supernatant placed in 96-well microtitre plates, 25 µL of 0.1% trypsin in shrimp salt solution (SSS); the mixture was incubated for 30 min. Then 25 µL of 0.3% *L-3, 4*-dihydroxyphenylalanine (*L*- DOPA) was added to the solution and incubated for 10 min. Optical density was read at 490 nm by a microplate reader. Units

of enzyme activity were expressed as the change in absorbance min^{-1} 100 μL^{-1} hemolymph (Joseph & Phillip 2007). One activity unit was equivalent to an increase of 0.001 in optical density.

Estimation of optimum level and statistical analysis. Data for respiratory burst activity and THC were analyzed by fitting quadratic regression equation used in fish to estimate protein and amino acids (Zeitoun et al 1976; Chiu et al 1988). This model was deemed appropriate for a hyperbolic data in which the response parameters reached a peak and declined at the highest level of the independent variable. In this method, a quadratic equation is used to fit the response data obtained from feeding a dietary series:

$$R = a + bI + cI^2$$

where R is the measured response; I is the dietary nutrient concentration; and a, b, and c are constants that are calculated to provide the best fit of the data. The value of I that produces the maximum response I_{max} is calculated as follows:

$$I_{max} = -0.5\ (b/c)$$

Data obtained from total hemocyte count (THC), respiratory burst activity and PO activities were tested for normality of distribution using Shapiro-Wilk test and homogeneity of variance using Levene's test. If the data did not pass the two tests, they were transformed until these tests were passed. The data were then analyzed using one-way ANOVA and if significant differences were observed, a post-hoc test was conducted (Tukey's test) to rank the experimental treatments. All probability values were set at a significance level of 0.05. Statistical analysis was carried out using the software SPSS 16.0.

Results

Total haemocyte count (THC). Total haemocyte count was highest among *L. vannamei* fed with 0.10 and 0.15% ulvan-incorporated diet which were statistically similar (Figure 1a). Similarly for *P. monodon*, THC values of shrimps fed with diets containing 0.10 and 0.15% ulvan were also highest and were statistically similar (Figure 1b). Among the ulvan-supplemented diet in either shrimps, dietary inclusion of 0.05% ulvan exhibited the lowest THC and were statistically similar with THCs of shrimps fed the control diet (without ulvan supplementation).

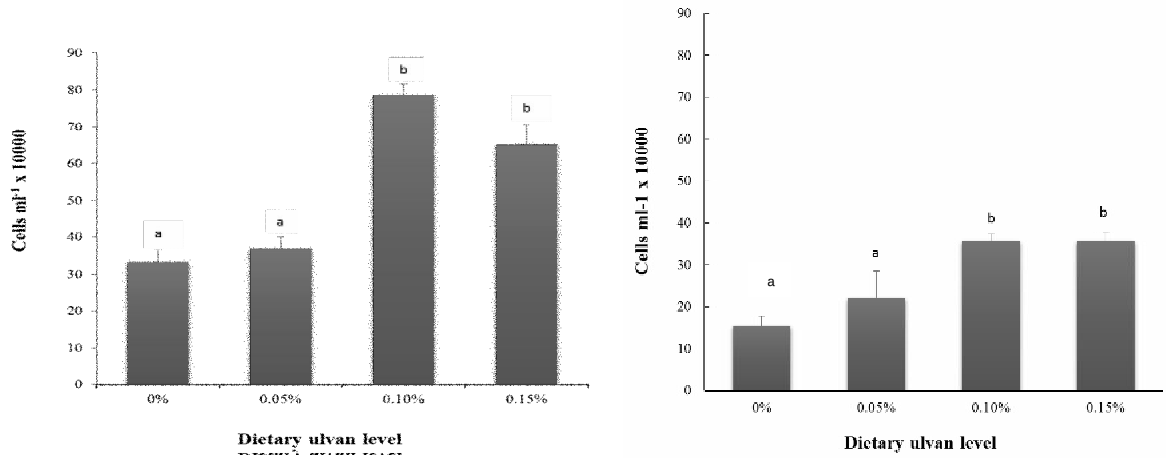
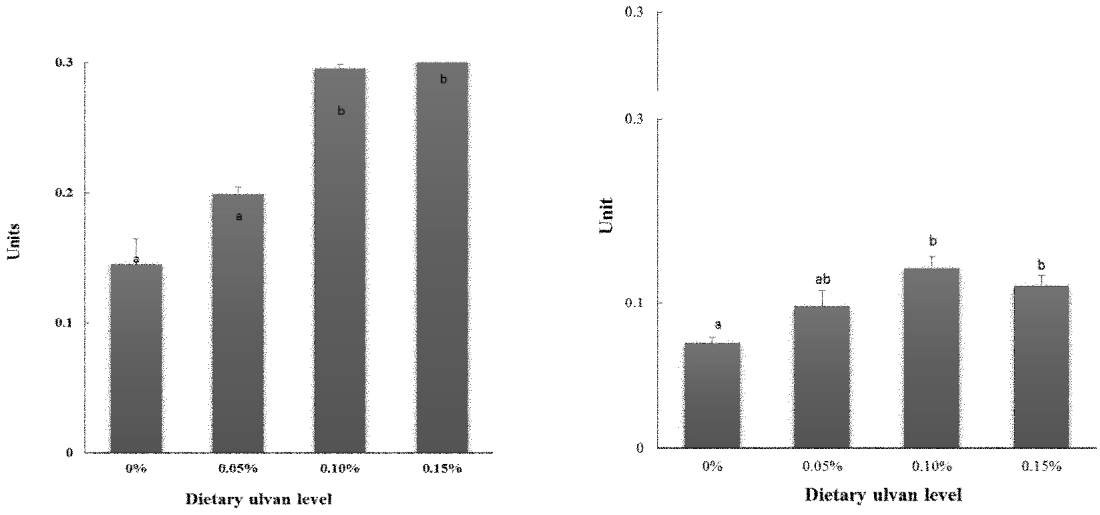


Figure 1. Total haemocyte count of *Litopenaeus vannamei* (a) and *Penaeus monodon* (b) fed with diets containing various ulvan inclusion levels.

Respiratory burst activity. In *L. vannamei*, respiratory burst activity were highest in shrimps fed diets with 0.10 and 0.15% ulvan which were statistically similar with each other, while respiratory burst activity in black tiger shrimps were also highest in those fed diets containing 0.10 and 0.15% ulvan (Figure 2). Shrimps fed diet containing 0.05% ulvan did not exhibit any improvement in respiratory burst activity over shrimps fed the control diet; both exhibited lower activities than did those fed diets containing 0.10 and 0.15% ulvan in both species of shrimps.



a b

Figure 2. Respiratory burst activity of *Litopenaeus vannamei* (a) and *Penaeus monodon* (b) fed with diets containing various ulvan inclusion level.

Phenoloxidase activity. Figure 3 shows the PO values of both shrimps fed with diets containing various dietary ulvan levels. Lowest PO values for both shrimps were exhibited in those fed the control diet and the diet containing 0.05% ulvan which were statistically similar with each other while the highest values were exhibited by those fed diets containing 0.10 and 0.15% ulvan which were statistically similar with each other. The trends were the same for both penaeid shrimps.

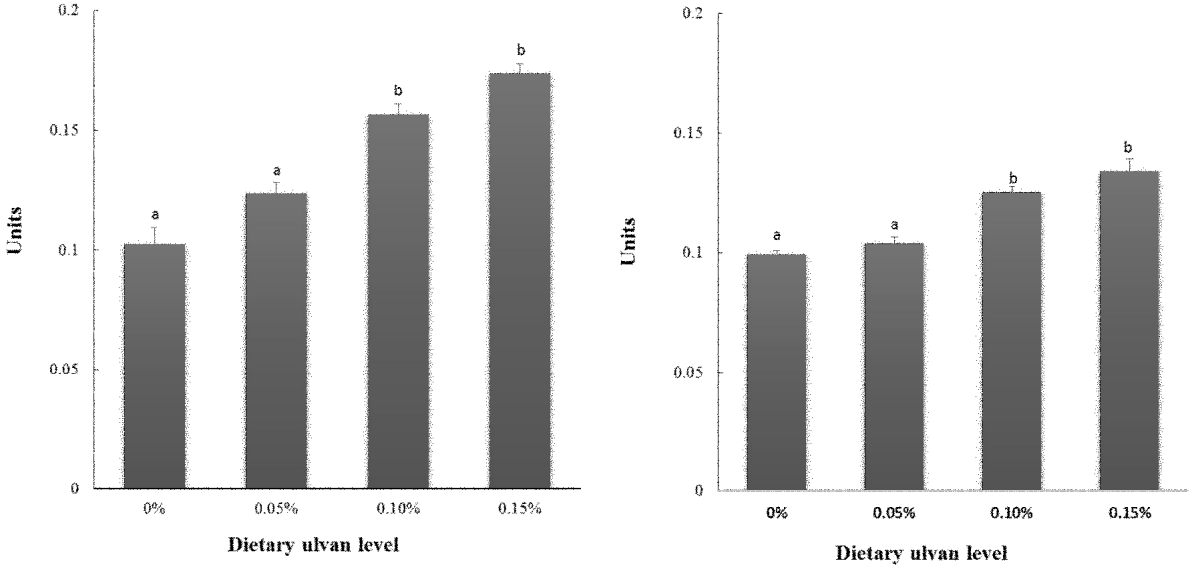


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Figure 3. Phenoloxidase activities of *Litopenaeus vannamei* (a) and *Penaeus monodon* (b) fed with diets containing various dietary inclusion levels of ulvan.

Estimation of optimum dosage of ulvan. The immune indices of the two shrimp species as affected by the dietary ulvan were fitted to a quadratic model (Figure 4) to estimate the optimum dosage. Using the immune parameters as response indices, the model shows that *L. vannamei* and *P. monodon* require a minimum amount of 0.21% and 0.15% of ulvan for maximum immune responses.

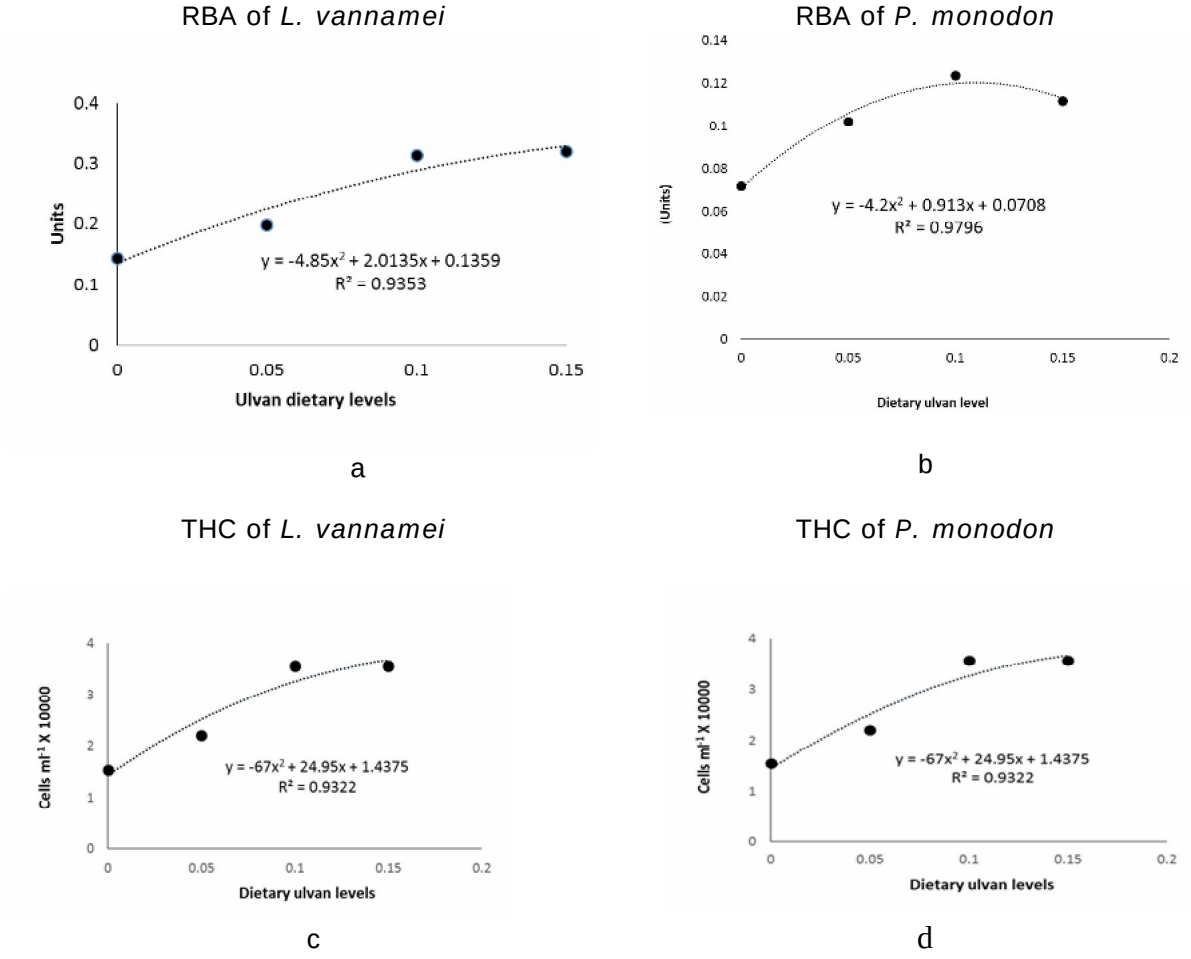

Figure 4. Quadratic model fitting of respiratory burst activity (RBA, a & b) and total hemocyte count (THC, c & d) responses to dietary ulvan for *Litopenaeus vannamei* and *Penaeus monodon*. Estimated Imax for *L. vannamei* using RBA and THC were both 0.21% dietary ulvan, while that for *P. monodon* were 0.11 and 0.19% dietary ulvan, respectively.

Discussion. Haemocytes play an important role in removing foreign particles such as bacteria from haemolymph by phagocytosis (Ratner & Vinson 1983). In crustaceans, THC is a stress indicator, but varies non-specifically according to the natural rhythms of the environment, as well as chemical and physico-chemical stress (Campa-Cordova et al 2002). In the present study, the reason why THC exhibited by *L. vannamei* was higher than those observed in *P. monodon* was still unknown. Increased in relative hemocyte count (RHC which is the ratio of THC of the shrimp fed test diet to the THC of shrimps fed with the control diet) after exposure to dietary ulvan could be related to the protective effects of the shrimp immune system (Chisholm & Smith 1995) since cellular responses are the main immune defense in invertebrates (Johansson & Söderhäll 1992; Sequeira et al 1996). The number of haemocytes can vary and even decreases dramatically during infections. The THC values observed in *L. vannamei* and *P. monodon* in the present study differed from each other and were lower than in those reported in other studies that reach up to 10^8 cells mL^{-1} but were higher than those reported by Manilal et al (2009). This indicated high disparity of THC among various shrimp species and a number of

factors affect this such as the haemocyte circulation system in which haemocytes are distributed both in vascular system and tissues. Increased THC values can either be from proliferation of the cells or from the movement of cells from tissues to hemolymphs. The decrease may be due to cell lysis or to increased movement from hemolymphs to tissues (Pipe & Coles 1995). THC also varies in relation with the moult cycle (Liu et al 2004).

The present study attempted to document the effects of ulvan on immune responses and was not designed to explain the mechanisms. However, literature have tried to explain that the respiratory burst capacity could be a response to changes in the lipid composition of cell membranes (Sung et al 1994; Muñoz et al 2000) and to the production of cell-activating factors (cytokines or chaperonins) that may improve the phagocytic capability of haemocytes (Itami et al 1998). It could be highly likely that this phenomenon might also be at work in the present study. Increased oxidant in cells of organisms under distress is expected after exposing shrimp to immunostimulants such as ulvan. This was confirmed in the results of the present study in which respiratory burst activities in shrimps fed with ulvan-incorporated diets were higher than those fed with the control diet. Castro et al (2004) have studied extracts from different seaweeds and among them were from *Ulva rigida* and *Enteromorpha* sp. which showed the best responses on the respiratory burst activity of turbot phagocytes. Priming effect of sulfated polysaccharides has been established for various immunostimulants (Sung et al 1994; Muñoz et al 2000; Downs et al 2001). The capacity of shrimp haemocytes to generate O_2^-, following immersion of shrimps in betaglucan or sulphated polysaccharide vary between 1.5–2.0 times higher than that of the control (Campa-Cordova et al 2002). This observation agreed very well with the observations in the present study with respiratory burst activity increased to a maximum of about 2.0 in *L. vannamei* and to about 1.7 in *P. monodon* over those of shrimps fed the control diet after 3 weeks of feeding.

The prophenoloxidase (proPO) system has been considered to play an important role in the defense system of crustaceans (Smith & Söderhäll 1983). Activation of the proPO system which is measured in terms of the phenoloxidase (PO) activity has been used by some investigators to measure immunostimulation in shrimp (Sung et al 1994; Devaraja et al 1998). The almost linear increase in *in vitro* PO activity of hemocytes with the dietary ulvan level as compared with that of the control in the present study suggested that the PO-activity was ulvan dosage-dependent. Velmurugan et al (2014) have revealed that the extracts from *Enteromorpha flexousa* increase the PO activity against WSSV of Indian white shrimp (*Penaeus indicus*). The proPO-depleted shrimps (expressed as decreased PO activity) exhibit lower haemocyte counts and significantly down-regulate expression of antimicrobial peptides (Velmurugan et al 2014).

Dietary ulvan was administered for 21 days in the present study before immune indices were measured. This period was deemed sufficient since in the observations of Sung et al (1994) and Dugger (1999) that twice-a-week exposure to food containing beta-*1,3* glucan is sufficient to maintain optimum, nonspecific immune cell activation in shrimp. In *L. vannamei*, only a single exposure with betaglucan is necessary to activate the haemocyte antioxidant response (i.e. increase the superoxide dismutase activity), as well as relative soluble protein content and relative hemocyte count.

The average optimum amount for maximum respiratory burst activity and maximum THC for *L. vannamei* and *P. monodon* were 0.21% and 0.15% dietary ulvan, respectively, as estimated from a quadratic fitting of the data. This difference could be due to differences in some physiological factors in the two penaeid shrimps.

Conclusions. Among the levels of dietary ulvan tested, 0.05% inclusion level was enough to prolong survival as shown by the challenge tests for both penaeid shrimps. Immunological parameters such as the total haemocyte count, respiratory burst and phenoloxidase activities were all enhanced in shrimps fed diets containing ulvan. Estimated optimum dietary ulvan level by the quadratic model fitting were 0.21% for *L. vannamei* and 0.15% for *P. monodon*. Results of the present study indicated that ulvan was an effective immunostimulant for *L. vannamei* and *P. monodon*.

Acknowledgements. The authors are grateful to the Philippine Department of Science and Technology (DOST) - Accelerated Science and Technology Human Resource Development Program (ASTHRDP) for providing the scholarship to Ms. Lauzon and to the DOST-Philippine Council for Agriculture, Aquatic and Natural Resources Development (PCAARD) for the research funding and to the Office of the Vice Chancellor for Research and Extension of the University of the Philippines Visayas for the additional research funding and publication support.

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Received: 02 December 2014. Accepted: 19 January 2015. Published online: 01 February 2015.
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How to cite this article:
Lauzon Q. D., Serrano Jr. A. E., 2015 Ulvan extract from *Enteromorpha intestinalis* enhances immune responses in *Litopenaeus vannamei* and *Penaeus monodon* juveniles. ABAH Bioflux 7(1):1-10.