



(43) International Publication Date
22 September 2016 (22.09.2016)

(10) International Publication Number
WO 2016/146736 A1

- (51) **International Patent Classification:**
A01K 1/02 (2006.01) *A01K 61/00* (2006.01)
- (21) **International Application Number:**
PCT/EP2016/055788
- (22) **International Filing Date:**
17 March 2016 (17.03.2016)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**
15159853.9 19 March 2015 (19.03.2015) EP
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- (81) **Designated States** (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,

BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

- (84) **Designated States** (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— of inventorship (Rule 4.17(iv))

Published:

— with international search report (Art. 21(3))

(54) **Title:** USE OF POLY-BETA-HYDROXYBUTYRATE AS HOUSING TO HOMOGENIZE GROWTH AND INCREASE SURVIVAL OF CRUSTACEANS IN AQUACULTURE SYSTEMS

(57) **Abstract:** The present invention relates to the culture of crustaceans in aquaculture systems. More specifically, the present invention relates to the use of poly-beta-hydroxybutyrate (PHB) to homogenize growth and/or to increase average survival of a population of crustaceans in an aquaculture system wherein said PHB serves as housing / habitat structure to said population in said aquaculture system.



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Use of poly-beta-hydroxybutyrate as housing to homogenize growth and increase survival of crustaceans in aquaculture systems

Technical field of invention

The present invention relates to the culture of crustaceans in aquaculture systems. More specifically, the present invention relates to the use of poly-beta-hydroxybutyrate (PHB) to homogenize growth and/or to increase average survival of a population of crustaceans in an aquaculture system wherein said PHB serves as housing / habitat structure to said population in said aquaculture system.

Background art

Because of the current high demand for shrimp and prawns, harvesting of wild stocks from the oceans can no longer be performed sustainably. Therefore, methods to produce shrimp and prawns under controlled conditions have been developed. Currently, the crustacean aquaculture industry is a high-value activity worldwide. The global annual production of crustaceans can be estimated to have reached 6.4 million metric tons and to be worth US\$ 30.8 billion in 2012 (Global Aquaculture Production – FishStat, accessed January 2015). With improved pond culture techniques, yield from traditional marine shrimp and prawn ponds has been raised to 500–800 kg/ha/year without supplementary feeding (Kungvankij and Chua, 1986). Yield can be further increased to 5–70 tons/ha/year through supplementary feeding and (super-) intensive pond management (Samocha, 2010).

The controlled culture of crustaceans implies keeping different developmental stages of the cultured animals in dedicated systems and under specific environmental conditions. In (semi-)intensive crustacean culture, substantial numbers of animals are lost during the larval and post larval stages. The reduction in survival at high densities results from a combination of factors, which include a limitation of favourable space and natural food sources, adverse shrimp behaviour such as cannibalism, a degradation of water quality, accumulation of undesirable sediment and the occurrence of diseases (Kautsky et al., 2000; Arnold et al., 2006).

Housing or shelter structures such as fiberglass window screens, plastic mesh, sheets and other plastic based 3D shapes have been added to the crustacean culture systems in an attempt to mitigate some of the negative effects of increased stocking density (Sandifer et al., 1987; Tidwell et al., 1998, 1999; Bratvold & Browdy, 2001). Also for freshwater prawns, housing structures in the form of aquatic weeds, grasses and tree branches have been placed in the ponds (FAO, 2002). The additional surface created by the housing structures decreases stocking density within the water column and

provides shelter for the animals during vulnerable periods. Several studies have indicated that the addition of extra housing structures in the ponds can improve the growth and survival of the cultured animals (Sandifer et al., 1987; Tidwell et al., 1998; Peterson & Griffith, 1999; Bratvold & Browdy, 2001; Moss & Moss, 2004; Arnold et al., 2006; Zarain-Herzberg et al., 2006; Ballester et al., 2007; Zhang et al., 2010). In addition to reducing stocking density, the addition of housing structures allows the animals to reside above polluted sediment thus creating a healthier living environment. Also, it has been indicated that housing structures could provide a natural food supplement for the cultured animals in the form of microbial biofilm growing on the structures, improve the water quality of ponds by the waste assimilation capacities of the microbial biofilm growing on the structures, and control the pathogenic bacteria as biofilms.

Polyhydroxyalkanoates (PHAs), naturally-occurring polyesters of various hydroxyalkanoates, are being produced industrially for use as natural, biodegradable, and biocompatible thermoplastics as alternatives for petroleum based plastics. PHAs are synthesized by a very broad range of microorganisms under unbalanced growth conditions and are accumulated in the cell in the form of intracellular inclusions serving as carbon and energy reserve. Poly-beta-hydroxybutyrate or poly-β-hydroxybutyrate or poly-3-hydroxybutyrate (PHB) is the best studied member of the family of PHAs. PHB is similar to polypropylene in its physical properties, but has the advantage of being biodegradable. PHB has been shown to be completely biodegraded by bacteria into water and carbon dioxide in natural environments, including water, soil, and compost (Lee et al., 2005).

Up to now, the use of PHB in aquaculture is limited to its application as a feed additive to improve growth and disease resistance of the cultured animals (Defoirdt et al. (2007), De Schryver et al. (2009), Nhan et al. (2010), Sui et al. (2012), Thai et al. (2014)) and to its use as a slow carbon release compound for denitrification (i.e. microbial conversion of nitrate into nitrogen gas) in the biofilters of recirculating aquaculture systems (RAS) (Boley et al., 2000).

Brief description of figures

Figure 1: Schematic representation of a housing structure unit used in the experimental tanks. The material out of which the housing structure is made comprises PHB-based plastic.

Figure 2: Wet body weight (g) frequency distribution of *P. monodon* postlarvae grown with the control PVC substrates (1) and experimental PHB substrates (2= PHBGreen, 3=PHBWhite). 'SE' = standard error and 'N' = number of shrimp.

Figure 3: Length (cm) frequency distribution of *P. monodon* postlarvae grown with the control PVC substrates (1) and experimental PHB substrates (2= PHBGreen, 3=PHBWhite). 'SE' = standard error and 'N' = number of shrimp.

5 Figure 4: Visit percentage (%) of non-exposed *P. monodon* postlarvae in/on the control PVC substrates and experimental PHB substrates monitored at different times. Values are means $\pm$ standard error (n=10).

Figure 5: Wet body weight (g) frequency distribution of *L. vannamei* postlarvae grown with the control PVC substrates (1) and experimental PHB substrates (2). 'SE' = standard error and 'N' =
10 number of shrimp.

Figure 6: Visit percentage (%) of non-exposed *L. vannamei* postlarvae in/on the control PVC substrates and experimental PHB substrates monitored at different times. Panel A: only 1 type of substrate was in the tank; panel B: two types of substrate were in the tank. Values are means $\pm$ standard error (n=10). * indicates a significant difference between substrate types at that time
15 point (T-test; $p < 0.05$).

Description of invention

The present invention relates to the surprising finding that the use of PHB as the basis for housing structures for crustaceans cultured in aquaculture systems is superior – when compared to non-PHB-based housing structures - in increasing homogenous growth and/or survival of the animals.

20 Therefore, the present invention relates in first instance to a method to increase homogenous growth and/or to improve survival of crustaceans cultured in aquaculture tanks or ponds, comprising the addition of housing structures comprising the polymer poly- β -hydroxybutyrate into said aquaculture tanks or ponds.

The term 'comprising the polymer poly- β -hydroxybutyrate' means that said housing structure is at
25 least partially made out of the polymer poly- β -hydroxybutyrate.

In other words, the present invention relates to a method as described above wherein said housing structure significantly increases the homogeneity in weight and/or in length, and/or, the average survival of said crustaceans when compared to crustaceans reared in systems containing non-poly- β -hydroxybutyrate -based housing structures.

5 The term 'poly- β -hydroxybutyrate or PHB' relates to the biopolymer of 3-hydroxybutyric acid produced by bacteria accumulated as vacuoles in the cytoplasm. The PHB is synthesized by bacteria mainly under conditions of nutrient limitation and carbon excess but can also be accumulated for the purpose of spore formation by *Bacillus* sp. In case the supply of nutrients is restored or at the commencement of sporulation, the PHB is depolymerized so that it can be used as a source of energy
10 and carbon for the bacteria. Alternatively, PHB may be produced by transgenic plants (Dalton et al., 2012). Since the first report on the rapid biodegradability of PHB, a lot of research groups have been trying to optimize PHB production at minimal costs for a more sustainable plastic production.

Hence, the present invention thus also relates to a method as described above wherein said PHB is extracted from bacterial or plant cells and is used for the production of bio-based plastic in any two
15 dimensional or three dimensional structural format adequate to provide housing structure for the crustaceans in the aquaculture systems.

More specifically, the present invention relates to a method as indicated above wherein said housing structure is made out of a polymer of pure poly- β -hydroxybutyrate, out of a co-polymer of poly- β -hydroxybutyrate with another polyhydroxyalkanoate other than poly- β -hydroxybutyrate (such as,
20 but not limited to, polyhydroxyvalerate or polyhydroxypropionate), out of a blend of poly- β -hydroxybutyrate, and/or a co-polymer of poly- β -hydroxybutyrate with another polyhydroxyalkanoate other than poly- β -hydroxybutyrate and/or another polymer which is biobased (such as polylactic acid...) and/or which is non-biobased (such as any petroleum-based plastic), or, is coated with any of said polymers or said polymer blends.

25 The term 'coating' or 'coated with' relates to adding a cover or a layer of PHB or any other of the aforementioned polymers or polymer blends to a non PHB based housing structure. Alternatively, the coating can be added on the walls of the tanks in which the crustaceans are reared.

The term 'housing structure' relates to any two or three dimensional structure that is placed in the water of the crustacean aquaculture system to create additional surface and/or shelter as described
30 above and on which crustaceans such as shrimp can attach and forage or in which

crustaceans/shrimp can enter, and which is made of – or comprises - PHB as described above. A non-limiting example of a housing structure is shown in Figure 1.

The term ‘increasing homogenous growth’ refers to the superior quality of PHB-based housing structures – as compared to non-PHB-based housing structures - to result in a more equal (or more homogenous) increase in weight and/or length between individual animals within a crustacean population raised in an environment containing said PHB-based housing structures. The latter ‘equal increase’ then refers to a smaller range between the shortest and the longest crustacean and/or a smaller range between the most lightweight and the most heavy crustacean in said population, or, refers to a Gaussian curve with a smaller variance that is constructed based on the length of all crustaceans in said population or a Gaussian curve with a smaller variance that is constructed based on the weight of all crustaceans in said population. The decrease in range between the shortest/most lightweight crustacean and the longest/most heavy crustacean is at least 8%, preferably at least 20%, more preferably at least 33% and most preferably more than 35%.

The term ‘survival’ refers to the relative number of living individual animals within a population having resided for a certain period in a tank or pond containing said housing structures as compared to the initial number of individual animals within this population. The term ‘improving survival’ refers to the superior quality of PHB-based housing structures – as compared to non-PHB-based housing structures - to result in a higher survival in a crustacean population raised in an environment containing said PHB-based housing structures. The improved survival when the crustacean population is raised in an environment containing PHB-based housing structures as compared to when the crustacean population is raised in an environment containing non-PHB based housing structures is significant as determined by a statistical test (for example t-test or one-way analysis of variance) with a p-value of 0.05.

The term ‘Crustacean’ means an animal belonging to the group (or phylum) of arthropods, and specifically includes such familiar animals as shrimp, prawns, crayfish and crabs. Crustaceans have an exoskeleton, which they molt to grow. They are distinguished from other groups of arthropods, such as insects, myriapods and chelicerates, by the possession of biramous (two-parted) limbs, and by the nauplius form of their larvae.

The present invention thus relates to a method as described above wherein said crustacean is a shrimp, a prawn, a crayfish or a crab. The term ‘crab’ specifically relates to the Chinese mitten crab

(*Eriocheir sinensis*) and the term 'crayfish' specifically relates to the European crayfish (*Astacus astacus*).

The present invention more specifically relates to a method as described above wherein said crustaceans are shrimp of the Penaeidae family (Türkay, 2013a) or prawns of the Palaemonidae family (Türkay, 2013b), or larvae or postlarvae of said shrimp or prawns.

Even more specifically, the present invention relates to a method as described above wherein said shrimp of the Penaeidae family is *Litopenaeus vannamei* (De Grave, 2013a) or *Penaeus monodon* (Fransen and De Grave, 2013) or *Penaeus chinensis* (De Grave, 2013b) or *Penaeus indicus* and wherein said prawn of the Palaemonidae family is *Macrobrachium rosenbergii* (Fransen, 2013b).

The term 'aquaculture tank or pond' refers to any water containing reservoir intended for the controlled production of said crustaceans. A water containing reservoir can take the form of any earthen based pond or any solid material based tank.

The present invention thus relates to a method as described above wherein said PHB is used to provide said housing to said crustaceans in said aquaculture tank or pond.

The present invention further relates to a method as described above wherein said housing structure is placed in the water at a volumetric ratio of minimally $0,5 \times 10^{-7}$ and wherein said volumetric ratio is the added volume of all housing structure material in the water (calculated as added surface of all housing structure material in the water times its thickness) over the volume of water within the tank or pond. Said volumetric ratio can be -for example- smaller than 1×10^{-2} , smaller than 1×10^{-3} , smaller than 1×10^{-4} , smaller than 1×10^{-5} , smaller than 1×10^{-6} , or even smaller than 1×10^{-7} .

Moreover, the present invention relates to a method as described above wherein said housing structure is applied in hatchery tanks (tanks used for housing the larvae of said crustaceans from hatching up to the post-larval stage when the shrimp are either transferred to a nursery tank or a grow-out tank or pond), nursery tanks (tanks used as an intermediary stage between the hatchery phase and the grow-out phase for housing the larvae and/or postlarvae of said crustaceans up to the moment of transfer to a grow-out pond or tank) or in the grow out tanks or ponds (water bodies used for housing the postlarvae of said crustaceans up to the adult stage when the shrimp are harvested from these water bodies).

The present invention will now be illustrated by the following non-limiting examples.

Examples

Example 1: The effect of using PHB as housing structure on the growth and survival of giant tiger prawn (*Penaeus monodon*) postlarvae.

1. Materials and methods

1.1 Construction of PHB housing material

PHB-based plastic sheets of 0,5 mm thickness were obtained from Metabolix Inc and cut in sections of 0,1 m x 0,1 m. These were folded to create cylindrical hollow tubes with a length of 0.1 m and a diameter of 0.025 m. The structure was fixed by the use of a cable tie. Three cylindrical PHB tubes were grouped together creating a single PHB housing unit (See figure 1). PVC tubes of the same dimensions and grouping were used as control housing structures. All housing units were preconditioned for 2 weeks in the recirculation filter unit of a tank containing adult tiger prawn to establish first microbial colonization on the surface of the structures.

1.2 Experimental animals

Giant tiger prawn (*Penaeus monodon*) postlarvae (PL) of 1 day old (PL1) were used as experimental animals. Test animals were held for 2 weeks in acclimatization tanks of 250 L at a density of 1 PL L⁻¹. The tanks were continuously supplied with UV-treated seawater with a mean temperature and salinity of 30 ± 2 °C and 32 ± 1 ppt, respectively. Aeration by means of an airstone attached to an air diffuser was constantly provided to assure levels of dissolved oxygen not lower than 5 mg L⁻¹. During the acclimatization period, the postlarvae were fed at 5% on wet body weight day⁻¹ with shrimp-formulated feeds containing 45% crude protein and 9% crude fat. Feed composition is given in Table 1.

1.3 Experimental set-up

The experimental system consisted of 15 fibreglass tanks of 60L filled with 40L seawater. These were stocked with the acclimatized *P. monodon* PL15 having an average weight of 30 ± 5 mg PL⁻¹. Stocking density in the tanks was 1 postlarva L⁻¹. The PHB-based and the PVC housing units (a unit consisting of 3 tubes) were introduced in the experimental tanks at 2 units tank⁻¹ resulting in a volumetric ratio of 5 x 10⁻⁴. This resulted in 10 replicate tanks containing PHB-based housing structures (of which 5 contained PHB in white colour and 5 contained PHB in green colour) and 5 replicate tanks containing PVC housing structures. The test animals were fed two times daily (9:00 am and 16:00 h) with the experimental shrimp diets (Table 1) at 5% on wet body weight day⁻¹. Each experimental tank was aerated using an air diffuser to maintain dissolved oxygen above 5 mg L⁻¹. Ambient temperature and

salinity in the experimental tanks averaged 30 ± 2 °C and 32 ppt, respectively. Light regime was set at a fixed 10 h light and 14 h dark. Water was exchanged whenever necessary to keep nitrogen levels below 0.5 mg $\text{NH}_4^+\text{-N/L}$, 0.2 mg $\text{NO}_2^-\text{-N/L}$ and 10 mg $\text{NO}_3^-\text{-N/L}$. The experimental trial lasted for 61 days.

5 1.4 Parameters analysed

Survival, total length, wet body weight, population weight range and population length range of the *P. monodon* PLs were evaluated at the end of the experimental trial (day 61). Survival was determined by counting the number of animals in each tank. The percentage survival was computed following the formula: $\text{Survival (\%)} = (X/N) \times 100$ where, X is the number of larvae present at the end of the experimental period, N is the number of larvae at stocking (i.e. 40 at day 0). Total length was measured from the tip of the rostrum to the edge of the telson after the specimen was placed on its back over a plastic calliper and gently stretched. The length range was defined as the difference between the shortest and longest shrimp in the population. The wet body weight of each shrimp PL was measured after excess moisture was removed from each specimen using a paper cloth. The size range was defined as the difference between the most lightweight and the most heavy shrimp in the population. The average daily weight gain (ADWG) in each tank was estimated according to the following formula: $\text{ADWG} = (W_2 - W_1) / \Delta t$, in which W_2 = final average weight, W_1 = initial average weight, and Δt = total days of culture.

A substrate preference test was performed at the end of the experimental period. At the end of the trial, one unit of the PHB-based or PVC housing structures was removed from each experimental tank and moved individually to new 60L tanks containing 40L seawater. Shrimp PLs originating from the same batch as the experimental animals, but maintained in the acclimatisation tanks and fed the experimental diet during the trial were tested for their preference towards the type of housing structure. The substrate units were positioned in one side of the tanks while 10 shrimp were introduced in the tanks opposite to the location of the substrate. The number of postlarvae spotted in/on the substrate after introducing them in the tanks was determined at different time points. The visit percentage (%) gives the fraction of PLs in the tank that was located in/on the substrate at each time point. Only the postlarvae that were in direct contact with the substrate were the ones considered and counted.

Table 1. The experimental feed composition (crude protein = 45% and crude fat = 9%)

Ingredients	Composition (%)	Amount incorporated in 500 g
Danish fishmeal	33.00	165.00
Squid liver powder	10.00	50.00
DSBM HP	7.00	35.00
Acetes	15.00	75.00
Wheat Pollard	2.50	12.50
Bread flour	13.00	65.00
Soybean Oil	1.00	5.00
Squid oil	2.00	10.00
Peruvian Fish oil	2.00	10.00
Lecithin	0.50	2.50
Vitamin mix	2.00	10.00
Mineral mix	2.00	10.00
Tasmix	0.50	2.50
Choline chloride	0.15	0.75
Vitamin C	0.05	0.25
Dicalphos	4.00	20.00
Cellulose	5.30	26.50

2. Results

2.1 Survival of the shrimp postlarvae

- 5 As shown in Table 2, the survival of the shrimp PLs cultured in the tanks containing PHB based housing structures was significantly higher than the survival of PLs cultured in the tanks containing the PVC housing structures.

2.2 Growth of the shrimp PLs

- 10 As shown in Table 2, the mean wet body weight, mean length and average daily weight gain at the end of the trial of the shrimp PLs cultured in the tanks containing PHB based housing structures was not significantly different from the average wet body weight, average length and average daily weight gain of the shrimp PLs cultured in the tanks containing PVC housing structures. In Figure 2, however, it can be observed that the weight of the shrimp PLs harvested at the end of the trial from the tanks containing the PHB based housing structures was more homogenous than the weight of
15 the shrimp PLs harvested from the tanks containing PVC housing structures. The more homogenous

shrimp size was also illustrated by the smaller standard error for the mean wet body weight and by the population weight range that was 48% and 33% decreased for shrimp PLS from the tanks containing white PHB based housing structures and green PHB based housing structures, respectively, in comparison with shrimp PLs from the tanks containing PVC housing structures (Table 2). The median weight and spread on the weight of the shrimp PLs from the tanks with the white PHB based housing structures was 495.2 mg and 192.6 – 903.4 mg, respectively; the median weight and spread on the weight of the shrimp PLs from the tanks with the green PHB based housing structures was 472.8 mg and 74.0 – 994.7 mg, respectively; and the median weight and spread on the weight of the shrimp PLs from the tanks with the PVC housing structures was 502.2 mg and 31.6 – 1407.3 mg, respectively. In Figure 3, it can be observed that the length of the shrimp PLs harvested at the end of the trial from the tanks containing the PHB based housing structures was more homogenous than the length of the shrimp PLs harvested from the tanks containing PVC housing structures. The more homogenous shrimp length was also illustrated by the smaller standard error for the mean length and by the population length range that was 49% and 30% decreased for shrimp PLS from the tanks containing white PHB based housing structures and green PHB based housing structures, respectively, in comparison with shrimp PLs from the tanks containing PVC housing structures (Table 2). The median length and spread on the length of the shrimp PLs from the tanks with the white PHB based housing structures was 4.9 cm and 3.6 – 6.0 cm, respectively; the median weight and spread on the weight of the shrimp PLs from the tanks with the green PHB based housing structures was 4.8 cm and 2.8 – 6.1 cm, respectively; and the median weight and spread on the weight of the shrimp PLs from the tanks with the PVC housing structures was 5.0 cm and 2.2 – 6.9 cm, respectively.

Table 2. Survival (%), mean wet body weight and mean length of *P. monodon* postlarvae grown with the control PVC substrates and experimental PHB substrates at the end of the experimental trial. Values are means $\pm$ standard error (n=5). Values in a row with different superscript letters are significantly different (One-way ANOVA; $P < 0.05$).

Parameters	Substrate types		
	Control	PHBGreen	PHBWhite
Survival (%)	67.3 $\pm$ 6.5 ^b	85.0 $\pm$ 2.5 ^a	88.7 $\pm$ 3.4 ^a
Mean length (cm)	4.88 $\pm$ 0.12 ^a	4.83 $\pm$ 0.06 ^a	4.86 $\pm$ 0.05 ^a
Mean wet body weight (mg)	545 $\pm$ 36 ^a	500 $\pm$ 17 ^a	496 $\pm$ 14 ^a
Average daily weight gain (mg day ⁻¹)	8.9 $\pm$ 1.1 ^a	7.9 $\pm$ 0.5 ^a	7.8 $\pm$ 0.4 ^a
Population weight range (mg)	1375.7	920.7	710.8
Population length range (cm)	4.7	3.3	2.4

2.3 Substrate preference by the shrimp PLs

In figure 4, it can be observed that there was no significant preference for a specific type of housing structure during 1 hour after introduction of the shrimp PLs in the experimental preference tanks. This can be considered as the acclimation period needed during which the animals are stressed from the transfer. From 1 hour onwards, however, the PHB based housing structures were more visited by the shrimp PLs than the PVC housing structures.

Example 2: The effect of using PHB as housing structure on the growth characteristics and survival of whiteleg shrimp (*Litopenaeus vannamei*) postlarvae.

1. Materials and methods

1.1 Construction of PHB housing material

PHB-based plastic sheets of 1 mm thickness were obtained from Biomer (Germany) and cut in sections of 0,1 m x 0,1 m. These were folded to create cylindrical hollow tubes with a length of 0.1 m and a diameter of 0.025 m. The structure was fixed by the use of a cable tie. Three cylindrical PHB tubes were grouped together creating a single PHB housing unit (See figure 1). PVC tubes of the same dimensions and grouping were used as control housing structures. All housing units were preconditioned for 2 weeks in the recirculation filter unit of a tank containing adult whiteleg shrimp to establish first microbial colonization on the surface of the structures.

1.2 Experimental animals

Whiteleg shrimp (*Litopenaeus vannamei*) postlarvae (PL) of 10 days old (PL10) were used as experimental animals. Test animals were held for 20 days in a recirculation system. The salinity and temperature in the recirculating system averaged 35 ppt and 28°C, respectively. The photoperiod was maintained in a 10h light - 14h dark cycle. During the rearing period, postlarvae were fed a commercial diet (CreveTec bvba, Belgium) containing 54% crude protein and 12% crude fat at a level of 10% on wet body weight day⁻¹. The daily ration was divided into equal amounts and offered two times a day (9:00, 16:00 h).

1.3 Experimental set-up

The experimental system consisted of 14 fibreglass tanks of 45L filled with 38L seawater. These were stocked with the acclimatized *L. vannamei* PL30 having an average weight of 106 ± 52 mg PL⁻¹.

Stocking density in the tanks was 1 postlarva L⁻¹. The PHB-based and the PVC housing units (a unit consisting of 3 tubes) were introduced in the experimental tanks at 3 units tank⁻¹ resulting in a volumetric ratio of 9 x 10⁻⁴. This resulted in 7 replicate tanks containing PHB-based housing structures and 7 replicate tanks containing PVC housing structures. The test animals were fed two times daily (9:00 am and 16:00 h) with a commercial diet (CreveTec bvba, Belgium) containing 54% crude protein and 12% crude fat at 5% on wet body weight day⁻¹. Each experimental tank was aerated using an air diffuser to maintain dissolved oxygen above 5 mg L⁻¹. Ambient temperature and salinity in the experimental tanks averaged 28 °C and 35 ppt, respectively. Light regime was set at a fixed 10 h light and 14 h dark. Water was exchanged whenever necessary to keep nitrogen levels below 0.2 mg NH₄⁺-N L⁻¹, 0.1 mg NO₂⁻-N L⁻¹ and 10 mg NO₃⁻-N L⁻¹. The experimental trial lasted for 55 days.

1.4 Parameters analysed

Survival, wet body weight and population weight range of the *L. vannamei* PLs were evaluated at the end of the experimental trial (day 55). Survival was determined by counting the number of animals in each tank. The percentage survival was computed following the formula: Survival (%) = (X/N)*100 where, X is the number of larvae present at the end of the experimental period, N is the number of larvae at stocking (i.e. 38 at day 0). The wet body weight of each shrimp PL was measured after excess moisture was removed from each specimen using a paper cloth. The size range was defined as the difference between the most lightweight and the most heavy shrimp in the population. The average daily weight gain (ADWG) in each tank was estimated according to the following formula: ADWG = (W2 - W1)/ Δt, in which W2 = final average weight, W1 = initial average weight, and Δt = total days of culture.

Two substrate preferences tests were performed at the end of the experimental period. For a first preference test at the end of the trial, one unit of the PHB-based structures was removed from 5 PHB experimental tanks and one unit of the PVC structures was removed from 5 PVC experimental tanks, and moved individually to new 5L transparent tanks containing 4L preheated (28 °C) seawater. Shrimp PLs originating from the same batch as the experimental animals, but maintained in the acclimatisation recirculation system and fed the experimental diet during the trial were tested for their preference towards the type of housing structure. The substrate units were positioned on one side of the tanks while 10 shrimp were introduced in the tanks opposite to the location of the substrate. The number of postlarvae spotted in/on the substrate after introducing them in the tanks was determined at different time points. The “visit percentage” (%) gives the fraction of PLs in the tank that was located in/on the substrate at each time point. Only the postlarvae that were in direct

contact with the substrate were the ones considered and counted.

For the second preference test at the end of the trial, one unit of the PHB-based structures was removed from 5 PHB experimental tanks and one unit of the PVC structures was removed from 5 PVC experimental tanks. One PHB-based unit and 1 PVC unit were moved together into new 5L transparent tanks containing 4 L preheated (28°C) seawater (i.e. 5 tanks in total). The substrate units were positioned at opposite sides of the tanks. Shrimp PLs originating from the same batch as the experimental animals, but maintained in the acclimatisation recirculation system and fed the experimental diet during the trial were tested for their preference towards the type of housing structure. Ten shrimp were introduced in the center of the tanks, and the number of postlarvae spotted in/on each substratum type was determined at different time points. The “visit percentage” (%) gives the fraction of PLs in the tanks that was located in/on each substrate type at each time point. Only the postlarvae that were in direct contact with the substrate were the ones considered and counted.

2. Results

2.1 Survival of the shrimp PLs

As shown in Table 3, the survival of the shrimp PLs cultured in the tanks containing PHB based housing structures was significantly higher than the survival of PLs cultured in the tanks containing the PVC housing structures.

2.2 Growth of the shrimp PLs

As shown in Table 3, the mean wet body weight and average daily weight gain at the end of the trial of the shrimp PLs cultured in the tanks containing PHB based housing structures was significantly higher than the mean wet body weight and average daily weight gain of the shrimp PLs cultured in the tanks containing PVC housing structures. Although it cannot be clearly visually deducted from the histogram (Figure 5), the weight of the shrimp PLs harvested at the end of the trial from the tanks containing the PHB based housing structures was more homogenous than the weight of the shrimp PLs harvested from the tanks containing PVC housing structures. The more homogenous shrimp size was illustrated by the smaller standard error for the mean wet body weight and by the population weight range that was 8.1% decreased for shrimp PLs from the tanks containing PHB based housing structures in comparison with shrimp PLs from the tanks containing PVC housing structures (Table 3). The median weight and spread on the weight of shrimp PLs from the tanks with the PHB based

housing structures was 884.8 mg and 217.5 – 1875.9 mg, respectively, while the median weight and spread on the weight of the shrimp PLs from the tanks with the PVC housing structures was 941.1 mg and 488.2 – 2012.5 mg, respectively.

- 5 Table 3. Survival (%) and mean wet body weight of *L. vannamei* postlarvae grown with the control PVC substrates and experimental PHB substrates at the end of the experimental trial. Values are means $\pm$ standard error (n=7). Values in a row with different superscript letters are significantly different (T-test; $P < 0.05$).

10	Parameters	Substrate types	
		PVC	PHB
	Survival (%)	73.9 $\pm$ 3.0 ^a	83.6 $\pm$ 2.4 ^b
	Mean wet body weight (mg)	891 $\pm$ 31 ^b	1009 $\pm$ 29 ^a
	Average daily weight gain (mg day ⁻¹)	14.3 $\pm$ 0.5 ^b	16.4 $\pm$ 0.5 ^a
15	Population weight range (mg)	1658.4	1524.3

2.3 Substrate preference by the shrimp PLs

In figure 6, it can be observed that there was a trend of preference for the housing structure based on PHB in the experimental preference tanks. In case there was only 1 type of substrate present in the observation tanks, this preference was significantly higher at time points 1h, 6h, 9h, and 15h. In case both substrate types were in the same observation tank, this preference was significantly higher at time points 6h, 9h, 12h, and 18h.

25 **Example 3: The effect of using PHB as housing structure on the growth characteristics and survival of giant freshwater prawn (*Macrobrachium rosenbergii*) postlarvae.**

Similar to experiment 1 and 2, postlarvae of giant freshwater prawn are housed in tanks containing either PHB based housing structures (5 replicate tanks) or PVC based housing structures (5 replicate tanks) for a period of 45-60 days under the experimental and environmental conditions as described in example 1 and 2, with the difference that the water is at a salinity of 12 ppt instead of seawater. After this culturing period, the survival, length and weight of the shrimp postlarvae is determined. Based on these data, the homogeneity in growth of the shrimp (weight, length and/or average daily weight gain) is determined.

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- 5 Zhang et al. (2010) Effects of artificial substrates on the growth, survival and spatial distribution of *Litopenaeus vannamei* in the intensive culture condition. Iranian Journal of Fisheries Sciences 9, 293-304

Claims

1. A method to increase homogenous growth and/or to improve survival of crustaceans cultured in aquaculture tanks or ponds, comprising the addition of housing structures comprising the polymer poly- β -hydroxybutyrate into said aquaculture tanks or ponds.
- 5 2. A method according to claim 1 wherein said housing structure is made out of a polymer of pure poly- β -hydroxybutyrate, out of a co-polymer of poly- β -hydroxybutyrate with a polyhydroxyalkanoate other than poly- β -hydroxybutyrate, out of a blend of poly- β -hydroxybutyrate and/or a co-polymer of poly- β -hydroxybutyrate with a polyhydroxyalkanoate other than poly- β -hydroxybutyrate and/or another polymer, or, is
10 coated with one of the aforementioned polymers.
3. A method according to claim 2 wherein said other polymer is a non-bio-based polymer.
4. A method according to claim 2 wherein said other polymer is a bio-based polymer.
5. A method according to any of claims 1-4 wherein said crustaceans are shrimp of the Penaeidae family or prawns of the Palaemonidae family, or larvae or postlarvae of said
15 shrimp or prawns.
6. A method according to claim 5 wherein said shrimp of the Penaeidae family is *Litopenaeus vannamei* or *Penaeus monodon* or *Penaeus chinensis* or *Penaeus indicus* and wherein said prawn of the Palaemonidae family is *Macrobrachium rosenbergii*.
7. A method according to any of claims 1-6 wherein said housing structure is placed in the
20 water at a volumetric ratio of minimally $0,5 \times 10^{-7}$ and wherein said volumetric ratio is the volume of the housing structure material over the volume of water within the tank or pond.
8. A method according to any of claims 1-7 wherein said housing structure is applied in hatchery tanks, in nursery tanks or in grow out tanks or ponds.
9. A method according to any of claims 1-8 wherein said housing structure increases the
25 homogeneity in weight and/or in length, and/or, the average survival of said crustaceans when compared to crustaceans reared in systems containing non-poly- β -hydroxybutyrate - based housing structures.
10. A method according to any of claims 1-9 wherein said increase in homogeneity in weight and/or length means a decrease in range between the shortest/most lightweight crustacean
30 and the longest/most heavy crustacean of at least 8%.
11. A method according to any of claims 1-9 wherein said increase in average survival means a significant increase as determined by a statistical test at a p-value of 0.05 applied to compare the survival of said crustacean population raised in an environment containing said PHB-

based housing structures to the survival of said crustacean population raised in an environment containing non-PHB based housing structures.

12. A method according to claims 2-6 and claims 8-9 wherein said coated with one of the aforementioned polymers is coating the walls of the tanks in which the crustaceans are reared.

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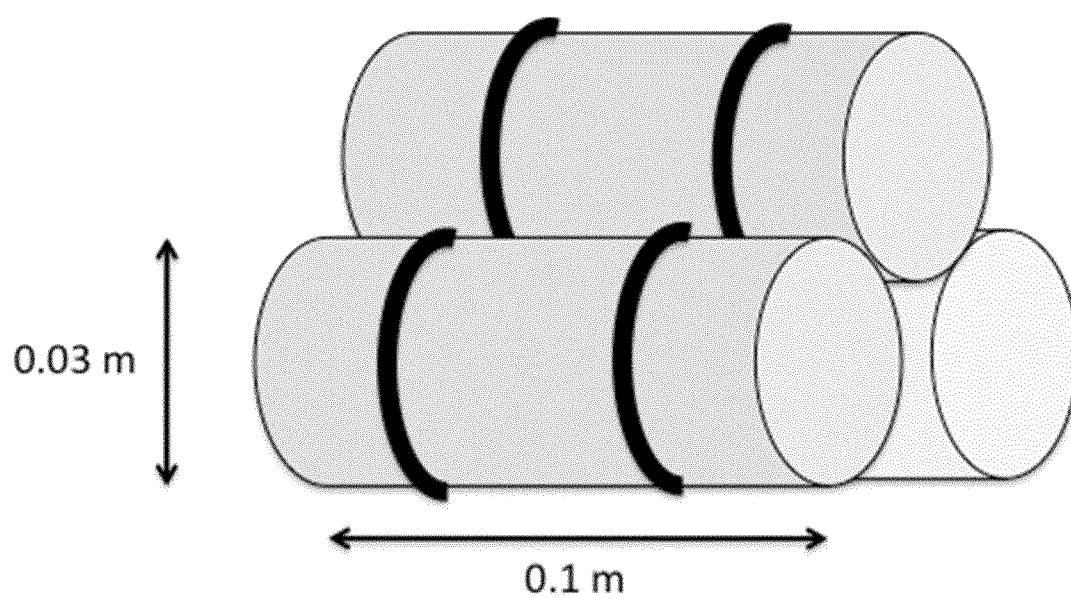


Figure 1

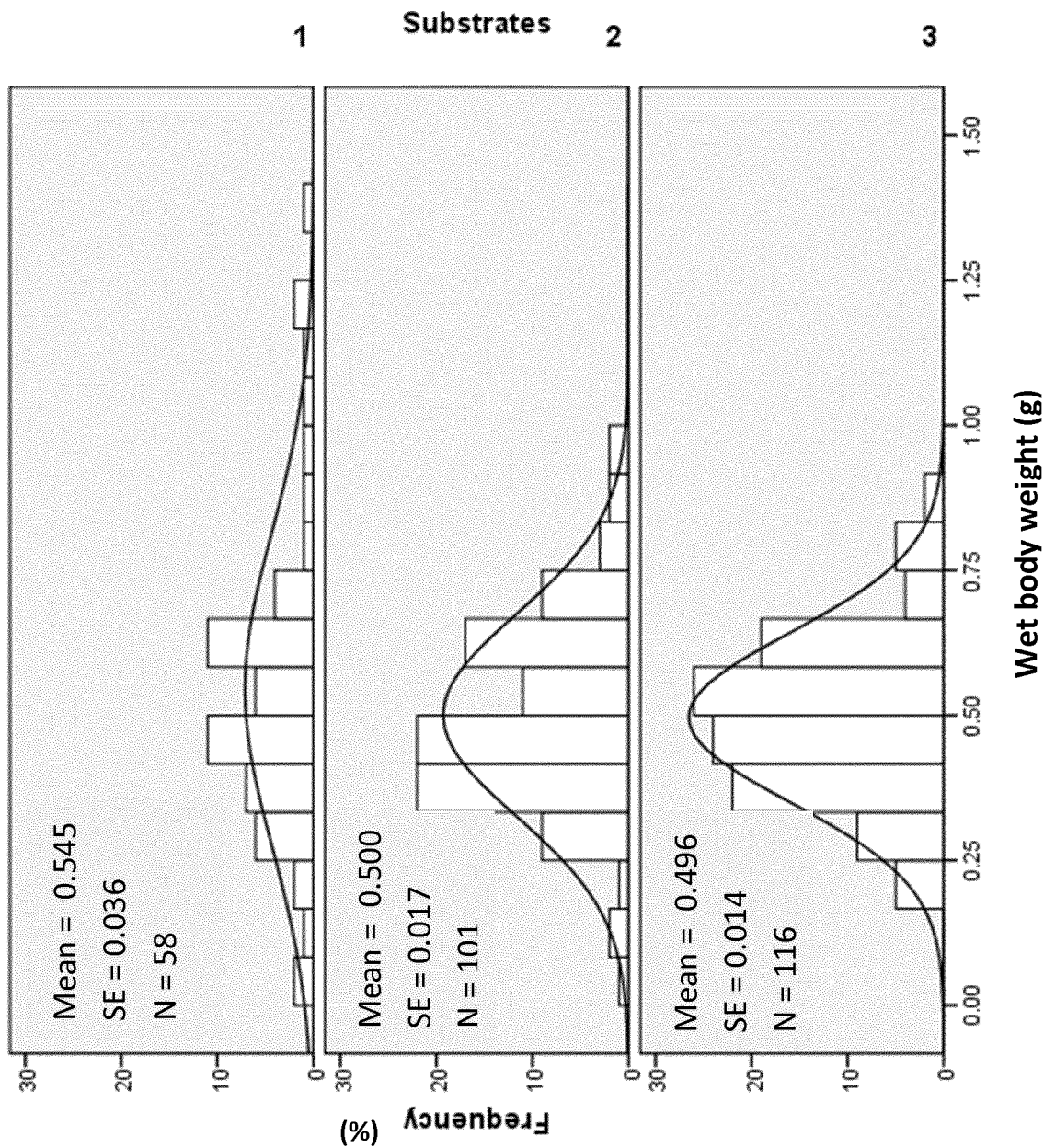


Figure 2

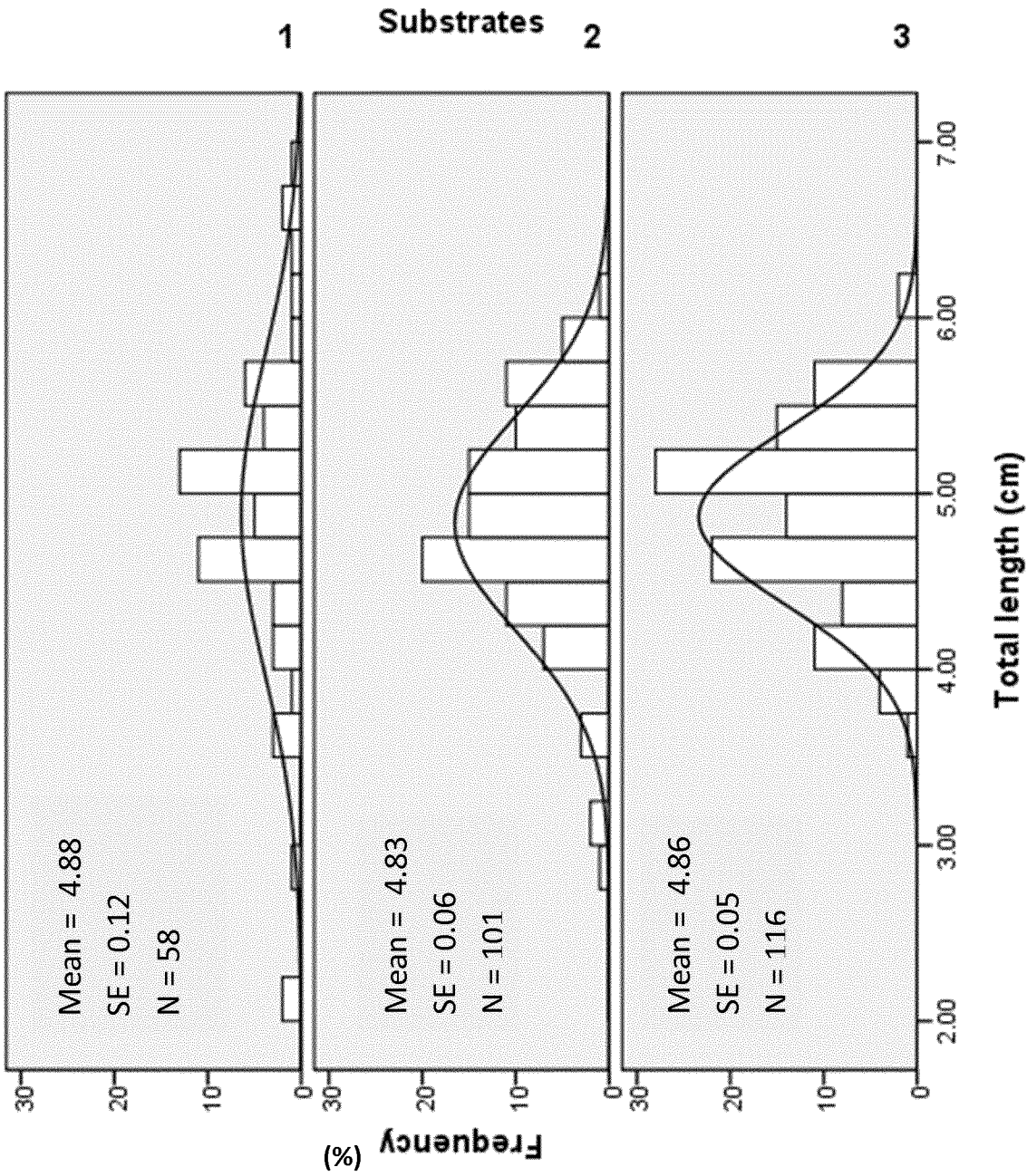


Figure 3

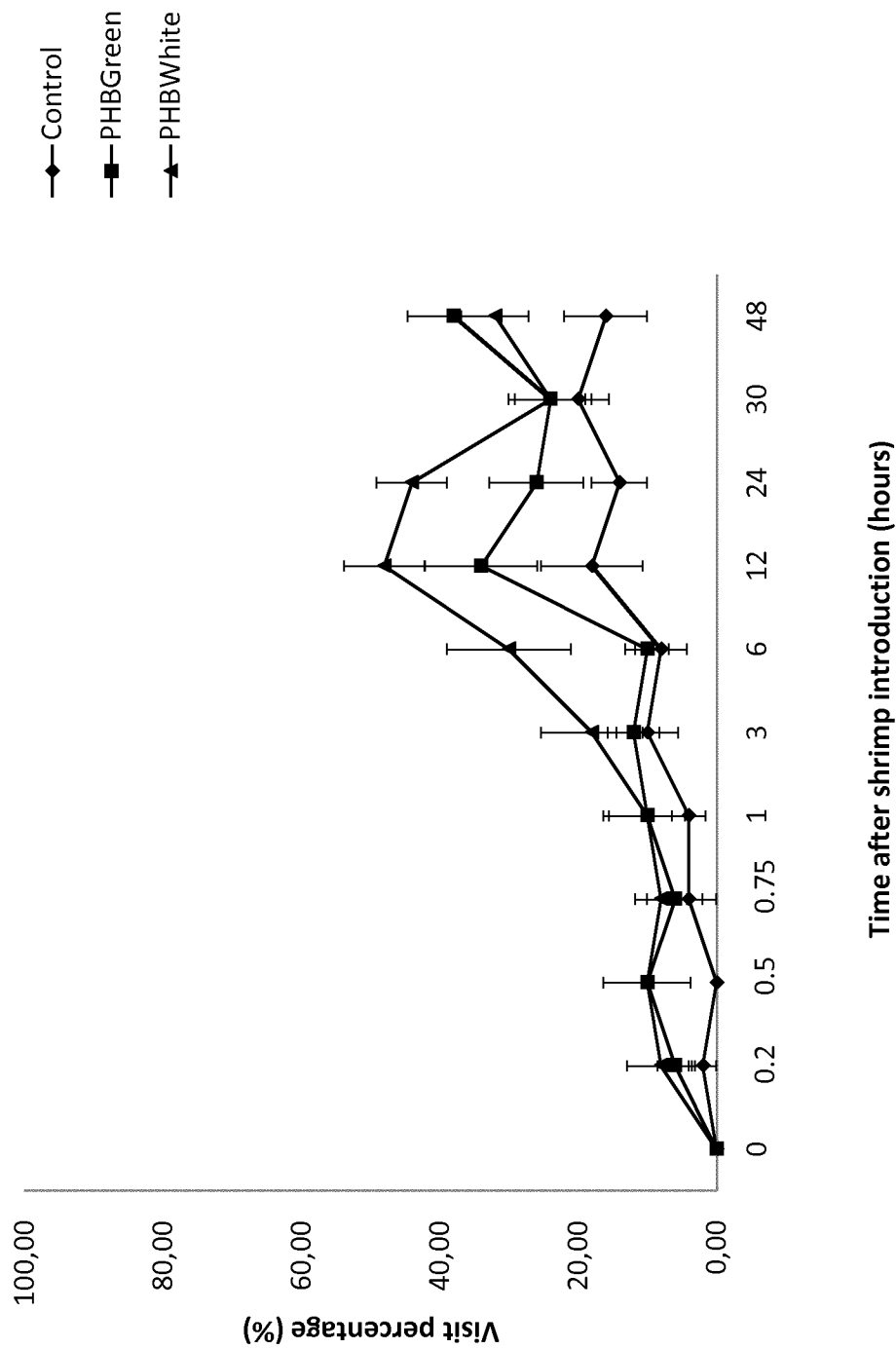


Figure 4

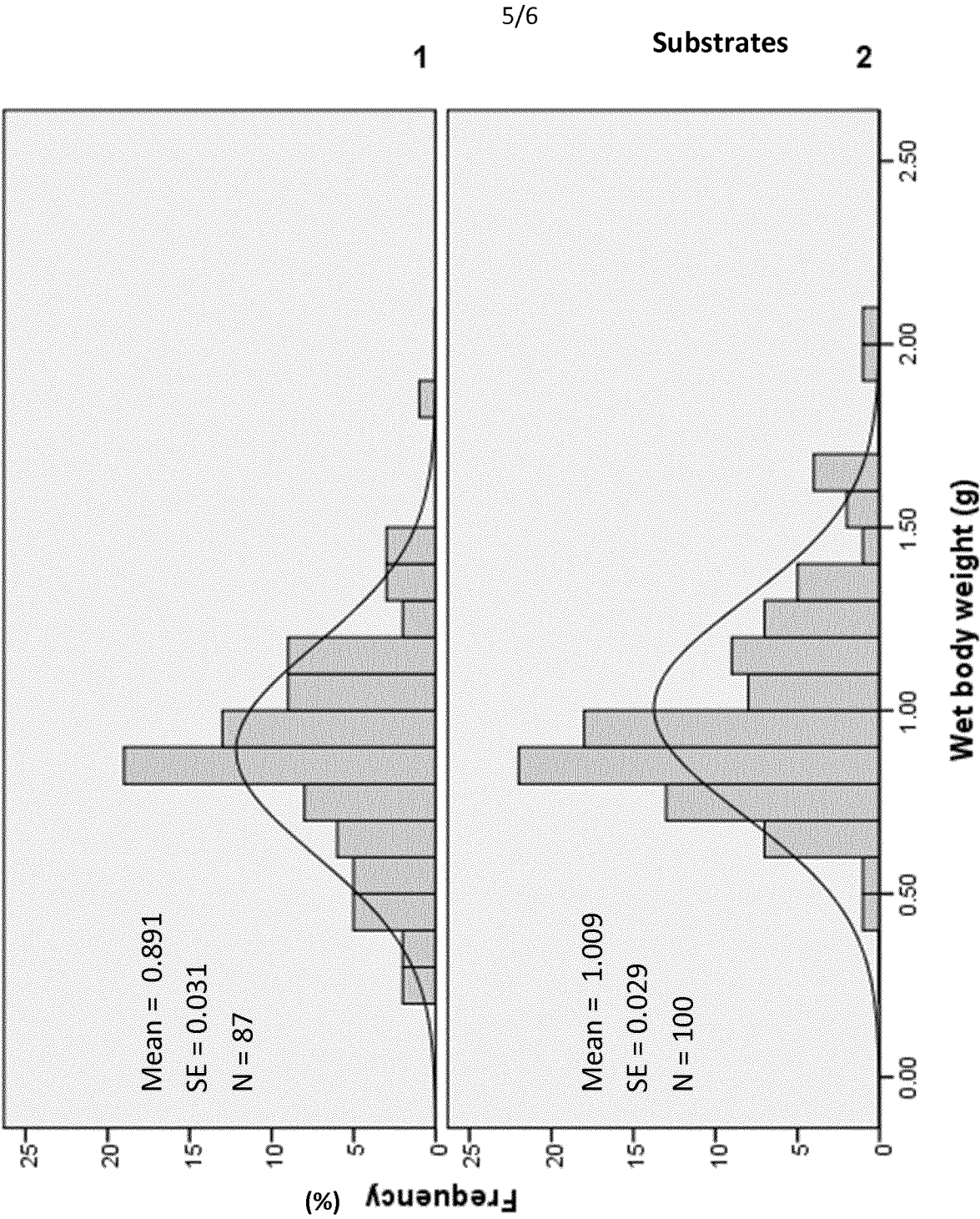


Figure 5

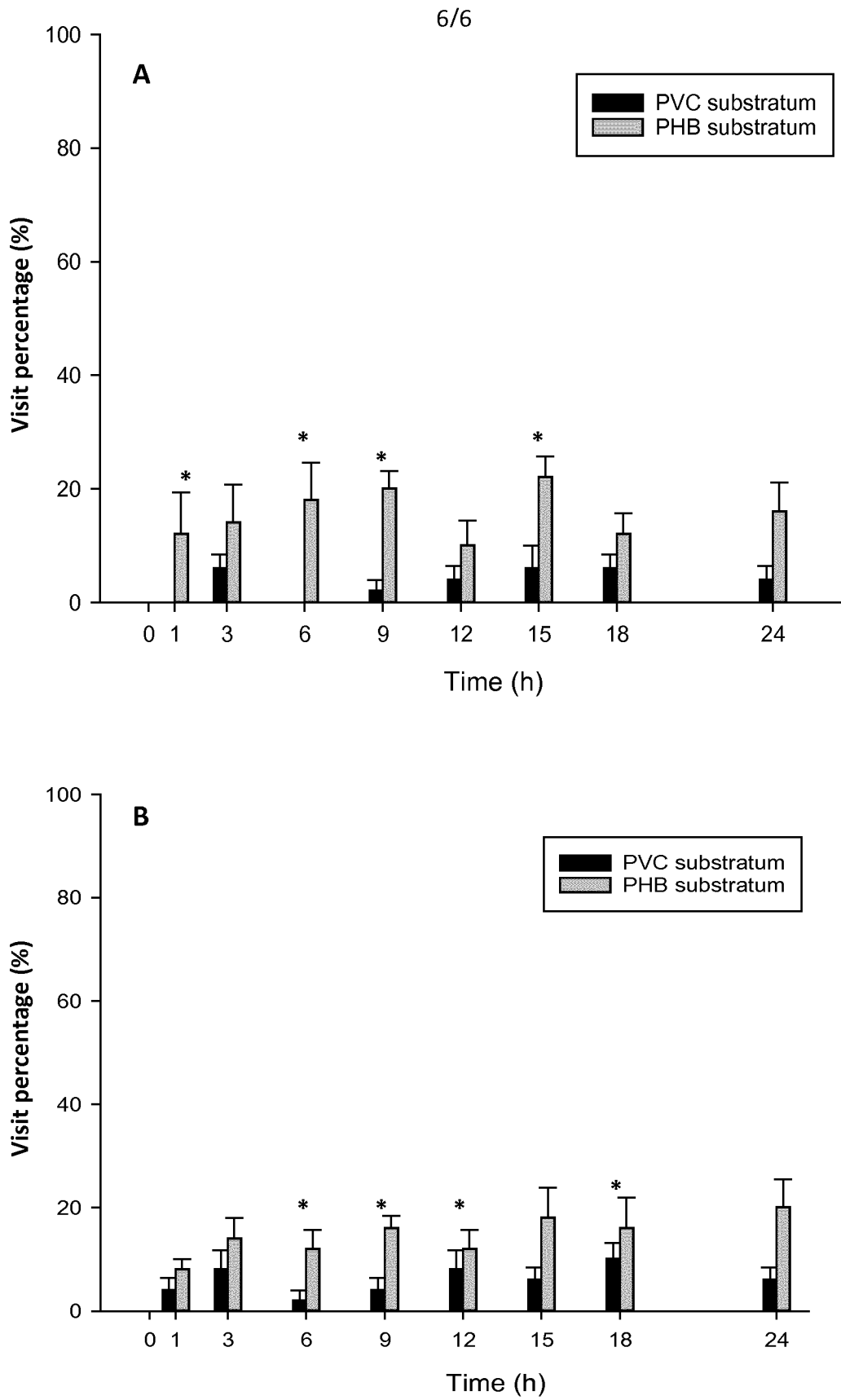


Figure 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/055788

A. CLASSIFICATION OF SUBJECT MATTER
INV. A01K1/02 A01K61/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2006/012670 A1 (BIOGRADE 999 PTY LTD [AU]; SCHEIRS JOHN [AU]) 9 February 2006 (2006-02-09) Whole doc., in particular p.8, l.15; p.11, l.5-18	1-12
A	US 2004/206696 A1 (RITTER GUNTER [DE]) 21 October 2004 (2004-10-21) para. [0009], [0106] ----- -/--	1-12



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

9 May 2016

Date of mailing of the international search report

17/05/2016

Name and mailing address of the ISA/

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Roscoe, Richard

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/055788

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	NHAN D T ET AL: "The effect of poly <2>-hydroxybutyrate on larviculture of the giant freshwater prawn Macrobrachium rosenbergii", AQUACULTURE, ELSEVIER, AMSTERDAM, NL, vol. 302, no. 1-2, 1 April 2010 (2010-04-01), pages 76-81, XP026976812, ISSN: 0044-8486 [retrieved on 2010-02-14] Whole doc., in particular Fig.2-5 -----	1-12
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2016/055788

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