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Relative contribution of natural productivity and compound feed to tissue growth in blue shrimp (*Litopenaeus stylirostris*) reared in biofloc: Assessment by C and N stable isotope ratios and effect on key digestive enzymes

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Abstract :

The aim of this study was to assess the relative contribution of natural productivity and compound food to the growth of the juvenile blue shrimp *Litopenaeus stylirostris* reared in a biofloc system. Two experiments were carried out based on the same protocol with three treatments: clear water with experimental diet (CW), biofloc with experimental diet (BF) and biofloc unfed (BU). Shrimp survival was significantly higher in biofloc rearing than in CW rearing. The contribution of the biofloc to shrimp diet was estimated through measurement of carbon and nitrogen stable isotope ratios in shrimp and food sources. Different isotopic compositions between feeds were obtained by feeding natural productivity with a mixture rich in fish meal and the shrimps with a pellet containing a high level of soy protein concentrate. Using a two source one-isotope mixing model, we found that the natural productivity of the biofloc system contributed to shrimp growth at a level of 39.8% and 36.9%, for C and N, respectively. The natural food consumed by the shrimps reared in the biofloc system resulted in higher gene expression (mRNA transcript abundance) and activities of two digestive enzymes in their digestive gland: α-amylase and trypsin. The growth of shrimp biomass reared in biofloc was, on average, 4.4 times that of those grown in clear water. Our results confirmed the best survival and promoted growth of shrimps using biofloc technology and highlighted the key role of the biofloc in the nutrition of rearing shrimps.

Statement of relevance

In this study, we have applied an original protocol to determine the respective contribution of natural productivity and artificial feeds on the alimentation of the juvenile blue shrimp *L. stylirostris* reared in biofloc system by using C and N natural stable isotope analysis. Moreover, we have compared, in shrimp digestive gland, the α-amylase and trypsin enzyme activities at biochemical and molecular levels for two different shrimp rearing systems, biofloc and clear water. In our knowledge, the use of molecular tool to study the influence of biofloc consumption on digest process of shrimp was never carried out. We think that our research is new and important to increase knowledge on biofloc topic.

Highlights

► Biofloc system promotes growth and survival of shrimps compared to clear water systems. ► Natural productivity contributes to shrimp growth for 39.8% and 36.9%, for C and N, respectively. ► Biofloc shrimps showed higher gene expression and activities of digestive enzyme compared to clear water shrimps.

Keywords : Biofloc, Shrimps, Stable isotope, Digestive enzyme activities, Digestive enzyme mRNA level

1. Introduction

Biofloc Technology (BFT) is a rearing system with zero or minimal water exchange which provides two critical services: recycling nutrients and maintaining the water quality and excretion, and providing supplementary food particles from biofloc (Burford *et al.*, 2004; Avnimelech, 2009). It is assumed that shrimp can consume the biofloc particles that are considered an important and complementary natural food source, leading to an improvement of the shrimp growth rate (Moss and Pruder 1995; Epp *et al.*, 2002; Tacon *et al.*, 2002; Burford *et al.*, 2004), and to a better feed efficiency (Browdy *et al.*, 2001; Schneider *et al.*, 2005; Wasielesky *et al.*, 2006; Hargreaves *et al.*, 2006). Moreover, a few authors have reported that natural productivity stimulates the activity of digestive enzymes (Xu and Pan, 2012, Xu *et al.*, 2013). However, despite evidence for the role of natural productivity in the nutrition of shrimps, very few studies have been carried out in order to quantify this role for shrimp reared in BFT (Ray, 2012).

In aquaculture, stable isotope analyses, considered a non-hazardous and non-invasive tool, can be used to estimate the relative contribution of different food sources (Phillips and Gregg, 2001; Gamboa-Delgado *et al.*, 2008). This method has already been used to evaluate the contribution of natural productivity within extensively or semi-intensively reared shrimp in earthen ponds (Anderson *et al.*, 1987, Parker *et al.*, 1989, Cam *et al.*, 1991) and also within net cages (Abreu *et al.*, 2007). However, in a biofloc system, there is a limitation of the stable isotopes method because the isotopic value of food sources should be precisely known and the difference between the isotopic signatures of different food sources must be sufficiently marked (Epp *et al.*, 2002). To overcome this limitation, the use of specific diets, especially live feeds, enriched or labelled with very high levels of ^{13}C or ^{15}N, have been applied as an alternative to radiolabels (Epp *et al.*, 2002; Burford *et al.*, 2004; Avnimelech, 2007; Avnimelech and Kochba, 2009). Cam *et al.* (1991) used natural stable isotopes to investigate the relative contribution of different food sources in an earthen pond system, although this method has the advantage, compared to the enriched isotopes method, of integrating measures of ingestion, assimilation and growth over longer time periods under normal feeding and environmental conditions (Le Vay and Gamboa-Delgado, 2011).

The aim of our study, achieved through two complementary experiments, is to assess the relative shares of the natural food from BFT and the compound diet of the juvenile blue shrimp *L. stylirostris*; this assessment was performed by crossing the isotope method by using C and N natural stable isotope analysis with a comparative method of two rearing systems: BFT and clear water. Under these conditions, we also compared the α-amylase and trypsin enzymes activities in the digestive gland of the reared shrimp in both rearing systems using biochemical and molecular methods.

2. Material and methods

2.1. Experiment 1: Relative dietary share of the compound feed and natural productivity of juvenile shrimp *Litopenaeus stylirostris* reared in biofloc rearing technology.

2.1.1. Shrimps and acclimatisation tanks

The 30 day-old shrimp post-larvae (*Litopenaeus stylirostris)* were obtained from the Ifremer hatchery (Saint-Vincent station, New Caledonia). Prior to experimentation, shrimps underwent an acclimatisation period of two weeks; for this purpose, they were stocked in 4000L tanks (height: 0.9m; diameter: 2.7m) and reared in clear water (100% renewal water

rate per day). The shrimp were fed with commercial feed (SICA© grower40) three times a day.

2.1.2. Biofloc preparation

The biofloc culture was established 30 days before the experiment with adult shrimps (mean weight: 20 g; biomass: 500g.m^{-2}). Shrimp were fed twice a day with commercial shrimp feed (SICA© grower40), and were removed before the beginning of the experiment. Each outdoor tank was continuously aerated with blown air delivered through a stone diffuser and were covered with shade nets to control the sunlight (70% inhibition of light).

2.1.3. Experimental Feed

The shrimps were fed an experimental diet formulated with a high level of soy concentrate protein (SCP) as the main source of protein. The diets were produced in the laboratory using the following procedure: the dry ingredients were ground in a grinder (Retsch®) with a 1mm screen. The meal obtained was mixed with oil and water (30%) in a horizontal mixer (Mainca®) until the consistency was suitable for pelleting. The mixture was then extruded through a 3 mm die in a meat grinder to form spaghetti. The spaghetti obtained was then dried in oven (Venticell® 222) at 60°C until the moisture content was reduced to 10%. After drying, the spaghetti was broken into 2 to 3 mm long pellets. The ingredient compositions of the experimental diet are shown in Table 1.

2.1.4. Experimental design and sampling

Three treatments were tested for a period of 30 days: 1) clear water + experimental pellet diet (CW); 2) biofloc + experimental pellet diet (BF) and 3) biofloc - unfed (BU). Each of the 12 outdoor circular polyester tanks (capacity: 536 litres; height: 0.7m; diameter: 1.2m) under a shade net (70% inhibition of light) were randomly assigned to a particular treatment (CW, BF or BU), such that each treatment contained 4 replicate tanks. In our study, the experimental unit is therefore represented by the tank.

Shrimp were caught in acclimatisation tanks (δ 2.1.1) using a cast net, and were then transferred and randomly distributed into the 12 experimental tanks. One hundred individuals (0.24 ± 0.04g) were placed in each tank (200 shrimps.m^{-2}). Each tank was continuously aerated with blown air delivered through a stone diffuser. The water renewal rate was of 300% per day in the CW treatment and 0% in the BF and BU treatments.

In order to obtain different isotopic signatures of two potential dietary sources: biofloc *vs.* experimental diet, we enriched biofloc with finely ground feed rich in fish meal with a high isotopic signature and fed the shrimp with the pelleted diet with soybeans as the main source of protein with a low isotopic signature. The feed used to enrich the natural productivity of biofloc treatments (BF and BU) was commercial shrimp feed (SICA© grower40) which was finely ground, sifted (<50 µm) and aerated in sea water for 24 hours previously to be distributed in the biofloc tanks, to prevent its direct consumption by the shrimps. The daily amount of food in solution (dry matter basis) to enrich biofloc represented 2% of the estimated shrimp biomass. In parallel, and with the exception of the unfed treatment (BU), shrimps were fed *ad libitum* with the experimental diet (§ 2.1.2). Experimental feed was supplied to shrimps three times a day (07:00 am, 01:00 pm and 05:00 pm) using feeding trays which were checked 2 hours after feeding; any unconsumed feed was removed.

Sampling for isotope analysis by mass spectrometry – Shrimps muscle and biofloc were sampled on day 0 and then once a week for the entire duration of the trial (4 times). For each muscle sample, ten shrimps were randomly taken from each of the 12 experimental tanks: the shrimps from each tank were pooled for analysis. Sampled shrimps muscle are rinsed with filtered seawater before being frozen for analysis. Biofloc was filtered through a 100µm

mesh from each biofloc tank, frozen in liquid nitrogen and kept at -80°C until analysis (2g of of wet biofloc particles were sampled, approximately). Furthermore, three samples of experimental diet were collected for isotope analysis.

2.1.5. Water quality analysis

Temperature and dissolved oxygen were recorded twice per day (08:00 am and 03:00 pm) with OxyGard Handy Gamma. The pH and salinity were recorded once per day (08:00 am) with a pH meter (Hach Lange HQ 40D) and conductimeter (WTW cond 315i), respectively.

Total ammonia nitrogen (NH_4^+-N) and nitrite-nitrogen (NO_2^--N) were analysed twice per week by a fluorescence method according to Holmes *et al.* (1999) and a spectrophotometric method according to Bendschneider and Robinson (1952).

Total suspended solid (TSS) were also measured twice weekly: water samples (50mL) were collected from each tank and filtered under vacuum pressure through pre-dried and pre-weighed GF/C filter paper (FW1). The filter paper containing suspended materials was dried in an oven until a constant weight was achieved (at 60°C for 24h). Dried samples (FW2) were weighed to 0.01 mg using a Mettler AC 100 balance. TSS is the difference between FW1 and FW2.

Chlorophyll a (Chl a) was determined using a spectrophotometer (Trilogy Turner Design) at wavelengths of 664 and 750nm, following the method of Holm-Hansen *et al.* (1965).

2.1.6. Stable isotope analysis and calculation

Analysis - All samples, centrifuged biofloc, shrimps and experimental diet, were freeze-dried and ground to a fine homogenous powder using a ball mill (Retsch MM400; Haan, Germany). Samples (0.3mg DW) packed in tin-capsules., were analysed with a continuous flow isotope ratio mass spectrometer (Delta V Advantage IRMS; Thermo Scientific, Bremen, Germany), coupled with an elemental analyser (Flash EA 1112 Thermo Scientific, Milan, Italy). Stable isotope results are presented as differences from universal reference standards (Vienna Pee Dee Belemnite for carbon, and Air N2 for nitrogen) using the following equation:

$$\delta(‰) = (R_{sample} - R_{standard})/R_{standard} \times 1000$$

where R is the ratio of heavier to light isotope ($^{13}C/^{12}C$ or $^{14}N/^{15}N$) of the sample and standard, respectively. Analytical precision based on an internal standard (acetanilide) interspersed among samples is <0.1‰ for C and N. C and N contents of samples were also measured and presented as % by weight.

Calculation – When the consuming organism reaches isotopic equilibrium with its diet (at the end of experimentation in our study), a difference in isotope values between the consumer and its diet is usually observed. Indeed, when an animal consumes a food item, that animal typically retains a greater portion of heavy C and N isotopes compared to the food item; this is a result of isotopic fractionation (Fry, 2006), thus producing an isotope discrimination factor, $\delta^{13}C$ (or $\delta^{15}N$) $_{body-diet}$.

To calculate this factor, the equation following was used: $\Delta\ (\%) = \delta_{tissue} - \delta_{diet.}$

Thus, the relative contribution of the two nutritional sources (biofloc *vs* experimental diet) can be determined using a two-source, one-isotope mixing model from Phillips and Gregg (2001). One of the model assumptions is that the consuming organism is in isotopic equilibrium with its diets. After a constant isotopic difference between the diet and shrimp was reached,

isotope values were corrected for discrimination factors before introduction into the mixing model. This was done by introducing the isotopic profiles of both shrimps fed only natural productivity and formulated feed into the model in order to calculate the relative contribution of the two nutritional sources in the co-feeding treatment. Carbon and nitrogen content in the natural productivity and formulated feed were significantly different: therefore, according to Fry (2006) and Gamboa-Delgado *et al.* (2008), in order to calculate the total amount of carbon and nitrogen contributed by each feeding source, the following equation was used:

$f_{total1} = (f_1.W_2)/(f_1.W_2 + f_2.W_1)$ and $f_{total2} = 1 - f_{total1}$

where f_{total1} = is the total percent contribution of source 1 in a two source mixing model,

$f_1 = (\delta^{13}C_{shrimp\ sample} - \delta^{13}C_{source2})/(\delta^{13}C_{source1} - \delta^{13}C_{source2})$ and $f_2 = 1 - f_1$

W_1 and W_2 represent the percent carbon content in each of the two sources.

The calculation procedures for N were the same as those for C.

2.2. Experiment 2: comparing the activity of digestive enzymes of juvenile shrimps *Litopenaeus stylirostris* grown in clear water and biofloc

The objective of this experiment was to compare shrimp grown in biofloc or in clear water systems for their α-amylase and trypsin activities and their corresponding transcripts abundance.

2.2.1. Shrimps and acclimatization

The 12 day-old shrimp post-larvae (*Litopenaeus stylirostris*) were obtained from the hatchery of the Aquaculture Technical Centre of Tahiti (Vairao, French Polynesia). Prior to experimentation, shrimps underwent an acclimatisation period of two weeks; for this purpose, they were stocked in 25 000L tanks (height: 1m; diameter: 5.74m) and reared in clear water (100% of renewal water rate a day). These shrimps were fed with commercial feed three times a day (SICA© grower40).

2.2.2. Experimental design and sampling

The experimental protocol was similar to that of the first experiment. The difference here was that the animals grown in clear water or in biofloc were fed with commercial feed (SICA© grower40), while biofloc received no specific enrichment. The protocol of the second experiment was as follows: three treatments were tested for a period of 30 days: 1) clear water + commercial pellet (CW); 2) biofloc + commercial pellet (BF) and 3) biofloc - unfed (BU). Each of the 12 outdoor circular polyester tanks (capacity: 250 litres; height: 0.7m; diameter: 0.8m) were randomly assigned to a particular treatment (CW, BF or BU), such that each treatment contained 4 replicate tanks. In our study, the experimental unit is therefore represented by a tank.

Shrimps were caught in acclimatisation tanks (§ 2.2.1) using a cast net, transferred and randomly distributed into the 12 experimental tanks. One hundred individuals (0.06 ± 0.02g) were placed in each outdoor tank (400 shrimps.m^{-2}). Each tank was continuously aerated with blown air delivered through a stone diffuser. The water renewal rate was of 300% per day in CW treatment and 0% in BF and BU treatments. The biofloc culture was established in the same manner as in experiment 1 (see § 2.1.2). Shrimps were fed *ad libitum*, with the exception of the unfed treatment (BU), with the commercial feed (SICA© grower40). Feed was supplied to shrimp three times a day (07:00 am, 01:00 pm and 05:00 pm).

Shrimp sampling was carried out on the last day (day 30) of the experiment. Digestive glands of shrimps were collected at different times of the day: before feeding (06:00 am), and one and three hours after feeding (08:00 am and 10:00 am, respectively). During the sampling day, feeding was carried out using a feeding tray which was collected 30min after the meal and the unconsumed food was discarded. At each of the sampling times, three shrimps per tank were caught and directly put in iced sea water (0°C) to stop the enzyme activity. Only shrimps in intermoult were used. Moulting stages were determined by microscopic examination of antennal scales according to the method of Drach and Tchernigovtzeff (1967). Then, the digestive glands (DGs) were removed and each one was separated into two longitudinal parts which were pooled for the 3 shrimps. For molecular analysis, DGs were immediately pooled in RNA later (Sigma®), refrigerated at 4°C for 12 hours and kept at -80°C until analysis. For biochemical analysis, DGs were immediately pooled and frozen in liquid nitrogen. A sample of biofloc was obtained by filtering through a 100µm mesh from each biofloc tank; concentrated biofloc was frozen in liquid nitrogen and kept at -80°C until analysis.

2.2.3. Water quality analysis

Water quality analysis was carried out in the same manner as experiment 1 (see paragraph 2.1.5).

2.2.4. Molecular analysis

Total RNA of digestive glands sampled during the second experiment was extracted using Trizol method (Invitrogen, USA) according to the manufacturer's instructions. The quantity and quality of each RNA sample were assessed by measuring their absorbance at 260 and 280 nm using a Nanodrop 1000 Spectrophotometer (Thermo Scientific) associated with the ND-1000 V3 7.0 software. A DNAse treatment to remove residual DNA was carried out using the Ambion DNA free kit, following the manufacturer's instructions. First-strand cDNA was synthesised with 500ng total RNA in each reaction system using the Roche transcriptor first strand cDNA synthesis, according to the manufacturer's protocol. All cDNA samples were diluted to 1/100 with nuclease-free water and stored at -20°C until they were used as templates in real-time quantitative PCR (qRT-PCR).

Primers for the amylase (Amy) and trypsin (Try) genes of *L. stylirostris* were designed by alignment of highly conserved regions from sequences registered for other shrimp species in GenBank (Table 2). Real-time qRT-PCR was carried out in an Stratagene Mx3000P machine (Agilent Technologies) using Brilliant® II SYBR® Green QPCR Master Mix (CAT#600828 - Agilent Technologies) following the manufacturer's recommendations. The reaction mixtures were in a volume of 25µL containing, 12.5µL SYBR Premix, 10µL cDNA (diluted of 1/100), and 1.25µL each of the 4µM forward and reverse primers. After initial denaturation at 95°C for 10 min, 40 cycles of amplification were carried out starting at 95°C for 30s, followed by 45s at 57°C, and 45s at 72°C, with a final extension at 95°C for 1 min, 30sec at 55°C and 95°C for 30s.

To determine the RT-PCR efficiencies of each primer pair used, standard curves were generated using five serial dilutions of a pool of one hundred cDNA samples from hepatopancreas. Primer ability was been validated when amplification efficiency varied between 90 and 110%. All analyses were run in duplicate. Relative gene expression levels were normalised to two specific house-keeping genes: Elongation factor (EF) and glyceraldehyde-3-phosphate-deshydrogenase (GADPH) (Table 2); each value was calculated with reference to CW shrimps before the first meal (relative expression = 1) according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.2.5. Biochemical analysis

All of the parameters were determined by biochemical assays with the microplate reader (Bioteck®). Prior to analysis, tissue and biofloc particles sampled during the second experiment were homogenised in Tris buffer 10 mM, 1 mM DTPA, pH 7.4. The homogenates were centrifuged at 4000 rpm for 10 min at 4°C and the supernatants kept at -80°C until they were analysed.

Proteins were estimated according to Bradford (1976), with bovine serum albumin as the standard. Trypsin was assayed by amidase activity using benzoyl-Arginine-p-nitroanalide (BAPNA) as a substrate following the methods of Erlanger *et al.* (1961) and García-Carreño *et al.* (1994). Assays were initiated by the addition of sample supernatant, and the release of p-nitroanalide was measured at 410 nm over 15 min. A positive control of 3 $mg.ml^{-1}$ trypsin (Sigma) was used. One activity unit was expressed as 1 µmol of p-nitroanilide released min^{-1}. The α-amylase activity was assayed by Bernfeld's (1955) method, using 1% soluble starch (Sigma) as a substrate in phosphate buffer 20 mM, pH 7, and reacting with 3.5-dinitrosalicylic acid. One unit of enzymatic activity was defined as 1 mg of maltose liberated in 15 min at 37°C. Units of specific enzyme activities were expressed in U.mg of $protein^{-1}$.

2.3. Statistical analysis

Statistical analysis of the data was carried out using *XLSTAT* software 2012. Percent data (survival rate) were normalised using an arcsine transformation before analysis. Normality of data distribution and homogeneity of variance were tested for zootechnical, isotopic, gene expression and activities data using the Shapiro-Wilk test and F-test. Zootechnical, gene expression and activities data were normally distributed and variances were homogenous. Hence, the effects of rearing treatments were tested using a one-way analysis of variance. Pairwise comparisons within the three rearing treatments were carried out using Tukey tests. Differences were considered significant at the level $p < 0.05$. Isotopic data were not normally distributed; the effects of rearing treatments were tested using the Kruskal Wallis test. Pairwise comparisons within the three rearing treatments were carried out using Dunn tests. Differences were considered significant at the level $p < 0.05$. Correlations between gene expression and activities data were tested using the critical value table for Spearman's rank correlation coefficient rho at the 5% alpha level.

3. Results

3.1. Zootechnical results (Table 3).

Experiment 1: Survival rates were significantly different between the 3 treatments with the lowest rate for CW treatment and the highest rates for the biofloc treatments. Compared to CW treatment, the survival rates were 30% and 45% higher in BU and BF treatments, respectively; the BU treatment exhibits an intermediate survival rate. Growth rate, Final weight and weight gain follow the same trend as the treatments: we observed an increase from CW to BF treatment, with BU treatment being in an intermediate position.

Experiment 2: As for the first experiment, survival rates were significantly different between the 3 treatments with the lowest rate for CW treatment and the highest rates for the biofloc treatments. Compared to CW treatment, the survival rates were 100% and 136% higher in BU and BF treatments, respectively. Growth rate, final weight and weight gain followed the same trend as that observed in the previous experiment with an increase from CW to BF treatment; BU treatment was in an intermediate position. However, the difference between the CW and BF treatments was much more pronounced in experiment 2.

3.2. Water quality analysis

Descriptive statistics of water parameters are given in Table 4. For the two experiments, no difference were observed for pH values, salinity, temperatures or dissolved oxygen concentrations between the treatments. Furthermore, all of the parameters measured were within acceptable ranges for the *Litopenaeus stylirostris* shrimp (Della Patrona and Brun, 2008). The concentration of TAN and NO_2^-–N were maintained at a low level in the BFT system during the rearing period. The biofloc productivities in terms of TSS level were near of $0.3 g.L^{-1}$ over the course of the experiment for the two experiments. In the BFT tanks, Chlorophyll a values were relatively high, indicating significant primary production.

3.3. $\delta^{13}C$ and $\delta^{15}N$ values and estimated biofloc contribution in the diet of shrimps

The results presented here are from Experiment 1, in which juvenile shrimps increased their body weight, according the treatment, by between 250% and 492% (Table 5).

The carbon (C) and nitrogen (N) concentration and both the $\delta^{13}C$ and $\delta^{15}N$ values of the two dietary sources, biofloc and experimental diet, are shown in Table 5. The mean C and N concentration and values of $\delta^{13}C$ and $\delta^{15}N$ obtained for biofloc were similar between the two treatments, BU and BF, but a significant difference was observed between biofloc and experimental diet ($p<0.0001$).

The evolution of both $\delta^{13}C$ and $\delta^{15}N$ values in muscle during the experiment are shown in Figure 1. The linearisation of the curves for the both isotopic ratios studied $\delta^{13}C$ and $\delta^{15}N$ is obtained from the 21st day of the experiment. Indeed, no significant differences were found for $\delta^{13}C$ and $\delta^{15}N$ values between the day 21 and day 28 for the three treatments ($p>0.05$ for C and N according to the three treatments). This result suggests that the isotopic equilibrium was reached from that point.

The isotopic discrimination factor (Δ = δmuscle-δdiet) calculated for both food sources, experimental diet and biofloc, shows that the dietary source affected the $\Delta^{13}C$ with values of 3.69 ± 0.12 and 2.48 ± 0.43 ($p=0.009$) and $\Delta^{15}N$ with values of 5.59 ± 0.19 and 2.77 ± 0.08 ($p=0.05$), for experimental diet and natural productivity, respectively.

The isotopic mixing model estimates the relative contribution of C and N from natural productivity and formulated feed to tissue growth (see 2.1.6). According to the model, 39.8% of the carbon and 36.9% of the nitrogen may have originated from the biofloc. Conversely, carbon and nitrogen from the experimental feed contributed to muscle growth by 60.2% and 63.1%, respectively.

3.4. Comparison of digestive enzyme activities and their gene expression in digestive gland of shrimps from treatments BFT and CW

The results presented here are from Experiment 2. Relative gene expression level and specific enzymatic activity means of three individual values of samplings (before meal, 1h and 3h after meal) are shown in Table 6. The relative expression in the digestive gland of shrimps from BF treatment exhibited a significantly higher level for α-amylase ($p<0.0001$) and for trypsin ($p=0.03$) compared to both of the other treatments, BU and CW. The same trend was observed for enzymatic activities, where α-amylase and trypsin activities in the digestive gland of shrimps from both biofloc treatments were two-fold higher than for shrimps from CW treatment ($p<0.0001$ for the both enzymes).

The evolution of digestive activities' enzyme levels and their relative expression over the course of the feeding trial is shown in Fig 2 and 3, respectively. Both trypsin (Fig 2A) and α-amylase (Fig 2B) activities presented the same trend; they did not change over time for BU

and BF treatment ($p>0.05$), while they increased significantly for CW treatment ($p=0.006$ for α-amylase; $p=0.015$ for trypsin) one hour after feeding, and then returned to the base level 3 hours after the meal. Furthermore, a treatment effect was observed: trypsin gene expression level (Fig 3A) was higher 1 hour ($p=0.002$) and 3 hours ($p=0.04$) after the meal for BF treatment compared to BU and CW treatment.

The α-amylase expression level (Fig 3B) was higher before the meal ($p=0.03$) and 3 h after the meal ($p=0.03$) for BF treatment compared to the other two treatments. However, no correlation was found between mRNA transcript abundance and digestive enzyme activities (for α-amylase $p=0.525$; for trypsin $p=0.101$). Finally, in the biofloc fraction we measured significant trypsin and α-amylase activities of 0.06 ± 0.02 $U.mg^{-1}$ (dry matter basis) and 0.54 ± 0.28 $U.mg^{-1}$ (dry matter basis), respectively.

4. Discussion

The present study with *Litopenaeus stylirostris* confirmed the beneficial role of BFT in promoting the growth and survival of reared shrimp, as has been shown by several authors for *Litopenaeus vannamei* and *Penaeus monodon* (Moss and Pruder 1995; Epp *et al.*, 2002; Tacon *et al.*, 2002; Burford *et al.*, 2004). Although the underlying mechanisms of BFT in promoting shrimp growth are largely unknown, it is expected that the beneficial effect of BFT has several interrelated causes.

In this frame, we studied the role of biofloc in the nutrition of shrimp by using natural C and N stable isotope analysis to quantify the biofloc (natural productivity) contribution to the carbon and nitrogen pool used for the growth of juvenile *Litopenaeus stylirostris.* The role of biofloc in shrimp nutrition was also assessed by comparing the α-amylase and trypsin enzymes activities at the biochemical and molecular levels, in the digestive gland of shrimp reared in clear water and in biofloc.

The trophic fractionation of isotopes is assumed to be relatively constant: about 3.4‰ per trophic level for nitrogen and 1‰ for carbon (De Niro and Epstein, 1978; Schroeder, 1983; Fry and Sherr 1984). However, this discrimination factor depends on animal species, development stage, tissues considered, and diet composition (Lochmann and Phillips, 1996). Gamboa-Delgado and Le Vay (2009) showed that discriminating factors for N range from 0.8 to 6.6, and for C range from 2.3 to 4.1, according to the inclusion rate of fish meal and soy protein concentrate in the diet for the shrimp. In our study, distinct discriminating factor was found between formulated feed and biofloc. The different dietary assimilation, excretion rate and protein quality of the two dietary sources could explain this difference (Olive *et al.*, 2003; Gamboa-Delgado *et al.*, 2008: Roth and Hobson, 2000; Waddington and MacArthur, 2008). In terms of experimentation, the existing isotopic fractionation between the animal and its food makes difficult the distinction between food sources in closed culture conditions where animals have simultaneous access to different sources of food that may have different discrimination factors. To circumvent this problem, in our study, we separated the treatments with one side using shrimps consuming only biofloc (BU) and the other with shrimps consuming only experimental diet (CW). Moreover, in order to clearly discriminate the isotopic signatures between the two food sources, we based our experimental protocol on the fact that different ingredients in shrimp feeds not only had diverse $\delta^{13}C$ or $\delta^{15}N$ values but also resulted in different apparent fractionation values (Anderson *et al.*, 1987; Gamboa-Delgado and Le Vay, 2009). Thus, in our protocol, we used fish meal and soy protein concentrate as the main ingredients to feed biofloc and shrimp, respectively; in doing so, we clearly distinguished between the two sources of food for shrimp, allowing us to estimate the discrimination factors for each food source. In these conditions, and based on estimates from a simple two source mixing model, the biofloc or natural productivity, enhanced with finely

ground dry feed in solution in water, contributed to shrimp growth at levels of 39.8% and 36.9% for the C and N, respectively.

Few studies in shrimp culture, and only those concerning extensive or semi-intensive farming, have used natural abundance carbon and nitrogen isotopes to estimate C and N contribution from natural productivity to growth of reared animals. Anderson *et al.* (1987) and Cam *et al.* (1991) applied the method in shrimp cultured in earthen ponds and showed that pond biota contributed between 53 and 77% and between 13% and 86% to the growth of *Litopenaeus vannamei* and *Penaeus japonicas,* respectively. Nunes *et al.* (1997) carried out investigations focusing on the analysis of stomach contents and stable carbon isotope ratios and attributed 75% of *Penaeus subtilis* shrimp growth to naturally occurring food under semi-intensive culture. The studies on the subject conducted in shrimp farmed in biofloc used labelled isotope tracers. Thus, Epp *et al.* (2002) stated that 31% of N and 50% of C assimilated for the growth of *L. vannamei* (average weight of 3.5g) came from the biofloc productivity. Meanwhile, using the same method, by adding ^{15}N-ammonium to the culture medium, Burford *et al.* (2004) estimated that N derived from the biofloc was retained at a level of up to 18-29% for 1-9g shrimp on total N ingested. Interestingly, Epp *et al.* (2002) and Burford *et al.* (2004) produced similar results to ours through a different methodology; differences between results are very likely due to the variability of the nutritional quality of biofloc obtained in different studies. Our results and those of the literature show that the share of natural productivity in the diet of shrimp is higher in extensive/semi-intensive earthen ponds than in biofloc intensive culture. This may be explained by the higher stocking densities used in BFT which were 50 to 120 shrimps.m-² in the studies of Epp *et al.* (2002) and Burford *et al.* (2004) and 200 shrimps.m^{-2} in our study, and between 10 and 20 shrimps.m-² in extensive/semi-intensive earthen pond (Anderson *et al.*, 1987; Cam *et al.*, 1991; Nunes *et al.*, 1997). Furthermore, natural productivity could also be enhanced in earthen ponds where the bottom consists of loose sediment that promotes benthic development (Burford *et al.*, 2004). With limited areas without earthen floors and high densities, the shrimp are forced to rely upon formulated feeds (Epp *et al.*, 2002).

Biochemical studies on digestive enzymes' activities have mainly focused on the influence of the size of the shrimp (Van Wormhoudt and Sellos., 1980, Lovett and Felder, 1990), its moulting stage (Van Wormhoudt and Favrel, 1988) or the composition of diet (Le Moullac *et al.*, 1997). The few studies focused on the direct influence of feeding on enzyme secretion in crustacean have shown that food intake in crustaceans induced digestive enzyme excretion (Barker and Gibson, 1977; Al-Mohanna *et al.*, 1985; Simon, 2009). Our present study follows that of Xu *et al.* (2013), who investigated the effects of biofloc on digestive enzyme activities of the white shrimp *Litopenaeus vannamei*. In our case, we compared the specific digestive enzyme activities and corresponding transcripts abundance between shrimps from both biofloc treatments (BU and BF) and clear water (CW). As for Xu *et al.* (2013), we observed higher amylase (carbohydrase) and trypsin (endoprotease) activities in both biofloc treatments compared to CW treatment. In their study, Moss *et al.* (2001) showed higher specific activity of most digestive enzymes in *L. vannamei* shrimps reared in a eutrophic pond compared to those reared in well water and hypothesised that this increased activity was due to natural productivity that served as a source of organic substrates. All of these studies converge and show that the natural productivity, regardless of the rearing method (semi-intensive in earthen pond or intensive in biofloc), represents a source of food that is constantly available and its consumption by shrimps at any time stimulates the enzyme activities associated with digestion. Conversely, artificial feed is only available at mealtimes and we have actually shown a significant post-prandial increase in enzyme activities in shrimp from the CW treatment (Fig 3A and B). Another hypothesis involving a contribution of exogenous enzymes from the biofloc consumed by shrimp may also explain the higher enzyme activities observed in animals reared in biofloc. Indeed, we showed that the biofloc particles exhibited significant trypsin and α-amylase activities, which may help the digestive function of the shrimp. Our results confirm several previous investigations which have shown

relevant extracellular enzymes produced by the microorganisms (bacteria, phytoplankton or zooplankton) from the biofloc, leading to the hypothesis that these exogenous enzymes may provide the shrimp with additional digestible abilities (Harris, 1993; Moss *et al.*, 2001; Xu and Pan, 2012). In addition, exogenous bacteria in shrimp gut might either stimulate endogenous enzymes produced by the host in some way, as has been shown with specific probiotic strains (Wang, 2007; Castex *et al.*, 2009).

At the level of gene expression of enzymes that were studied here, it appears that shrimp with access to two food sources in the BF treatment presented a higher level of mRNA compared to animals fed with a single dietary source (BU and CW). This result can be explained by the assumption that shrimp from the BF treatment, with access to two dietary sources, ingested more protein and more carbohydrate than shrimps from other treatments. Indeed, several authors have shown that the amount and the nature of dietary protein modulated the transcription and translation of trypsin mRNA (Péres *et al.*, 1998; Wang *et al.*, 2006). For their part, Huvet *et al.* (2003) showed in *Crassoteras gigas* higher amylase expression in oyster with “high food condition” compared to animals with “low food condition”. In order to explain our results, we therefore hypothesise that a higher amount of nutritive substrate available for the shrimp in treatment BF could modulate the gene expression of α-amylase and trypsin enzymes.

5. Conclusion

Our study shows that juvenile shrimp *L. stylirostris* reared in biofloc get 37-40% of their food from the natural productivity, which in turn stimulates digestive enzyme activities; this increased activity may contribute to promote the growth of shrimps reared in biofloc. Concomitantly, we showed that survival rates of the shrimps reared in biofloc were greatly improved; however, the relationship which may exist between the effects of biofloc on nutrition and on survival rate is still unclear and needs further investigation.

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References

Abreu, P.C., Ballester, E.L., Odebrecht, C., Wasielesky Jr, W., Cavalli, R.O., Granéli, W., Anesio, A.M., 2007. Importance of biofilm as food source for shrimp (*Farfantepenaeus paulensis*) evaluated by stable isotopes ($\delta^{13}C$ and $\delta^{15}N$). Journal of Experimental Marine Biology and Ecology, 347(1), 88-96.

Al-Mohanna, S.Y., Nott, J. A., Lane, D.J.W., 1985. Mitotic E-and secretory F-cells in the hepatopancreas of the shrimp *Penaeus semisulcatus* (Crustacea: Decapoda). Journal of the Marine Biological Association of the United Kingdom, 65(04), 901-910.

Anderson, R.K., Parker, P.L., Lawrence, A., 1987. A 13C/12C tracer study of the utilization of presented feed by a commercially important shrimp *Penaeus vannamei* in a pond growout system1. Journal of the World Aquaculture Society, 18(3), 148-155.

Avnimelech, Y., 2007. Feeding with microbial flocs by tilapia in minimal discharge bio-flocs technology ponds. Aquaculture, 264(1), 140-147.

Avnimelech, Y., 2009. Biofloc Technology - A practical guide Book. Baton Rouge, Louisiana, United States, The World Aquaculture Society.

Avnimelech, Y., Kochba, M., 2009. Evaluation of nitrogen uptake and excretion by tilapia in bio floc tanks, using ^{15}N tracing. Aquaculture, 287(1), 163-168.

Barker, P.L., Gibson, R., 1977. Observations on the feeding mechanism, structure of the gut, and digestive physiology of the european lobster *Homarus gammarus* L.)(Decapoda: Nephropidae). Journal of Experimental Marine Biology and Ecology, 26(3), 297-324.

Bendschneider, K. & Robinson, R.J., 1952. A new spectrophotometric method for the determination of nitrite in sea water. J. mar. Res., 11, pp. 87–96.

Bernfeld, P. (1955). [17] Amylases, α and β. Methods in enzymology, 1, 149-158.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry, 72(1), 248-254.

Browdy, C.L., Bratvold, D., Stokes, A.D., Mcintosh, R.P., 2001. Perspectives on the application of closed shrimp culture systems. Proceedings of the special session on sustainable shrimp culture, aquaculture 2001. The World Aquaculture Society, Baton Rouge, LA, pp 20–34.

Burford, M.A., Thompson, P.J., McIntosh, P.R., Bauman, R.H., Pearson, D.C., 2004. The contribution of flocculated material to shrimp (*Litopenaeus vannamei*) nutrition in a high-intensity, zero exchange system. Aquaculture 232, 525–537.

Cam. D.. Rollet. P.-E.. Mariotti. A.. Guillaume. J.. 1991. The relative contribution of natural productivity and formulated food in the nutrition of *Penaeus japonicus* reared in semi-intensive conditions. Aquat. Living Resour. 4. 175–180.

Castex, M., 2009. Evaluation du probiotique bactérien Pediococcus acidilactici MA18/5M chez la crevette pénéide *Litopenaeus stylirostris* en Nouvelle-Calédonie In l'Institut des Sciences et Industries du Vivant et de l'Environnement, pp. 386. Agro Paris Tech, Paris.

Della Patrona, L., Brun, P., 2008. L'élevage de la crevette bleue en Nouvelle-Calédonie. *Litopenaeus stylirostris*. Ifremer, Département LEAD (Laboratoire Ecosystème et Aquaculture Durable) en Nouvelle-Calédonie.

De Niro, M.J., Epstein, S., 1978. Influence of diet on the distribution of nitrogen isotopes in animals. Geochim. Cosmochim. Acta, 42 (1978), pp. 495–506.

Drach, P., Tchernifovtzeff, C., 1967. Sur la méthode de détermination des stades d'intermue et son application générale aux crustacés. Vie Milieu 18, 596-609.

Erlanger, B. F., Kokowsky, N., Cohen, W., 1961. The preparation and properties of two new chromogenic substrates of trypsin. Archives of biochemistry and biophysics, 95(2), 271-278.

Epp, M.A., Ziemann, D.A., Schell, D.M., 2002. Carbon and nitrogen dynamics in zero-water exchange shrimp culture as indicated by stable isotope tracers. Aquaculture Research, *33*(11), 839-846.

Fry, B., Sherr, E. B., 1984. delta super (13) C measurements as indicators of carbon flow in marine and freshwater ecosystems. Contributions in Marine Science, 27, 13-47.

Fry, B., 2006.Stable isotope ecology. Springer, Heidelberg, 308 pp.

Gamboa-Delgado, J., Cañavate, J. P., Zerolo, R., Le Vay, L., 2008. Natural carbon stable isotope ratios as indicators of the relative contribution of live and inert diets to growth in larval Senegalese sole (*Solea senegalensis*). Aquaculture, 280(1), 190-197.

Gamboa-Delgado, J., Le Vay, L., 2009. Natural stable isotopes as indicators of the relative contribution of soy protein and fish meal to tissue growth in Pacific white shrimp (*Litopenaeus vannamei*) fed compound diets. Aquaculture, 291(1), 115-123.

García-Carreño, F. L., Hernandez-Cortes, M. P., Haard, N. F., 1994. Enzymes with peptidase and proteinase activity from the digestive systems of a freshwater and a marine decapod. Journal of Agricultural and Food Chemistry, 42(7), 1456-1461.

Hargreaves, J.A., 2006. Photosynthetic suspended-growth systems in aquaculture. Aquaculture Engineering 34, pp 344-63.

Harris, J.M., 1993. The presence, nature, and role of gut microflora in aquatic invertebrates: a synthesis. Microbial ecology, 25(3), 195-231.

Holmes, R.M., Aminot, A., Kerouel, R., Hooker, B.A., Peterson, B.J., 1999. A simple and precise method for measuring ammonium in marine and freshwater ecosystems. Canadian Journal of Fisheries and Aquatic Sciences 56(10): 1801-1808.

Holm-Hansen, O., Lorenzen, C.J., Holmes, P.E., Strickland JDH, 1965. Fluorometric determination of chlorophyll. Journal du Conseil. Conseil International Pour l'Exploitation de la Mer. 30 pp. 3–15.

Huvet, A., Daniel, J.Y., Quere, C., Dubois, S., Prudence, M., Van Wormhoudt, A., Sellos, D., Samain, J.F., Moal, J., 2003. Tissue expression of two α-amylase genes in the Pacific oyster *Crassostrea gigas*. Effects of two different food rations. Aquaculture, 228(1), 321-333.

Le Moullac, G., Klein, B., Sellos, D., Van Wormhoudt, A., 1997. Adaptation of trypsin, chymotrypsin and α-amylase to casein level and protein source in *Penaeus vannamei* (Crustacea Decapoda). Journal of Experimental Marine Biology and Ecology, 208(1), 107-125.

Le Vay, L., Gamboa-Delgado, J., 2011. Naturally-occurring stable isotopes as direct measures of larval feeding efficiency, nutrient incorporation and turnover. Aquaculture, 315(1), 95-103.

Livak, K. J., Schmittgen, T. D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{\Delta\Delta CT}$ Method. Methods, 25(4), 402-408.

Lochman, R., Phillips, H., 1996. Stable isotopic evaluation of the relative assimilation of natural and artificial foods by golden shiners *Notemigonus crysoleucas* in ponds. Journal of the World Aquaculture Society, 27(2), 168-177.

Lovett, D. L., Felder, D. L., 1990. Ontogenetic change in digestive enzyme activity of larval and postlarval white shrimp *Penaeus setiferus* (Crustacea, Decapoda, Penaeidae). The Biological Bulletin, 178(2), 144-159.

Moss, S.M., Pruder, G.D., 1995. Characterization of organic particles associated with rapid growth in juvenile white shrimp, *Penaeus vannamei* Boone, reared under intensive culture conditions. Journal of Experimental Marine Biology and Ecology, 187(2), 175-191.

Moss, S. M., Divakaran, S., Kim, B. G., 2001. Stimulating effects of pond water on digestive enzyme activity in the Pacific white shrimp, *Litopenaeus vannamei* (Boone). Aquaculture Research, 32(2), 125-131.

Nunes, A. J. P., Gesteira, T. C. V., Goddard, S., 1997. Food ingestion and assimilation by the southern brown shrimp *Penaeus subtilis* under semi-intensive culture in NE Brazil. Aquaculture, 149(1), 121-136.

Olive, P.J., Pinnegar, J. K., Polunin, N.V., Richards, G., Welch, R., 2003. Isotope trophic-step fractionation: a dynamic equilibrium model. Journal of Animal Ecology, 72(4), 608-617.

Parker, P. L., Anderson, R. K., & Lawrence, A., 1989. A $\delta^{13}C$ and $\delta^{15}N$ tracer study of nutrition in aquaculture: *Penaeus vannamei* in a pond growout system. In Stable isotopes in ecological research (pp. 288-303). Springer New York.

Péres, A., Infante, J. Z., Cahu, C., 1998. Dietary regulation of activities and mRNA levels of trypsin and amylase in sea bass (*Dicentrarchus labrax*) larvae. Fish Physiology and Biochemistry, 19(2), 145-152.

Phillips, D. L., Gregg, J. W., 2001. Uncertainty in source partitioning using stable isotopes. Oecologia, 127(2), 171-179.

Ray, A.J., 2012. Management of biological and chemical constituents for the advancement of intensive, minimal-exchange, biofloc-based shrimp (*Litopenaeus vannamei*) aquaculture. Dissertations. Paper 713. http://aquila.usm.edu/theses_dissertations/713

Roth, J. D., Hobson, K. A., 2000. Stable carbon and nitrogen isotopic fractionation between diet and tissue of captive red fox: implications for dietary reconstruction. Canadian Journal of Zoology, 78(5), 848-852.

Schneider, O., Sereti, V., Eding, E.H., Verreth, J.A.J., 2005. Analysis of nutrient flows in integrated intensive aquaculture systems. Aquacultural engineering, 32(3), 379-401.

Schroeder, G.L., 1983. Sources of fish and prawn growth in polyculture ponds as indicated by δC analysis. Aquaculture, 35, 29-42.

Simon, C.J., 2009. Digestive enzyme response to natural and formulated diets in cultured juvenile spiny lobster, *Jasus edwardsii*. Aquaculture, 294(3), 271-281.

Tacon, A.G.J., Cody, J.J., Conquest, L.D., Divakaran, S., Forster, I.P., Decamp, O.E., 2002. Effect of culture system on the nutrition and growth performance of Pacific white shrimp *Litopenaeus vannamei* (Boone) fed different diets. *Aquaculture Nutrition*, *8*(2), 121-137.

Van Wormhoudt, A., Sellos, D., 1980. Aspects biochimiques de la croissance: acides nucléiques et enzymes digestives chez *Palaemon serratus* (Crustacea Natantia). Oceanologica Acta, 3(1), 97-105.

Van Wormhoudt, A., Favrel, P., 1988. Electrophoretic characterization of *Palaemon elegans* (crustacea, decapoda) α amylase system: Study of amylase polymorphism during the intermolt cycle. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, 89(2), 201-207.

Waddington, K., MacArthur, L., 2008. Diet quality and muscle tissue location influence consumer-diet discrimination in captive-reared rock lobsters (*Panulirus cygnus*). Marine Biology, 154(3), 569-576.

Wang, C., Xie, S., Zhu, X., Lei, W., Yang, Y., Liu, J., 2006. Effects of age and dietary protein level on digestive enzyme activity and gene expression of *Pelteobagrus fulvidraco* larvae. Aquaculture, 254(1), 554-562.

Wang, Y.B., 2007. Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei.* Aquaculture 269, 259-264.

Wasielesky Jr, W., Atwood, H., Stokes, A., Browdy, C.L., 2006. Effect of natural production in a zero exchange suspended microbial floc based super-intensive culture system for white shrimp *Litopenaeus vannamei.* Aquaculture, 258(1), 396-403.

Xu, W.J., Pan, L.Q., 2012. Effects of bioflocs on growth performance, digestive enzyme activity and body composition of juvenile *Litopenaeus vannamei* in zero-water exchange tanks manipulating C/N ratio in feed. *Aquaculture*, *356*, 147-152.

Xu, W. J., Pan, L. Q., Sun, X. H., Huang, J., 2013. Effects of bioflocs on water quality, and survival, growth and digestive enzyme activities of *Litopenaeus vannamei* (Boone) in zero-water exchange culture tanks. Aquaculture Research, 44(7), 1093-1102.

Tables

Table 1: Diet ingredients and proximate composition of both diets.

(1)Vitamin Premix for shrimp from BEC feed solutions PTY, LTD - ingredients: vitamin AD3 1000/200, vitamin B1 98% Thiamine, Mononitrate, vitamin B2 Riboflavin 80%, vitamin B3 99% Niacin, vitamin B5 98% D-Calpan, vitamine B6 98% Pyridoxine, Vitamin B9 97% Folic acid, vitamine D3 500, vitamin E 50 ADD, vitamin K3 43.7%;(2) Vitamin C: Rovimix Stay-C 35 from DSM Cie; (3) Trace elements from SICA Cie (SAEML NORD AVENIR).

Diet ingredients (g,k^{-1} diet)	**Experimental diet**	**Commercial diet (SICA G40)**
Soy protein concentrate	453	Feed ingredients indicated by the company : Wheat, soybean meal, fishmeals, squid meal, crustacean meal, vitamins, minerals, binder
Crab meal	151	
wheat flour	337	
Soy oil	20.5	
fish oil	10	
Shrimp vitamin premix (1)	2.5	
Vitamin C (2)	3	
shrimp trace mineral premix (3)	2.5	
phosphate	20.5	
Proximate composition (%)		
Protein	38.9	39.5
Lipids	5.5	7.9
Ash	12.2	13.9

Table 2: Primers used for quantitative RT-PCR assay of *Litopenaeus stylirostris* trypsin, α-amylase, elongation factor 1-α and Glyceraldehyde-3-phosphate-deshydrogenase.

Gene name	Sequence 5' - 3'	Size	Primer size	Tm	Genbank ascension
Trypsin - F	GTGTCGCGATGCTTATGGC	95pb	19	60	
Trypsin - R	CAGAGTCACCCTGGCACG		18	60	
Amylase - F	TGGAAGTGGTCGGACATCG	70pb	19	59	
Amylase - R	CCTGAACGCCGGCGAAT		17	60	
Elongation factor 1α - R	CGTTCCGGTGATCATGTTCTTGATG	382pb	35	60	AY117542
Elongation factor 1α - F	GGTGCTGGACAAGCTGAAGGC		31	60	
Glyceraldehyde-3-phosphate-deshydrogenase - F	CGTTGGACACCACCTTCA	146pb	18	59	AI770197
Glyceraldehyde-3-phosphate-deshydrogenase - R	GTGTGCGGTGTCAACATGGA		20	55	

Table 3: Final survival rate and growth parameters at the end of the grow-out period in the both experiments.

S= significance. Values are means ±s.d (n=4). Stars indicate "significant" differences between treatment (* $p<0.05$. ** $p<0.01$; *** $p<0.001$). n.s. non-significant. Different superscripts indicate significant differences.

Zootechnical parameters	Experiment 1 - Treatments			S	Experiment 2 -Treatments			S
	CW	BU	BF		CW	BU	BF	
Survival rate (%)	^{a}64.20±13.10	^{ab}83.10±15.00	^{b}93.50±13.00	*	^{a}42.17± 15.35	^{ab}88.40 ± 14.20	^{b}99.30 ± 1.30	*
Growth rate (g.wk^{-1})	^{a}0.06 ± 0.01	^{ab}0.16 ± 0.09	^{b}0.22 ± 0.06	**	^{a}0.04 ± 0.01	^{a}0.05 ± 0.01	^{b}0.21 ± 0.03	***
Final weight (g)	^{a}0.60 ± 0.06	^{ab}0.93 ± 0.29	^{b}1.18 ± 0.34	**	^{a}0.21 ± 0.04	^{a}0.25 ± 0.02	^{b}0.88 ± 0.19	***
Weight gain (%)	^{a}250	^{ab}388	^{b}492	**	^{a}350	^{a}417	^{b}1467	***

Table 4: The overall means ± s.d of measured water parameters for the two experiments.

Parameters \ Treatments	Experiment 1			Experiment 2		
	CW	BU	BF	CW	BU	BF
temperature ± SD (°C)	25.99 ± 1.19	25.10 ± 1.56	25.16 ± 1.56	24.79 ± 2.32	24.29 ± 2.07	24.38 ± 2.02
DO± SD (mg.L^{-1})	6.28 ± 0.47	6.57 ± 0.60	6.53 ± 0.43	7.06 ± 0.40	7.46 ± 0.83	7.39 ± 0.59
pH ± SD	8.19 ± 0.04	7.99 ± 0.08	7.90 ± 0.10	8.01 ± 0.17	7.94 ± 0.20	7.90 ± 0.16
Salinity ± SD (‰)	34.58 ± 0.18	34.22 ± 0.88	34.26 ± 0.98	34.60 ± 0.20	34.44 ± 1.26	34.48 ± 1.08
TAN ± SD (mg.L^{-1})	0.00 ± 0.00	0.04 ± 0.10	0.13 ± 0.27	0.00 ± 0.00	0.02 ± 0.09	0.00 ± 0.00
NO_2^--N ± SD (mg.L^{-1})	0.00 ± 0.00	0.04 ± 0.14	0.09 ± 0.27	0.00 ± 0.00	0.02 ± 0.02	0 .04 ± 0.07
TSS ± SD (g.L^{-1})	0.02 ± 0.00	0.30 ± 0.04	0.36 ± 0.04	0.02 ±0.05	0.26 ± 0.18	0.27 ± 0.13
Chla ± SD (µg.L^{-1})	1.03 ± 1.51	133.35 ± 17.98	160.94 ± 52.95	0.98 ± 0.25	139.90 ± 111.00	156.90 ± 114.32

Table 5: Isotopic value ($\delta^{13}C$ and $\delta^{15}N$) and Carbon and nitrogen percentage in the two nutritional sources.

S= significance. Values are means ±s.d (n=4). Stars indicate "significant" differences between treatment (* p<0.05. ** p<0.01; *** p<0.001). n.s. non-significant.

	Treatments	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	C (%)	N (%)	C/N
Biofloc	BU	^{a}-19.53 ± 0.33	^{a}10.02 ± 1.35	^{a}20.44 ± 2.42	^{a}3.04 ± 0.60	6.72 ± 0.82
	BF	^{a}-19.74 ± 0.30	^{a}10.21 ± 0.58	^{a}20.60 ± 2.21	^{a}3.25 ± 0.56	6.34 ± 0.48
Compound diet	BF and CW	^{b}-24.50 ± 0.04	^{b}1.41 ± 0.05	^{b}41.08 ± 6.04	^{b}6.04 ± 0.08	6.80 ± 0.06
S		***	***	***	***	n.s

Table 6: Means values of α-amylase and trypsin relative expression levels and specific enzymatic activities for the three treatments (experiment 2).

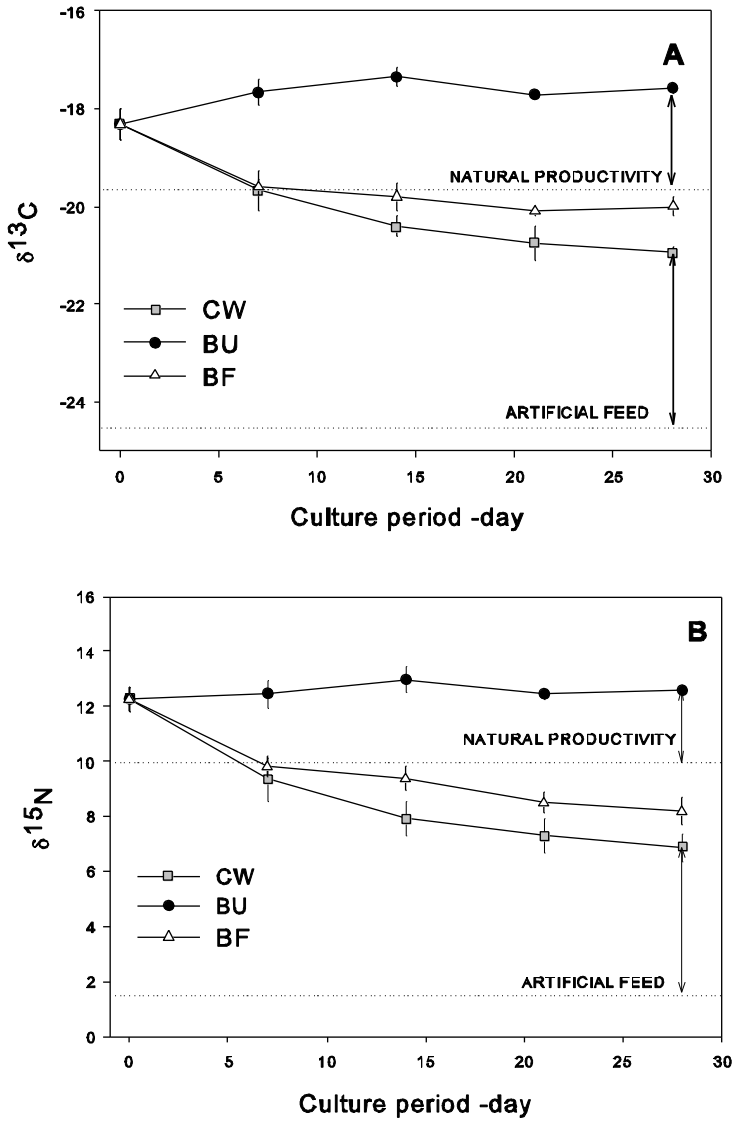
S= significance. Values are means ±s.d (n=4). Stars indicate "significant" differences between treatment (* p<0.05. **p <0.01; *** p<0.001). n.s. non-significant.

	Enzyme	CW	BU	BF	S
Relative expression level	α-amylase	^{a}1.59 ± 0.60	^{a}1.71 ± 0.63	^{b}2.64 ± 0.77	***
	trypsin	^{a}0.85 ± 0.45	^{a}0.90 ± 0.73	^{b}1.46 ± 0.84	*
Specific enzymatic activities (U.mg of protein^{-1})	α-amylase	^{a}10.09 ± 4.92	^{b}19.27 ± 9.38	^{b}19.32 ± 4.08	***
	trypsin	^{a}2.78 ± 1.55	^{b}7.99 ± 2.85	^{b}7.32 ± 1.65	***

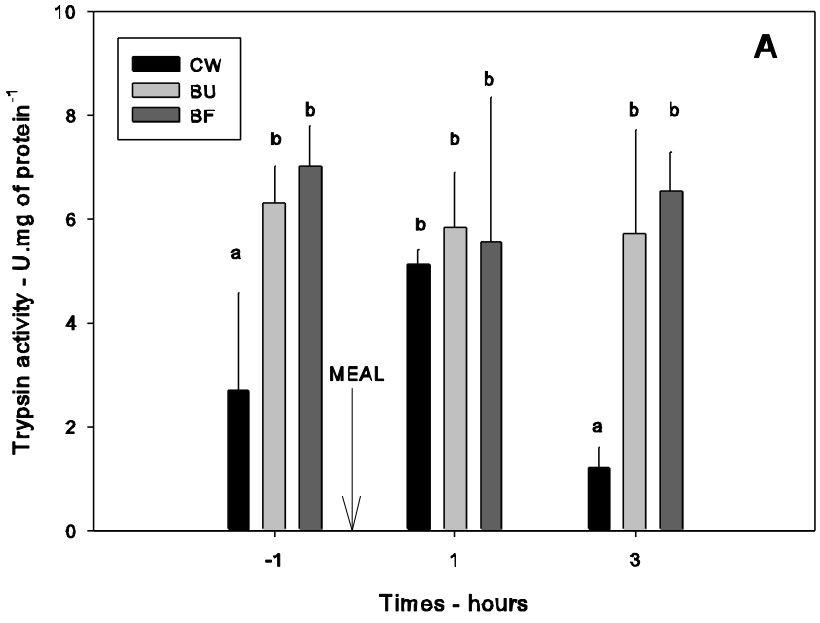
Figures

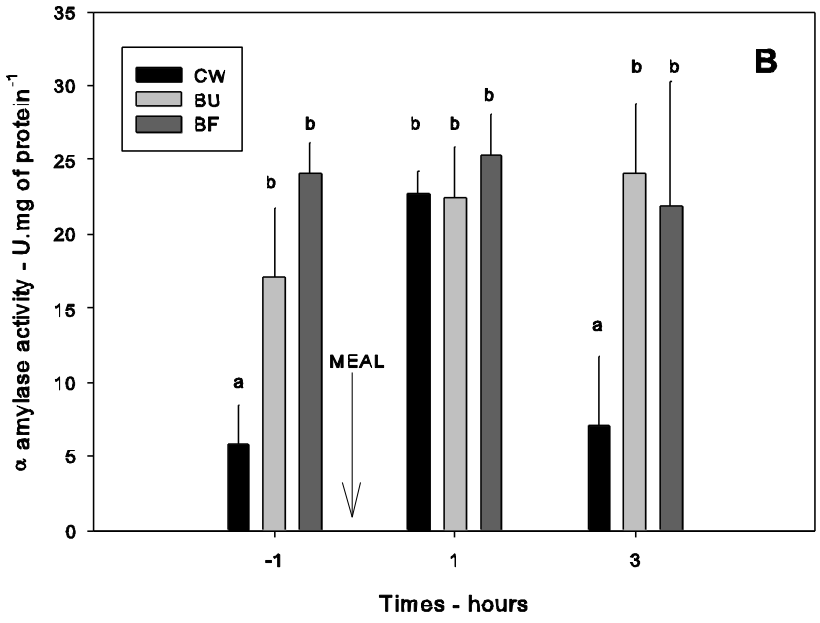
<u>Figure 1:</u> Carbon (A) and Nitrogen (B) isotopic changes in muscle of shrimp according treatments: CW, BU or BF.

Arrows represent isotopic discrimination factors between both dietary sources and between shrimps fed natural productivity and experimental diet only



<u>Figure 2:</u> Evolution of trypsin (A) and α-amylase (B) activities over the course of the feeding trial.





<u>Figure 3:</u> Evolution of trypsin (A) and α-amylase (B) relative expression levels over the course of the feeding trial.