RESEARCH ARTICLE

Influence of body size, temperature, and diet concentration on feeding of *Styela clava*

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As solitary filter feeder, *Styela clava* shows superior water purification capacity in the integrated multi-trophic aquaculture (IMTA) model consisting of *S. clava*, microalgae, and sea cucumber, which should be an ideal system to reduce organic and inorganic pollution in the water. It is of great significance to study the physiological energetics of *S. clava* for the application of this IMTA system. The physiological energetics of *S. clava* was determined in a simulated natural culture system to provide information for comparing to the energetic data derived from feeding ecological studies. The ascidians were grouped into five cohorts based on body size in terms of their wet weight of 1.5-2.0 g, 2.5-3.5 g, 5.0-6.5 g, 8.0-9.5 g, and 11.0-14.0 g, respectively. Clearance rate (CR), ingestion rate (IR), and assimilation efficiency (AE) of ascidian were estimated using flow-through feeding chambers. The results showed that the CR and IR increased exponentially with the ascidians' body size at different temperatures. Body size showed little effect on AE at the same water temperature. However, significant differences were observed between different temperature regions in the range of 12-28°C regardless of body size. *S. clava* performed far better in feeding behavior at 20°C than that at higher or lower temperatures. Within the range of diet concentration (as particulate organic matter, POM) from 2.11 to 6.11 mg POM/L, the CR and IR increased exponentially with POM. This study provides basic data for the application of ascidian in the IMTA system.

Keywords: *Styela clava*; feeding; clearance rate; ingestion rate; assimilation efficiency; diet concentration.

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Introduction

The large amount of organic or inorganic waste produced from highly intensive coastal marine cultivation has had serious impacts on the environment. Perhaps the most effective solution is the application of integrated multi-trophic aquaculture (IMTA) system to reduce organic and inorganic pollution in the surrounding area [1-2], which may facilitate sufficient use of space and bait, quick cycling of substances in the water body, a stable culture system, less pollution, and control of culture scale [3-5].

The solitary ascidian, *Styela clava*, a kind of benthic filter-feeding macro-invertebrates, is a dominant member of the benthic community in Bohai and western Yellow Seas of China with densities of up to 8,100 individuals/m^2. The average size of *S. clava* is about 100 mm, and the maximum can reach 158 mm. Like other suspension-feeders, ascidians could affect the water environment through filtering phytoplankton, biodeposition, and nutrient cycling [6].

S. clava meets all the requirements for biological remedy advanced by Gifford *et al.* [7]. *S. clava* can filter particulate matters ranging from 0.1 μm to 750 μm, such as colloid, bacteria, phytoplankton, organic detritus, dissolve organic matter, and effectively filter organic particulate matter to extract edible part [8]. *S. clava* lives in almost all sea waters and fresh waters all over the world, and occupies a large proportion of biomass, especially in the coastal waters [9]. *S. clava* is rich in protein, capable of removing organic nutrients in the water body, and the ideal organism for the aquaculture water body bioremediation along the coast.

The physiological energetics of each species is the basis for the study and application of the IMTA system. The physiological energetics of filter-feeders has been extensively studied using the biodeposition method in the last 20 years [10-14]. Some achievements have been successfully applied to bivalves' aquaculture to estimate the potential growth and output such as aquaculture scallops and environmental load [15-17]. Since *S. clava* is not a traditional economic organism, the research on the ascidian *S. clava* mainly focuses on its unique evolutionary status at present. Little is known about its feeding physiology [18]. This study focused on physiological energetics of *S. clava* at a laboratory scale to investigate the influence of body size, water temperature, and diet concentration on the indexes of ingestion rate (IR), clearance rate (CR), and assimilation efficiency (AE). The results of this study could provide basic data for the application of ascidian in the IMTA system including *S. clava*, microalgae, and sea cucumber.

Material and methods

Sample collection and groups

S. clava used in the experiments were collected from the intertidal zone (37°22′ N, 121°25′ E) in Yantai, Shandong Province, China in May. The ascidians were taken down along with their substratum to avoid hurting the animals and transported back to the laboratory within 1 hour. All debris and epibiota were cleared carefully from the adherences. The ascidians were transferred into aquariums to acclimatize the laboratory environment at room temperature and fed the microalgae, *Isochrysis galbana*, prior to the experiments. After acclimatization at least 10 days, the temperature of water was adjusted to experimental temperature gradually at the rate of 0.5°C per day. The experimental ascidians were grouped into five experimental groups based on their body sizes in terms of their wet mass of 1.5-2.0 g, 2.5-3.5 g, 5.0-6.5 g, 8.0-9.5 g, 11.0-14.0 g, respectively, and five experimental temperatures of 12, 16, 20, 24, 28°C were tested.

Experimental instrument

Filtered seawater of ambient salinity (30±2 g/L) was first pumped into a reservoir (400 m^3), and then delivered into a 400 L higher tank continually and gravity-fed through delivery lines to twelve 10.0 L flow feeding chambers (Figure 1). All tanks were held in an air-conditioned room, and each chamber equipped with a thermostat to maintain experimental temperature. The flow rate of 30 L/h was adjusted among the 12 chambers by maintaining water pressure in the overhead tank. In front of the inflow aperture of each chamber, baffles were fixed 1.5 cm to the bottom to direct the water exceed the ascidian's inhalant siphons. Outflow apertures were fixed 2 cm from the top of each chamber to maintain the water volume at 10 L. Each experiment group was comprised of five duplicate culture chambers (contained 20 ascidians in each chamber) and two controls. Control group was the chamber without animal to determine the suspended particles' concentration in the system. Before testing, at least 1 h adaptation time was reserved for the experimental animals in the feeding chambers.

Experimental feeding diet

The microalgae, *Chaetoceros debilis*, was selected as feeding diet to *S. clava* in this study

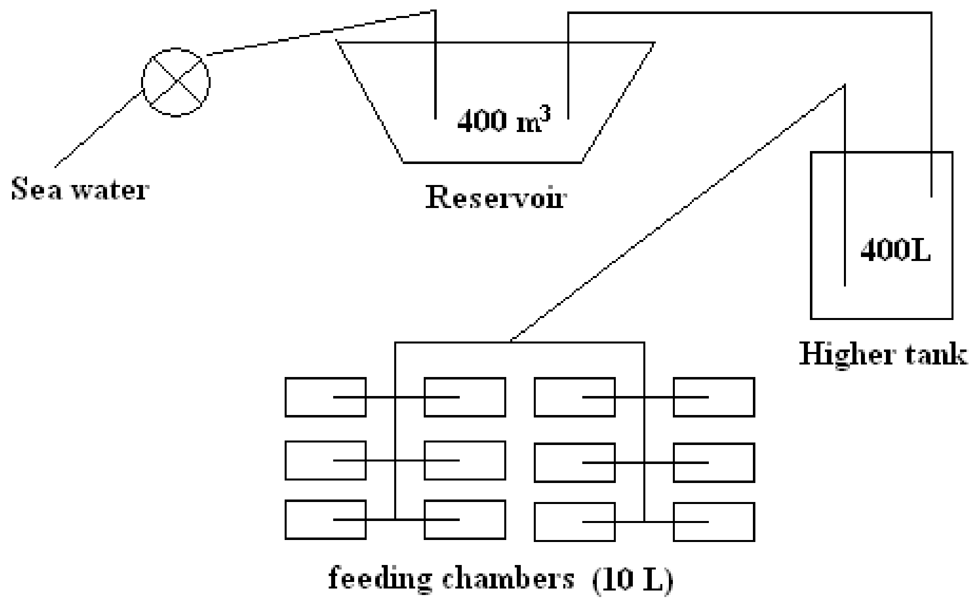

Figure 1. Sketch showing the design of the culture system.

and maintained by the Institute of Ocean Biochemical Engineering at Yantai University, Yantai, Shandong Province, China. Microalgae was cultured with f/2 Medium (Ally Biotechnology Co., Ltd, Shanghai, China) at 21-23°C under an illumination intensity of 1.10 mW/cm^2 for 24 h. The diet concentrations were microscopic monitored in the inflow and outflow water during the experiments, and the diet concentration in inflow water was regulated by adding cultured microalgae in the higher tank. The diet concentration was indicated by the content of total particulate matter (TPM) and particulate organic matter (POM) in the seawater of the chamber. TPM and POM were determined according to the following procedures described by Bayne and Newell [19], Sun *et al*. [10], and Dong *et al*. [20]. First, a certain volume of water sample (500-1,000 mL) was vacuum filtered through GF/C glass-fiber filters (mesh 0.45 µm) (Jiangsu Lvmeng Scientific Instrument Co. LTD, Taizhou, Jiangsu, China). The filters were burned in muffle furnace (Tianrun Electronic Technology Co. LTD, Tangshan, Hebi, China) at 450°C for 6 h before being weighed (W_0). The filtered material was then rinsed with 0.5 mol/L NH_4COOH (Sinopharm Group, Beijing, China) to remove salts before dried at 110°C for 12 h and weighed to get the first dry weight (W_{110}). It was re-weighed after being burned at 450°C for 6 h to give the second dry weight (W_{450}). Finally, TPM and POM were calculated using the following equations:

$$POM = W_{110} - W_{450}$$

$$TPM = W_{110} - W_0$$

Replicate inflow/outflow water and fecal samples were taken from each culture and control chamber over a 10 h experimental period at consecutive 1 h intervals to measure both TPM and POM.

Experimental design

In this study, three separate one-factor experiments were designed to investigate the influence of body size, temperature, and diet concentration on feeding of *Styela clava*. The diet concentration was balanced at 3.06±0.07 mg TPM/L in the inflow water to exam the effects of ascidian's body size and temperature on feeding activity. The experiments were carried at the temperatures of 12±0.1, 16±0.1, 20±0.1, 24±0.1, and 28±0.1°C, respectively. Eight diet concentrations (2.11±0.15, 3.06±0.17, 4.01±0.08, 5.09±0.15, 6.11±0.17, 10±0.18, 15±0.23, and 20±0.19 mg POM/L) and medium-size ascidians of 8.0-9.5 g wet weight were used to test the influence of diet concentration. For each experiment, the dry weights of the ascidians were precisely measured at last after they were dried at 60°C. Three factors including clearance rate (CR), ingestion rate (IR), and assimilation efficiency (AE) of ascidian were employed in this study to evaluate the influence of body size, temperature, and diet concentration on feeding of *Styela clava*.

Clearance rate refers to the volume of water filtered per hour by an individual animal (L/ind·h) and is estimated using the formula below [21]:

$$CR = V \times \ln[(C_{e0} - C_{e0} \times S_{ed}) / C_{et}] / \left(N \times t\right)$$
$$S_{ed} = \left(C'_{e0} - C'_{et}\right) / C'_{e0}$$

where V is the experimental seawater volume (L), N is the number of animals used in the experiment (ind), t is the duration of the experiment (h), C_{e0} and C_{et} are the diet concentration (mg TPM/L) in the inflow and outflow water of the culture chamber, respectively. S_{ed} refers to the variation coefficient in diet concentration of controls, where C'_{e0} and C'_{et} are the diet concentration (mg TPM/L) in the inflow and outflow water of the control chamber, respectively.

Ingestion rate refers to the amount of POM ingested per hour by the individual ascidian (mg/ind·h), and is calculated as below [22]:

$$IR = V \times \left(C_{e0} - C_{e0} \times S_{ed} - C_{et}\right) / \left(N \times t\right)$$

Where V, N, and t are the same as the CR equation mentioned above. C_{eo} and C_{et} are the diet concentration (mg TPM/L) in inflow and outflow water of the culture chamber, respectively. S_{ed} refers to the variation coefficient in diet concentration of controls and its computation is the same as above.

Assimilation efficiency is defined as the percentage of assimilated matter through the animal's digestive system in the ingested amount of TPM and the efficiency with which organic material is absorbed from ingested food material, and is calculated as following [23]:

$$AE = 100 \times \left(F' - E'\right) / \left[(1 - E') \times F'\right]$$

Where F' is the proportion of organic matter in the diet and E' is the proportion of organic matter in the feces.

Statistical analysis

All the results of CR, IR, and AE were calculated as mean ± standard deviation (SD) in this study. SPSS software (version 11.0) (IBM, Ammon, New York, USA) was employed. A probability of $p<0.05$ was taken as an acceptable level of significance. The data were analyzed statistically for differences using one-way analysis of variance (ANOVA) to test for differences among all means. Tukey's multiple comparison tests were used to detect differences between groups. Nonlinear regression was performed, and the regression curves were compared using an analysis of covariance (ANOCOVA).

Results

Effects of body size and temperature

The diet concentration was balanced at 3.06±0.07 mg TPM/L in the inflow water of the experiment. The CR, IR, and AE of the ascidians were measured (Figures 2-4). The calculated relationships between body size (W, dry mass) and CR as well as IR both demonstrated power increase with increasing body size. Within the range of experimental water temperature, CR, IR, and AE all increased with increasing temperature up to 20°C, and then decreased with further increasing temperature. Significant differences were observed between different temperatures ($p<0.05$). The results of variance analysis showed that CR and IR were very significantly influenced

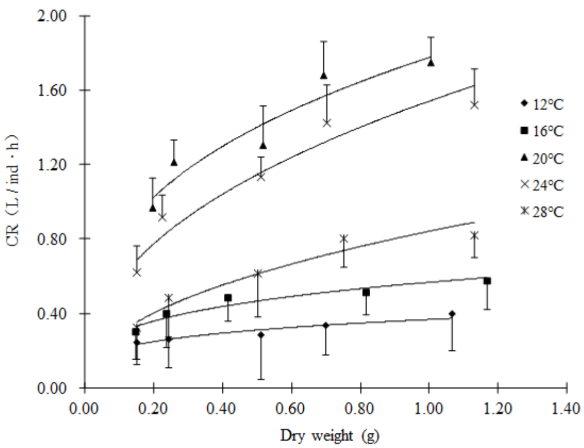

Figure 2. Relationships among temperature, body weight, and clearance rate of *Styela clava*.

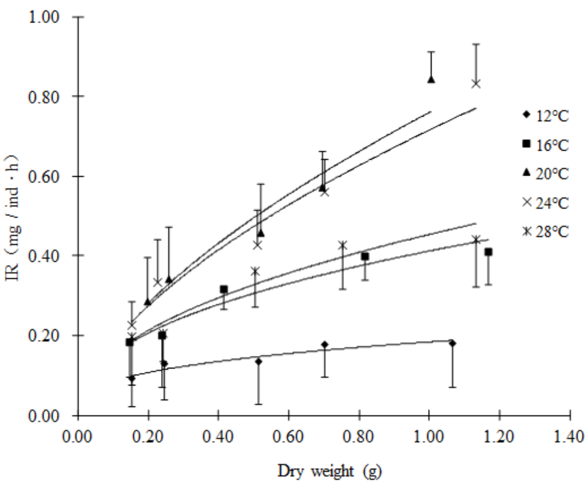

Figure 3. Relationships among temperature, body weight and ingestion rate of *Styela clava*.

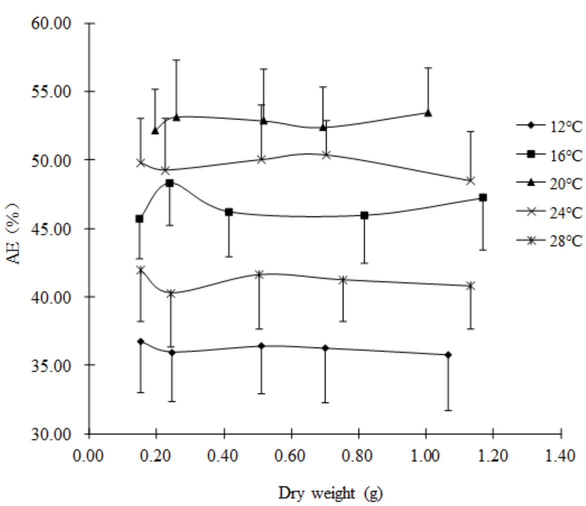

Figure 4. Relationships among temperature, body weight, and assimilation efficiency of *Styela clava*.

by ascidian's size and temperature (p<0.01). The AE showed no significant difference between different body size at same temperature (p>0.05). However, significant differences were observed between different temperature regions regardless of body size (p<0.01).

Effect of diet concentration

For this experiment, five diet concentrations (2.11±0.15, 3.06±0.17, 4.01±0.08, 5.09±0.15, and 6.11±0.17 mg POM/L) and medium-size ascidians of 8.0-9.5 g in wet mass were used. The results clearly demonstrated that ascidian's CR and IR increased with increasing diet concentration in a power manner (Figure 5 and 6). Both temperature and diet concentration significantly affected ascidian's CR and IR (p<0.01). To determine the influence of higher diet concentrations on ascidian's CR and IR, the diet concentration was extended up to 20 mg POM/L (Figure 5 and 6). Within a certain limit of diet concentration (from 2.11 to 10.06 mg POM/L), both CR and IR of the ascidian were increased with the increase of diet concentration. At the critical diet concentration of 10.06 mg POM/L, both CR and IR of the ascidians reached highest levels.

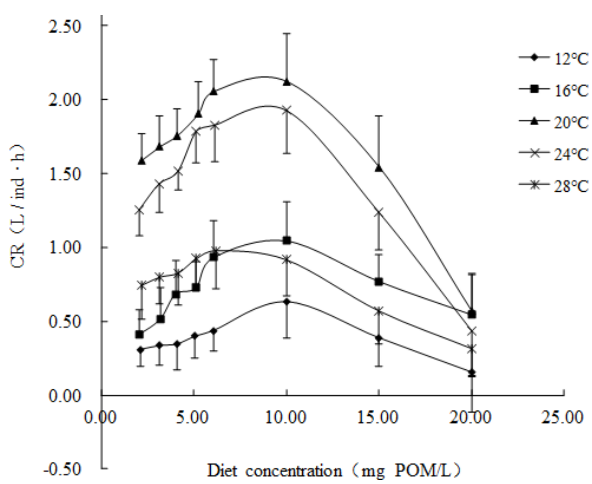

Figure 5. Effects of temperature, diet concentration on clearance rate of *Styela clava*.

At values above the critical diet concentration, the CR of ascidian decreased with increasing diet concentration, while the IR kept steady, which showed little difference with diet concentration increase ($p>0.05$). Within the whole scope of diet concentration tested (from 2.11 to 20.12 mg POM/L), both CR and IR of ascidian were quadratic correlated with the diet concentration. As to AE of the ascidian, with the increase of diet concentration, no significant variation was found ($p>0.05$), with values fluctuating around mean levels of 36% (12°C), 45% (16°C), 52% (20°C), 50% (24°C), and 41% (28°C), respectively (Figure 7).

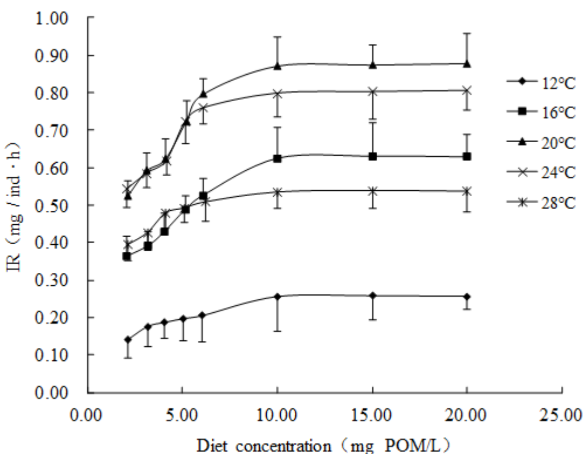

Figure 6. Effects of temperature, diet concentration on ingestion rate of *Styela clava*.

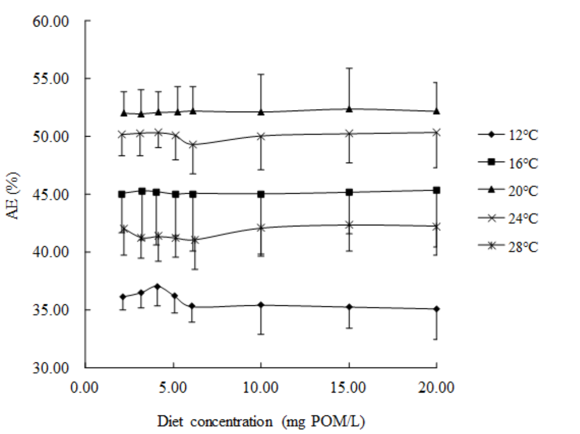

Figure 7. Relationships among temperature, diet concentration and assimilation efficiency of *Styela clava*.

Discussion

The IMTA model may result in sufficient use of space and bait, quick cycling of substances in the water body, a stable culture system, less pollution, and control of culture scale [4, 24]. The IMTA model consisting of *S. clava* – microalgae – *S. japonicus* takes full advantage of mutual benefit among the cultured organisms. As filter-feeding periphyton, *S. clava* may be fixed to suspension to filter suspended particles and pathogenic microorganisms as well as to keep the water clean. Further studies are needed for the complicated interactional mechanisms among different organisms of this model with the physiological ecology of ascidians as one of the contents to be studied.

The physiological ecology of marine organisms is important for understanding their adaptations to particular habitat conditions. Physiological functioning in marine invertebrates is usually dependent on environmental and organism features, including temperature, salinity, and body size. However, physiological rate independence from temperature is sometimes observed in invertebrates that experience highly variable environmental conditions [25]. Most physiological studies of filter-feeding animals have been done on bivalves to show how these species respond to changes in the physiological environment. However, for ascidian *S. clava*, despite its abundance, virtually little is known about its metabolic physiology. The physiological data obtained in the present study provided valuable information on the metabolic processes underlying the effect of temperature and body size on the development of *S. clava*, which should be important for the application of this IMTA system.

Effect of body size

The relationship between body size (W) and CR as well as IR for bivalves could be described by the generalized formula $R=aW^b$ as described by Bayne and Newell [19], and the mean value of b was 0.62±0.13 within the range between 0.66 and 0.82 according to Winter [26]. The b values of the ascidian *Styela clava* in our experiments were 0.33-0.62 for IR and 0.24-0.46 for CR, which were somewhat lower than bivalves. This difference might possibly attribute to the different species and physiological conditions of animals, as well as different experimental environments used in the experiments. The ascidians are able to adjust their IR to meet nutritional and energetic demands regardless of individual size. This is in agreement with the results reported by Winter on *Modiolus modiolus* [26] and Vahl on *Mytilus edulis* [27].

Effect of temperature

The experimental results clearly showed the significant effect of temperature on feeding of the ascidian *Styela clava*. CR, IR, and AE of the ascidians at critical temperature (20-24°C) were all significantly higher than that at higher or lower temperatures. This phenomenon was also observed by Robbins [28] and Petersen *et al*. [25]. The metabolism level and growth efficiency of the ascidian increased with the increase of water temperature in the fitting temperature range [29]. The ascidian had to increase its IR, CR, and AE to complement its energy demand. But when the temperature was not in the appropriate range, the ascidians were probably on its deviant physiological condition, and resulted in the decrease of CR, IR, and AE.

Effect of diet concentrations

Diet concentration is one of the major factors influencing the feeding physiology of filter-feeding animals [30-32]. Our results showed that the power relationships between diet concentration and CR as well as IR of *S. clava* were only within a certain concentration range. Above the critical diet concentration, CR of the ascidian decreased dramatically and fell nearly to zero, while IR kept steady with increasing diet concentration, which indicated that the ascidian *Styela clava* was capable of stabilizing IR with increasing diet concentration and maintaining a stable IR value even at higher diet concentration. This is in agreement with findings on other bivalves [33]. Generally, two major mechanisms were used to regulate IR for filter-feeding animals as reduce CR and increase pseudofeces excretion [34]. The first mechanism plays the primary role at higher organic content, and the second mechanism has a limited role in the feeding regulation of *Styela clava* since no pseudofeces produced were found in these experiments. Armsworthy *et al*. [35] observed the feeding structures and processes of the ascidian *Halocynthia pyriformis* by endoscopy and found an increase in squirting frequency at a higher diet concentration to facilitate rejecting of unwanted material. Mucus velocity was much lower at higher diet concentration than that at lower concentration, while the overall distance of mucus travel and the probability of clogging reduced at a higher diet concentration. Ascidian appeared to compensate for episodic changes in the quantity and quality of available food particles by altering siphon-opening diameter, squirting frequency, structure and transport of mucus, and retention efficiency to maintain IR constant. Our results indicated that AE of the ascidian *S. clava* was independent of diet concentration in the range of this experiment, which was similar to many other filter-feeding bivalves such as *Cerastoderma edule*, *Mercenaria mercenaria*, and *Mytilus edulis* [32, 34, 36, 37]. The increase of AE was also found for *Placopecten magellanicus* and *Mya arenara* induced by organic content in the TPM, but no effect of TPM concentration was found in this experiment. The results implied the relationship between the food quality and feeding physiology of filter-feeding animals.

Acknowledgement

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