Iranian Journal of Fisheries Sciences 19(4) 1981-1993 2020
DOI: 10.22092/ijfs.2018.117925

Nutrient removal from aquaculture effluent using settling ponds and filter-feeding species (*Amphibalanus amphitrite* and *Saccostrea cucullata*): an *in-situ* study

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Received: November 2017 Accepted: December 2017

Abstract

The potential application of settling ponds and two fouling filter-feeder species *Amphibalanus amphitrite* and *Saccostrea cucullata* to bioremediate semi-intensive shrimp farm effluent through 6 hours residence time was investigated. Settling pond reduced total nitrogen, total phosphorus and chlorophyll *a* to 80.5%, 77.8% and 94.3% of the initial concentrations (2.47±0.07 mg L^{-1}, 0.154±0.006 mg L^{-1}, and 24.44±2.02 µg L^{-1}), respectively. Among *S. cucullata*, *A. amphitrite* and combination of both species, oysters showed the highest efficiency in nutrient removal. Total nitrogen, total phosphorus and chlorophyll *a* diminished respectively to 70.6%, 67.7% and 40.9% of the initial concentrations in oyster treatments. These proportions were respectively 81.5%, 63.2% and 72.4% for ponds containing barnacles, and 69.3%, 71.2% and 44.9% of the initial amounts in the combination of the two species treatment. Among three different densities used for treatments, medium density of oysters (0.54 oysters per liter) showed comparable effectiveness in nutrients and phytoplankton removal to the high density. Lower ammonia production along with imposing less costs and effort, as well as relatively equal ability; suggest the medium density of *S. cucullata* as the most suitable choice. Our results suggest that applying settlement ponds, and particularly with filter-feeder species such as *S. cucullata*, might mitigate the adverse impacts of shrimp wastewater, including coastal eutrophication, on adjacent ecosystems.

Keywords: Aquaculture effluent, Coastal ecosystem, Filter-feeding, Persian Gulf, Bioremediation

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Introduction

Aquaculture in general and shrimp culture in particular has undergone a remarkable growth during the last decades (FAO, 2016). Increase in areas under shrimp culture and changing systems to semi-intensive and intensive have resulted in the introduction of many pollutants into the adjacent ecosystems (Trott *et al.*, 2004; Herbeck *et al.*, 2013). Organic and inorganic nitrogen (N) and phosphorus (P) discharging from aquaculture facilities, could be used by and stimulate excessive growth of autotrophic and heterotrophic organisms (Tyrrell, 1999; Middelburg and Nieuwenhuize, 2000) and might cause coastal eutrophication and marine habitat destruction (Trott and Alongi, 2000; Herbeck *et al.*, 2013). To mitigate these adverse effects, different physicochemical (e.g., reverse osmosis, ion exchange and activated carbon absorption) and biological (e.g. constructed wetlands, biofloc technology and biofilters) methods have been used (Liu *et al.*, 2007; Lin *et al.*, 2010; Mook *et al.*, 2012; Song *et al.*, 2016). Limitations of physicochemical wastewater treatment processes such as accumulation of removed nutrients and pollutants in the systems, precipitation of salts on the membranes and biofouling problems (Mook *et al.*, 2012), in conjunction with high costs of establishment and maintenance, as well as requiring power and complicated equipment make these techniques less desirable in aquaculture wastewater treatment. As limitations of settling ponds and biological methods are less, these techniques can be considered economically and environmentally more feasible, especially in less developed countries, to remediate aquaculture wastewaters.

The particulate matter suspending in shrimp effluent owing to their characteristics (e.g. high organic proportion, and proper size) (Kinne *et al.*, 2001; Jackson *et al.*, 2003; Herbeck *et al.*, 2013), could be efficiently assimilated via filter-feeding process (Erler *et al.*, 2004; De Azevedo *et al.*, 2015). Many previous studies have focused on the ability of filter-feeder organisms, including bivalves, crustaceans and annelids as bio-filters of wastewaters (Jones *et al.*, 2001; Kinne *et al.*, 2001; Milanese *et al.*, 2003; Erler *et al.*, 2004; Giangrande *et al.*, 2005; Gifford *et al.*, 2007; Kinoshita *et al.*, 2008; Ramos *et al.*, 2009; De Azevedo *et al.*, 2015).

When selecting species to employ as bioremediator, more appropriate choices are sessile, native and ubiquitous species (Gifford *et al.*, 2007). In the present study, barnacle, *Amphibalanus amphitrite* (Darwin, 1854) and oyster, *Saccostrea cucullata* (Born, 1778) as two common and abundant fouling species residing around the shrimp culture sites in the northern Persian Gulf, were selected to bio-remediate shrimp farm effluents. Fouling filter-feeders, in addition to having the capacity of filtering suspended particles from the water column (Wisely and Reid, 1978; Cranford *et al.*, 2011), are relatively independent of the sediments type and grain size of treatment ponds because they permanently attach to hard

[DOR: 20.1001.1.15622916.2020.19.4.25.3]

substrates. Furthermore, these species can be collected from the wild relatively easily. By choosing the appropriate substrate type and placing them in a suitable location for settlement, a target biofouling species can be provided (Faimali *et al*., 2004; Tanyaros, 2011) and transferred to the intended area. These characteristics, in general, suggest that these species can be potentially suitable options for bioremediation of shrimp culture effluents.

The present study aimed to investigate the capability of settling ponds with or without bio-filters (*S. cucullata* and *A. amphitrite*) on N, P and Chl-*a* removal from shrimp effluent. Moreover, we tried to determine the most appropriate combination of the species, effective species densities, and more efficient effluent retention time in the treatment ponds to achieve the highest performance.

Materials and methods

Location

The present study was carried out in the Shif shrimp site (SSS) adjacent to the Bushehr Bay in the northern coasts of the Persian Gulf (Fig. 1). The shrimp culture areas in SSS is about 361 ha having around 86.6 million shrimps in stock (stocking density of approximately 24 shrimp m^{-2}). The Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931) is cultured in the SSS using semi-intensive farming system.

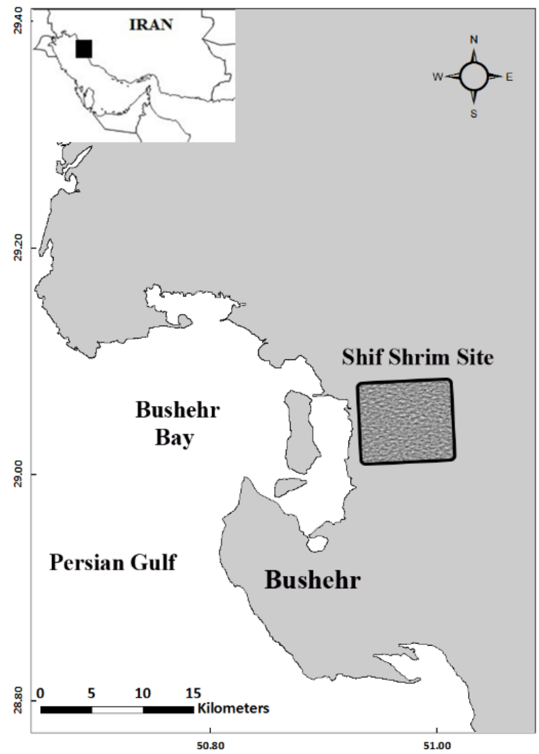

Figure 1: Location map of Shif shrimp site adjacent to the Bushehr Bay, Iran.

Experimental design

To measure the efficiency of the oyster, *S. cucullata* and the barnacle, *A. amphitrite* in N and P removal from shrimp farm effluent, adult specimens of barnacles and oysters were collected in July-August 2016. The oysters were collected from the wild populations. To collect the barnacles, PVC panels (15×15 cm) were deployed at 1 m depth for a period of two months to have enough adult barnacles attached to them.

Specimens were then transferred to the treatment ponds two weeks prior to the experiments to acclimatize to the environmental conditions. A set of ponds with the capacity of 500 L were constructed closed to the effluent channel of the SSS. Three treatments (barnacle, oyster and a combination of the two) with three densities for each

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treatment (Table 1) were applied. One pond remained without specimen and applied as settling pond (control). Since the size and weight of the individuals of the two species are extremely different (Table 1), the filtration by equal densities could not be comparable. Therefore, low, medium and high densities of each species were chosen regarding their size and weight. The experiments were conducted in three series and three replicates were used for each treatment in each series (i.e. in total nine replicates per treatment).

Water samples were taken before the treatments and after one, three and six hours from the beginning, to analyze the amount of nitrite (NO_2-N) and nitrate nitrogen (NO_3-N), ammonia (NH_3-N), total nitrogen, total phosphorus and chlorophyll *a*. Moreover, temperature, salinity, dissolved oxygen, and pH were measured at the beginning and the end of treatments.

Table 1: Densities (low, medium and high) applied for *Amphibalanus amphitrite* and *Saccostrea cucullata* (individual per liter) at each treatment and the mean (± SE) length and weight of each species.

Species	Weight (G)	Length (cm)	Low	Medium	High
***A. mphitrite* (AA)**	1.20±0.31	0.88±0.26	10	20	30
***S. cucullata* (SC)**	36.15±7.03	6.01±1.18	0.27	0.54	0.80
AA + SC	-	-	5+0.13	10+0.27	15+0.40

Analytical procedures

To measure Chl-*a* content, a certain volume of water was filtered through Whatman glass fiber filters (GF/F). After extracting phytoplankton from water sample, applied filters were immediately frozen. The pigments extraction was conducted using aqueous acetone and spectrophotometry was subsequently applied to determine the optical density of the extract (Clesceri *et al.*, 1998).

Ammonia nitrogen was determined in accordance with the oxidation method by Parsons (2013). This photometric technique was employed for measurement of ammonia through the oxidation reaction with hypochlorite in an alkaline medium. Nitrite and nitrate were measured using colorimetric and cadmium reduction methods, respectively (Clesceri *et al.*, 1998). Total nitrogen measurement was carried out by oxidative digestion of total nitrogen compounds of the shrimp effluent sample to nitrate, and subsequent measuring of nitrate (Clesceri *et al.*, 1998). All digestible organic and inorganic forms of nitrogen were converted to nitrate through alkaline oxidation at temperatures of 100 to 110 °C. The ascorbic acid method was applied to determine total phosphorus of water samples (Clesceri *et al.*, 1998). Phosphorus, in this procedure, is released from phosphorus compounds as liberated orthophosphate; afterwards, orthophosphate in the water sample was measured by colorimetric analysis.

Statistical analyses

The normality and homogeneity of the data were checked using the Shapiro-

Wilk's and Levene's tests, respectively. Prior to analysis, non-normal data were transformed to meet the assumption of normality. Statistically significant differences among variables after one, three and six hours of retention, with different treatments (barnacle, oyster and combination of the two) and different densities of each treatment (low, medium and high) were tested using three-way repeated measures ANOVA, followed by Bonferroni post-hoc test. When the data did not meet the assumption of sphericity (Mauchly's test for sphericity), *p*-values were corrected using the Greenhouse-Geisser correction. All statistical tests were performed using SPSS software (ver. 21).

Results

Effluent characteristics

During the course of the experiments, average water temperature, salinity, pH and dissolved oxygen concentration in the shrimp farm effluent were 27.7±0.3 °C, 40.8±0.2 ppt, 7.52±0.11 and 7.27±0.61 mg L^{-1}, respectively.

The mean concentration of nitrite/nitrate (NOx), total ammonia (TAN), total nitrogen (TN), total phosphorus (TP) and Chl-*a* in shrimp farm effluent, settling ponds and barnacle and oyster treatments at different densities are presented in Table 2.

Nitrite/nitrate and total ammonia

Changes in NOx concentrations, as inorganic forms of nitrogen in shrimp pond effluent, among three different treatments and settling ponds were not significantly different ($p>0.05$, Fig. 2A, B, C). Different densities exhibited no significant effect on NOx concentrations ($p>0.05$). Nevertheless, a significant reduction in NOx occurred by increasing residence time in all ponds ($p<0.05$, Fig. 2A, B, C).

Among treatments (oyster, barnacle and the combination) no significant difference in terms of TAN was detected ($p>0.05$), although all treatments were significantly different from settling pond ($p<0.05$). Effluent TAN concentration with a mean of 0.262±0.027 mg L^{-1} (Table 2) showed a significant increase in all treatments after 6 h ($p<0.05$, Fig. 2D, E, F). In settling ponds (control), in contrast, TAN concentration decreased after 6 h.

Total nitrogen

The amount of TN in the shrimp effluent was initially 2.47±0.07 mg L^{-1} (Table 2). All treatments with different densities and settling ponds exhibited significant difference from shrimp effluent (pre-treatment samples) in terms of TN ($p<0.05$, Fig. 2G, H, I). Among treatments, oyster and combination of species showed a significant difference in TN removal from barnacle treatment ($p<0.05$). Settling pond, oyster, barnacle and the combination of the two species, reduced the TN concentration to 80.5%, 70.6%, 81.5% and 69.3% of the initial concentration respectively, at high density and after 6 h.

Total phosphorus

In comparison to shrimp effluent, settling pond as well as all treatments

[DOR: 20.1001.1.15622916.2020.19.4.25.3]

reduced TP significantly ($p<0.05$). Among three treatments, barnacles showed the highest efficiency in TP reduction compared to oyster and combination ($p<0.05$). The highest reduction in TP was observed in barnacle ponds with the high density in which TP content reached 63.2% of the initial amount (i.e. 0.154±0.006 mg L^{-1}) after 6 h (Fig. 2L). Oyster and combination of the two species in the highest density and 6 h of filtration decreased the TP to the 67.7% and 71.2%, respectively (Fig. 2K, J). Only medium and high densities of treatments were significantly different from settling ponds in TP removal terms ($p<0.05$).

Chlorophyll a

Chlorophyll *a* concentration in all treatments were significantly different from the effluent Chl-*a* content ($p<0.05$), but settling ponds did not show any significant difference from effluent in terms of Chl-*a* ($p>0.05$). Oysters reduced the Chl-*a* content of the effluent more effectively compared to the other treatments ($p<0.05$). The initial concentration of Chl-a in the effluent reduced continuously with time, being the lowest at the highest density of oysters (40.9% of initial concentration after 6 h, Fig. 2M). A similar trend of Chl-*a* reduction with increasing the density and filtration time of the combination of the two species (44.9% of initial concentration after 6 h, Fig. 2N) and barnacles (72.4% of initial concentration after 6 h, Fig. 2O) was observed but with a lower rate. Settling pond by decreasing Chl-*a* content to 94.3% of the initial amount did not efficiently reduce it after 6 h.

Table 2: Concentrations of different water quality parameters (mean±SD) in the shrimp effluent (pre-treatment sample), settling ponds and various treatments after six hours of filtration (different letters indicating significance difference between cases). Data for one and three hours after filtration are not presented in the table and can be followed in Fig. 2.

Treatment	NOx (mg L^{-1})	Ammonia (mg L^{-1})	Total N (mg L^{-1})	Total P (mg L^{-1})	Chlorophyll *a* (µg L^{-1})
Effluent	0.035±0.004[a]	0.262±0.027[a]	2.47±0.07[a]	0.154±0.006[a]	24.44±2.02[a]
Settling pond	0.029±0.010[b]	0.206±0.038[b]	2.04±0.25[b]	0.129±0.017[b]	22.47±4.73[a]
Oyster-Low	0.031±0.008[b]	0.328±0.066[c]	2.11±0.27[b]	0.114±0.007[b]	14.40±6.13[bc]
Oyster-Med	0.025±0.008[b]	0.374±0.067[c]	1.82±0.19[c]	0.100±0.006[c]	10.73±4.89[b]
Oyster-High	0.027±0.007[b]	0.387±0.083[d]	1.74±0.19[c]	0.096±0.005[c]	09.53±4.80[b]
Comb.-Low	0.029±0.001[b]	0.264±0.007[c]	2.29±0.13[b]	0.113±0.005[b]	15.30±1.48[c]
Comb.-Med	0.025±0.001[b]	0.264±0.020[c]	2.12±0.08[b]	0.102±0.002[c]	10.63±1.23[b]
Comb.-High	0.025±0.001[b]	0.293±0.013[d]	1.89±0.09[c]	0.099±0.004[c]	12.80±1.54[b]
Barnacle-Low	0.029±0.003[b]	0.248±0.025[c]	1.87±0.13[c]	0.104±0.005[b]	18.37±2.15[ac]
Barnacle-Med	0.028±0.002[b]	0.257±0.030[c]	1.82±0.14[c]	0.099±0.006[c]	18.13±2.44[ac]
Barnacle-High	0.029±0.001[b]	0.260±0.034[c]	1.84±0.09[c]	0.085±0.006[d]	16.40±2.07[c]

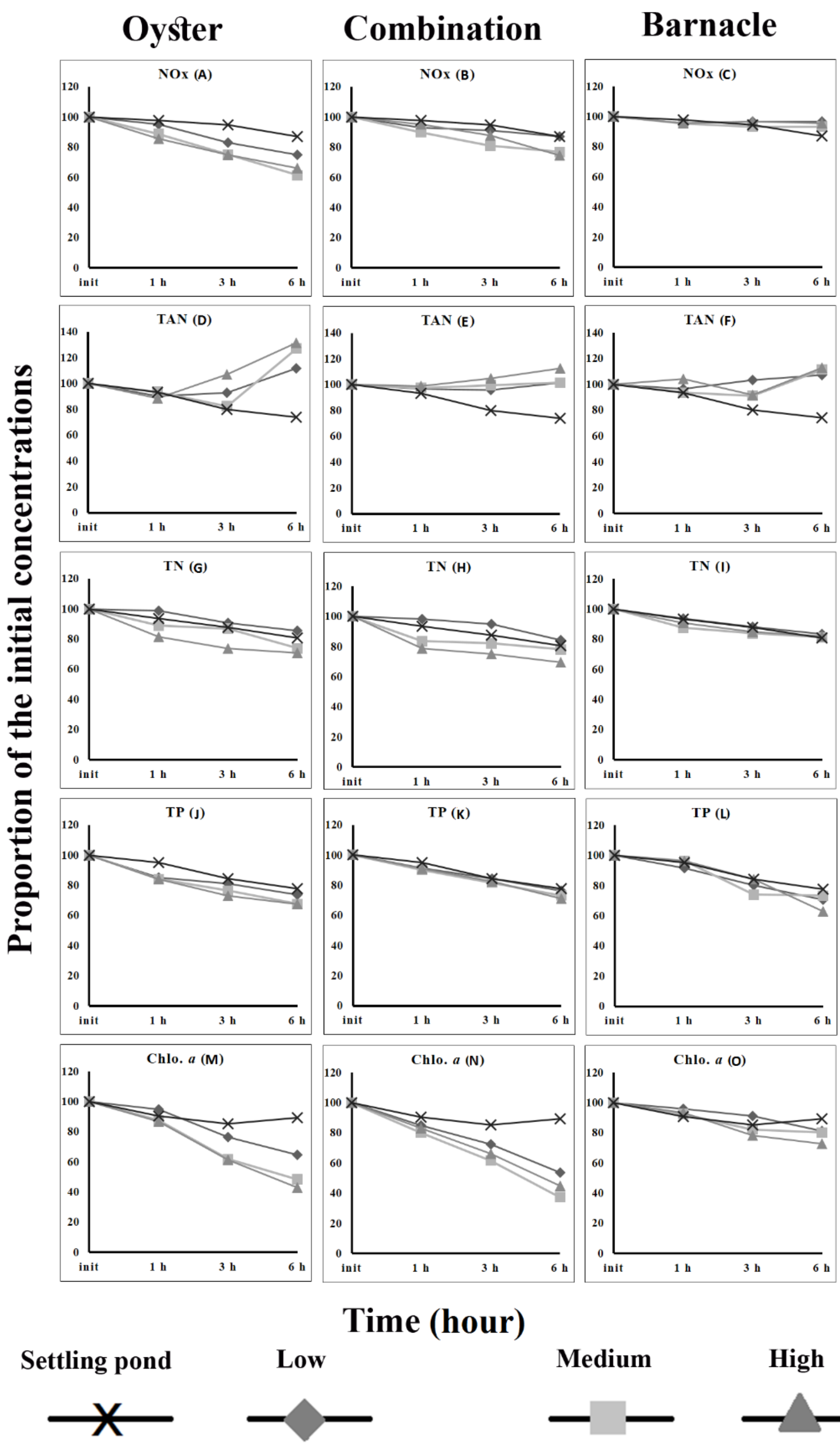

Figure 2: Changes (proportions of the initial concentrations in %) in NOx (A, B, C), ammonia (D, E, F), total nitrogen (G, H, I), total phosphorus (J, K, L) and Chlorophyll *a* (M, N, O), in the experimental tanks containing oysters, combination of the two species and barnacles respectively, during the course of experiments.

Discussion

Settling pond, in the present study, exhibited a considerable effect in reduction of TN, TP and Chl-*a* in shrimp farm wastewater. This approach was, however, not as effective as ponds containing bio-filters. Nutrients were lower in the ponds contacting barnacles,

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oysters and combination of the two species among which, oysters showed the highest efficiency in nutrients and Chl-*a* removal. The results showed that TN, TP and Chl-*a* diminished respectively to 70.6%, 67.7% and 40.9% of the initial concentrations in oyster treatments. The observed higher ability of *S. cucullata* in TN, TP and Chl-*a* removal compared to *A. amphitrite* may be partially due to the filtration mechanism in bivalves in which a huge part of organic and inorganic suspended matter is removed from the water column in the forms of faeces and pseudo-faeces (Shumway *et al.*, 1985).

The capability of oysters in nutrient removal from aquaculture wastewaters is different among various species. Jones and Preston (1999) indicated that oyster (*Saccostrea commercialis*) filtration, at a density of 24 individuals per 34 L tank, resulted in a reduction of TN, TP and Chl-*a* concentrations to 80%, 67% and 8% of the initial concentrations after a period of 2 h. Kinne *et al.* (2001) used the oyster *Crassostrea virginica* to remediate effluents of an intensive shrimp farm. The authors demonstrated that this oyster can effectively eliminate Chl-*a*. Jones *et al.* (2001) using an integrated treatment process, applied *S. commercialis* to remediate shrimp farm effluent. Based on the results, this species could reduce total Kjeldahl nitrogen, TP and Chl-*a* to 67.3%, 62.9% and 8.5% of the contents at the beginning within 24 h. *Crassostrea rhizophorae* and *C. gigas* in a study of Ramos *et al.* (2009), decreased Chl-*a* content to 0.0% and 17.6% of the initial concentration in shrimp wastewater after 6 h. In addition to filter-feeding, oyster shells of living or dead specimens have the capability to remove phytoplankton and nutrients from the water column (Kwon *et al.*, 2004; Caffrey *et al.*, 2016). As well as settling ponds, oysters can be used in raceways to filter phytoplankton from water (Kinne *et al.*, 2001) and even may show a higher efficiency than being used in settling ponds (Jones *et al.*, 2002). In contrast to oysters, barnacles did not exhibit a remarkable performance in nutrient removal. The potential use of barnacle, *Balanus* (*Amphibalanus*) *amphitrite* for eliminating P and N from the Salton Sea was studied by Geraci *et al.* (2008). The authors deployed hard substrates in the water column for barnacle larval settlement, and periodically harvested the adult individuals grown on the provided substrates. The results indicated that this species did not have enough efficiency to remove the huge amount of N and P from this water body.

Changes in nutrients and Chl-*a* concentrations in settling pond seem to be due to microbial activities and physical precipitation (Erler *et al.*, 2004). The microbial community, including eukaryotes and prokaryotes, could consume a considerable part of organic (e.g. urea, amino-acids) and inorganic (e.g. ammonia, nitrate) nutrients derived from shrimp culture process (Wheeler and Kirchman, 1986; Middelburg and Nieuwenhuize, 2000). These microorganisms can assimilate

[DOR: 20.1001.1.15622916.2020.19.4.25.3]

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nutrients, therefore transform dissolved forms to particles with larger sizes (i.e. microbial biomass), which could be assimilated through filter-feeding or precipitated in settling ponds (Teichert-Coddington *et al.*, 1999; Castine *et al.*, 2012). In the present study, precipitation of phytoplankton, N and P compounds in settling ponds was not as effective as bio-filters in terms of nutrients and Chl-*a* removal. In order to increase the efficiency of settling ponds in nutrient removal from aquaculture effluents, using biological methods in conjunction with settlement ponds is recommended (Castine *et al.*, 2012). Filter-feeders can be used to extract nutrients from water rather than being precipitated in settlement ponds floors, through consuming a part of bioavailable nutrients. As *S. cucullata* is an edible oyster, employing this species can be considered as a new source of income for shrimp farmers. Growing farm numbers and stocking density of farms might result in the introduction of more nutrients to the adjacent ecosystems (Thomas *et al.*, 2010). In addition, at the final phase of shrimp growth, discharging nutrients from culture ponds would remarkably increase (Teichert-Coddington *et al.*, 1999; Costanzo *et al.*, 2004). Given these facts, settling ponds alone may not have required efficiency, and using oysters as the method with the highest efficiency should be contemplated.

Despite the reduction in TN, the concentration of TAN increased in settling ponds and ponds containing oysters and barnacles. Ammonia excretion by barnacles and oysters (White and Walker, 1981; Jones *et al.*, 2002) and ammonia production through mineralization of organic matter (Hargreaves, 1998; Erler *et al.*, 2007), along with grazing phytoplankton, as ammonia consumers, by filter-feeders (Hargreaves, 1998), seems caused a rise in ammonia concentration. Since ammonia is considered as a toxic substance for aquatic organisms (Martinelle and Häggström, 1993; Barbieri, 2010) and higher oyster densities could lead to more ammonia release, therefore, lower densities of oysters might be a more suitable choice. Based on the results, medium density of oysters (0.54 oysters L^{-1}) could have the relatively equal efficiency compared to the high density (0.80 oysters L^{-1}). Therefore, as the medium density of oysters produces less TAN and lower stocking density of oysters requires less effort and imposes fewer costs, using the medium density seems to be sufficient.

In conclusion, there is a potential to apply settling ponds with 6 h retention time to reduce nutrients and phytoplankton loads of shrimp effluent. Nevertheless, to achieve higher efficiency in nutrients and phytoplankton removal using oyster *S. cucullata* within settling ponds is recommended.

Acknowledgements
The present study was supported by a grant from Shahid Beheshti University. Iran Shrimp Research Centre staffs are gratefully acknowledged for their support and participation in the study.

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