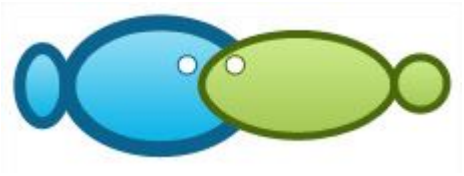

Determination of an appropriate ratio of N:P for optimisation of algal development in fertilizer ponds

Nguyen V. Hoa, Dao T. Nhi

Department of Coastal Aquaculture, College of Aquaculture and Fisheries, Cantho University, Cantho, Vietnam. Corresponding author: N. V. Hoa, nvhoa@ctu.edu.vn

Abstract. The purpose of this study is to examine the effect of nitrogen and phosphorus ratios ($N:P_s$) on the development of marine algae in fertilized ponds, which are considered to be natural food for *Artemia* farming. Phytoplankton composition and abundance were determined through qualitative and quantitative assessments. The experiment comprised four treatments, in triplicate (i.e. treatments 1, 2, 3 and 4, with ratios of N:P = 3, N:P = 6, N:P = 9 and N:P = 12, respectively); fishmeal (2^{nd} grade) was used at the rate of 30 g m^{-3}, while urea and DAP were combined at the rate of 6 g m^{-3}, with urea and DAP accounting for 4.93 g and 1.07 g, respectively. Study results showed that the average density of algae varied from $637x10^3$ to $2,434x10^3$ cells mL^{-1}. Algae density produced by the N:P = 6 treatment was the highest (i.e. $2,434x10^3$ cells mL^{-1}), and there were statistically significant differences ($p < 0.05$) among the treatments. In total, 52 algal species were found: 21 belonging to Bacillariophyta; 21, to Chlorophyta; 7, to Cyanophyta; 1, to Dinophyta; and 2, to Euglenophyta. Additionally, algal compositions were 38, 32, 35 and 27 species observed in four different treatments (N:P = 3, N:P = 6, N:P = 9 and N:P = 12, respectively). The dominant genera were typically *Nitzschia, Thalassiosira* (Bacillariophyta), *Tetraselmis* (Chlorophyta) and *Oscillatoria, Lyngbya* (Cyanophyta). As results demonstrated that algae developed well at treatment N:P = 6, this could be considered a suitable treatment for fertilizer pond management applied in *Artemia* farming.

Key Words: N:P ratio, phytoplankton, fertilizer pond.

Introduction. Nitrogen and phosphorus are the two key elements for algae development in most water bodies. Kim et al (2007) concluded that high nitrogen levels and low N:P ratios were favourable for cyanobacterial dominance in a shallow hypertrophic reservoir, and that a higher level of nitrogen concentration in the water column is likely to induce critical P-limitation on phytoplankton growth. As reviewed by Rasdi & Qin (2014), N-limitation usually results in low protein content and high carbohydrate or lipid storage, while P-limitation can also shift the relative contents of protein, lipid and carbohydrate in algae cells. Additional nitrogen at appropriately high levels (i.e. 18.53, 24.7 and 30.88 $mg.L^{-1}$) when culturing *Spirulina platensis* helped to enhance maximal biomass (4.90±0.12, 4.79±0.11 and 4.35±0.28 g L^{-1}, respectively) compared to low nitrogen levels (6.18 and 12.35 mg L^{-1}) and, therefore, to lead their respective lower biomass (3.06±0.27 and 3.46±0.04 $g.L^{-1}$, respectively) at day 8 (Tran 2013). However, it is well-known that the Redfield ratio of N:P = 16:1 (Redfield et al 1963) is commonly used to evaluate nutritional status (N, P) in a number of marine ecosystems; when N and P appear differently from the above ratio, this will lead to an imbalance in N or P in the water body and affect the growth of algae. Lagus et al (2004) observed that *Chaetoceros* sp. could develop at low nutrient levels, but required an N:P ratio of at least 38.1-39.1. Similarly, *Chlorococcales* sp. was found to be well-developed at N:P = 20-50:1, while Cyanophyta was dominant at N:P = 5-10:1 (Bulgakov & Levich 1999). The same observation was made by Smith et al (2006), who recorded that the N:P ratio is a factor affecting algal community structure. Another research study on the ratio of N:P in soil and water environments related to the development of *Chaetoceros calcitrans* proposed that an N:P ratio in the range of 4-44:1 is considered suitable for the optimal growth of the alga (Thu et al 2008). The authors also suggested that the differences in algae

density among culture environments was caused primarily by the effect of nitrogen sources and the diffusion of phosphorus from the bottom soil, as well as the providing of other nutrients into the culture environment, which in turn, lead to a difference in the ratio of N:P in the water environment. According to Rasdi & Qin (2014), when manipulating the N:P ratio to 5:1, 10:1, 20:1, 30:1, 60:1 and 120:1, respectively, and based on an F/2 formula for the study of *Tisochrysis lutea* and *Nannochloropsis oculata*, they found algal growth at different N:P ratios to be similar in both algal species. However, the N:P ratio of 20:1 favoured algal growth and protein content, while in the N:P ratio of 120:1, algal growth and protein synthesis were reduced, but lipid was increased in both algae species. They also concluded that N:P ratio manipulation is an effective strategy for changing the biochemical composition of algae, and that N or P limitation tends to lower the polyunsaturated fatty acid (PUFA) content in algae. Fong et al (1993) studied the N vs P limitation of algal biomass in shallow coastal lagoons via set-ups comprising different N:P ratios (i.e. 1, 5, 15, 30 and 60) and four concentrations (i.e. low, medium, high and very high); N and P were provided by nitrate ($NaNO_3$) and orthophosphate ($NaHPO_4$), at a salinity of 30 ‰ . They found that the N- vs P-limitation in shallow coastal lagoons was influenced not only by the supply rate per nutrient, but also by season and the potential composition of the algal community, and that these in turn are possibly constrained by many physical factors, including depth of the water, mixture patterns and climatic condition.

A high content of HUFA (highly unsaturated fatty acids) in *Artemia* cysts of various samples revealed a distinct variability among different strains and within strains, both between and within years (Leger et al 1986; Lavens et al 1989). Vos et al (1984) recorded varying fatty acid profiles when San Francisco Bay *Artemia* were inoculated in various salt ponds in Asia. In addition, several authors already demonstrated that zooplankton organisms, including *Artemia*, mainly reflect the fatty acid pattern of their food (Leger et al 1986). Therefore, manipulating the N:P ratio for fertilizer ponds is very crucial and may help in shaping the desired algae, which are appropriate in terms of dimension, development and, especially, HUFA content, as required for the production of high quality *Artemia* cysts. *Artemia* cysts are considered as excellent live food for a number of shrimp/fish larvae, especially for marine shrimps (Sorgeloos et al 1986; Lavens & Sorgeloos 1996). The aim of this study is to examine the effect of nitrogen and phosphorus ratios (N:Ps) on the development of marine algae in fertilized ponds, which are considered to be natural food for *Artemia* farming.

Material and Method. This study was conducted in the Artemia Experimental Field Station (Can Tho University) in the Vinh Chau solar saltworks, Soc Trang province (Mekong Delta) during January 8 to February 4, 2016. Materials used in this investigation comprised the following: chicken manure, fish meal (2nd grade) (see Table 1), urea (46%), DAP (diammonium phosphate, N:P:K = 18:46:0%), phytoplankton net (mesh size: 25-30 µm), basket, pet bottles (1 L), graduated cylinder 100 mL, formalin (38-40%), microscope (Olympus bran, option of 4/10/40/100x Plan anti fungus objectives. Model: CX22RFS1. Made in China), Bürker chamber (Depth = 0.100 mm; Surface of smallest square = 0.0400 mm^2; minimal cell concentration =106 cells mL^{-1}. Made in Germany), pipet (Biohit, 1-5 mL, made in Germany), lame (Glass, 5 inches, made in Germany) and lamella (Glass, 24x24 mm – 7501004 – Isolab, made in Germany), thermometer (Glass, 0-100°C, made in China), refractometer (Atago, 0-250 ppt, Master 528M, made in Japan), Secchi disc, scale (Sartorius CP224S-230VAC CP-Series Analytical Balances, 220g x 0.1 Mg 220 V. Made in China), screening and refrigerator (Toshiba 250L, Made in Japan).

Table 1

Nitrogen and phosphorus in chicken manure and (2^{nd} grade) fish meal (mg g^{-1} dried weight)

Item	*N (mg g^{-1})*	*P (mg g^{-1})*
Chicken manure	2.32±0.67	5.59±2.82
Fish meal (2^{nd} grade)	8.24±0.08	1.17±0.04

Note: values (n = 3) represent mean and standard deviation.

The experiment was set up with 4 treatments (three replicates each): treatment 1: 330 g urea and 120 g DAP (N:P = 3:1); treatment 2: 381 g urea and 69 g DAP (N:P = 6:1); treatment 3: 402 g urea and 48 g DAP (N:P = 9:1); and treatment 4: 412.5 g urea and 37.5 g DAP (N:P = 12:1), respectively. In addition, the amount of fish meal (2^{nd} grade) in the 4 treatments was fixed at the rate of 30 g m^{-3}. Manipulation of N:P in the different treatments was based on the N, P contents in urea and DAP, as the total amount of fertilizer was constantly maintained at 6 g m^{-3}.

The experiment was conducted in twelve 150-m^2 earthen ponds, with water depths of 50 cm and salinity of 30 g L^{-1}, and lasted for 11 days (until the algae collapsed).

Ponds were emptied, and trash fishes as well as predators were removed; pond bottoms were sun-dried for 2–3 days and then refilled with seawater (salinity, 30 g L^{-1}), and water level was maintained at 50 cm from the bottom before experimental set-up. Predators were eliminated by saponin (15 mg L^{-1}) in order to remove small crustaceans (e.g. copepod, mysis). Saponin was applied at noon time or in the early afternoon (at high temperature) in order to enhance its toxicity. Before experimental set-up fresh seawater was filled into the ponds via screening of 1 mm mesh size to prevent trash fishes and predator penetration prior experimental set-up.

The total amount of urea and DAP was 6 g m^{-3}, wherein urea and DAP were combined in accordance with the set-up. Periodic fertilization occurred on day 1, day 4, day 7 and day 10 throughout the experiment. Fish meal (2^{nd} grade) was applied once per week and on day 0 (starting) and day 7 of the experiment at the rate of 30 g m^{-3}.

Parameter monitoring. Water quality and green water were evaluated through appropriate sampling methodology.

a. Quantitative sampling. Water sampling was conducted at 8 am daily and at five sampling points: four in the pond corners and one in the middle of the pond. A two-litre sample was collected at each point, followed by a one-litre subsample that was taken after mixing well; samples were preserved by 2-4% formaldehyde and analysed within a month.

b. Qualitative sampling. Green water was sampled during every three-day interval by using a phytoplankton net (mesh size, 25-30 µm) and by pushing the sampling net in zigzags. After sampling, algae stuck on the net were washed and collected into the sampling bottle attached beneath. Algae samples were kept in bottles and preserved with 2-4% formaldehyde.

c. Water parameters. A litre of pond water was sampled and kept at 4ºC prior to analysis. Periodic sampling was conducted every 3 days for environmental parameters of TAN (Total Ammonia Nitrogen), NO_3- (nitrate), PO_4^{3-} (phosphate), TN (Total Nitrogen), TP (Total Phosporus) (APHA 1998) and chlorophyll *a*. For chlorophyll a analysis, water samples were filtered using filter paper (GFF 25 mm, 0.2 µm) in the field station and analysed in the chemical laboratory (APHA 1998).

Analytical methodology

Quantitative sample. Algae were counted using a microscope with a Bürker chamber. Counting protocol and determination of algal density were in accordance with Coutteau (1996):

$$\text{Number of cells } (mL^{-1}) = [(n_1+n_2)/160]*10^6*d,$$

where: n_1 and n_2 are the number of cells in the upper and lower chambers, respectively, and d is the dilution factor.

Qualitative sample. Collected samples, after preservation with formaldehyde, were examined under a microscope (magnification, 40×) in order to classify the algal species

(Shirota 1966). Algal images may be needed for description of their morphological, size measurement and identification.

Appearance frequency followed the scale of Scheffer & Robinson (1939). When algae appeared to be > 60% in the specimen, they would be marked as 'a lot of/so many' with the sign of (+++); when they appeared in the range of 30-60%, they would be marked as 'many' with the sign of (++); and when they were < 30%, they would be marked as 'seldom' with the sign of (+).

Environmental parameters were measured by appropriate methods: TAN was analysed by the phenate method (APHA et al 1998); NO^{3-}, by the sulfosalicylic acid method; PO_4^{3-}, by the $SnCl_2$ method (APHA et al 1998); TN, by the Macro-Kjeldahl method (TKN, TP water sample); TP, by the Macro-Kjeldahl method (TKN, TP water sample); and chlorophyll *a*, via colorimetric analysis by colour spectrum (Nusch 1980), after extraction by acetone.

Physical parameters were measured daily at various points. Temperature was measured twice per day – at 7 am and 2 pm – by thermometer; salinity was recorded once per day – at 7 am – by refractometer; turbidity of green water was measured once per day – at 2 pm – by Secchi disc; and, lastly, pH was recorded with a pH meter every three days at 7 am.

Data analysis. Excel 2010 spreadsheet software was used to calculate means and standard deviations. Tukey HSD test in STATISTICA 7 was applied in order to statistically compare treatments, at a significance level of $p < 0.05$.

Results

Water quality. Average temperatures in the morning (7 am) in treatments 1, 2, 3 and 4 were 25.4±1.2°C, 25.3±1.2°C, 25.4±1.1°C, 25.3±1.2°C, respectively. At 2 pm, temperature varied according to treatment, as 29.4±1.8°C, 30.5±1.7°C, 29.5±1.7°C and 29.7±1.2°C, respectively. Temperature throughout the experiment was rather stable, and a non-significant difference ($p > 0.05$) among treatments was found; thus, it was considered to be an appropriate temperature for algae to develop.

The salinity variation of treatments 1, 2, 3 and 4 were 34.4±1.1 g L^{-1}, 34.5±1.0 g L^{-1}, 34.4±1 g L^{-1} and 34.4±12 g L^{-1}, respectively (Figure 1). Increased salinity towards the end of the experiment was denoted as linked with the occurrence of elevated air temperature and evaporation.

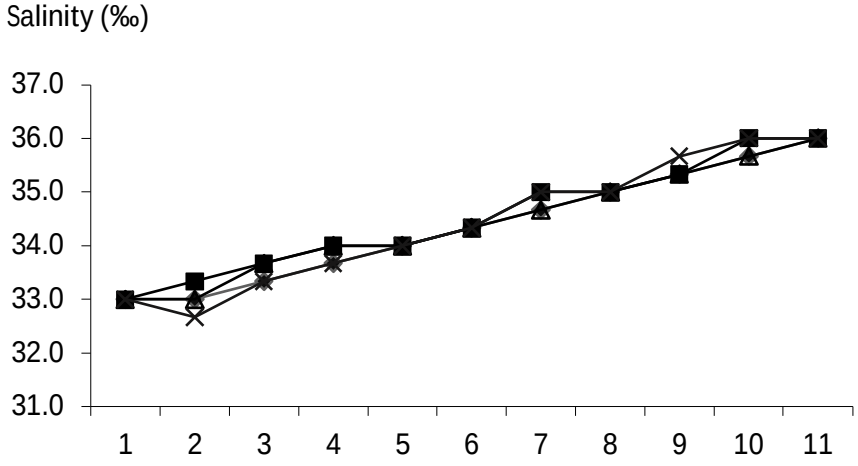

Figure 1. Variation of salinity throughout the experiment.

Average transparency of treatments 1, 2, 3 and 4 were 33.3±11.7 cm, 32.0±13.1 cm, 33.0±11.6 cm and 33.4±11.9 cm, respectively (Figure 2). The lowest transparency was 18.3±1.53 cm in treatment 2 (N:P = 6), when algae displayed their highest density (2.43±0.07 x 10^6 cells mL^{-1}) at day 7.

Variation of pH in treatments 1, 2, 3 and 4 were 9.09±0.2, 9.11±0.22, 9.08±0.23 and 9.12±0.16, respectively. The value of average pH was highest – 9.43±0.05 – at day 7 of the experiment. In general, pH was elevated in accordance with the development of algae by time, but stayed within the suitable range for algae to develop.

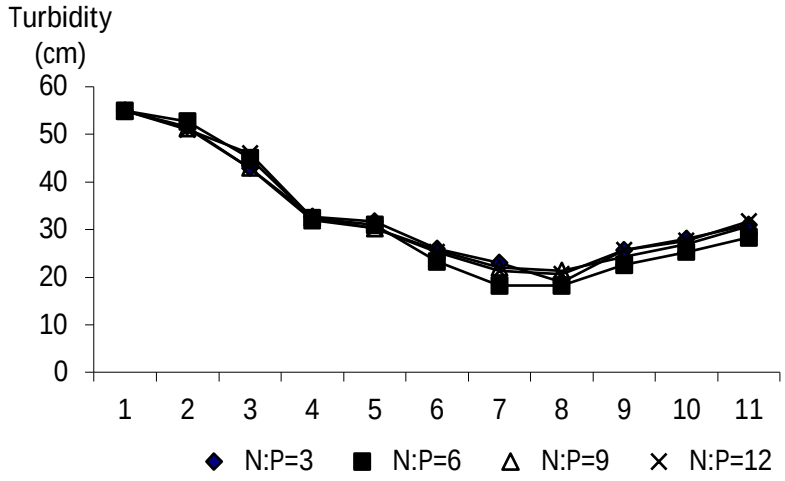

Figure 2. Variation of turbidity in different treatments.

The initial concentration of $N\text{-}NH_4^+$ was 0.14 mg L^{-1}, and after fertilization, $N\text{-}NH_4^+$ values of all treatments were increased; it was clearly observed that $N\text{-}NH_4^+$ had a negative correlation to algal development (i.e. high level of $N\text{-}NH_4^+$ at low algal density and vice versa). There were significant differences in $N\text{-}NH_4^+$ among treatments ($p < 0.05$) from day 4 onwards; average $N\text{-}NH_4^+$ of treatments 1, 2, 3 and 4 throughout the experiment were 1.53±0.9 mg L^{-1}, 2.0±1.3 mg L^{-1}, 2.58±1.6 mg L^{-1} and 3.02±19 mg L^{-1}, respectively. The treatment of N:P = 12 (treatment 4) had the highest average level of $N\text{-}NH_4^+$ (i.e. 4.14±0.02 mg.L^{-1}) at day 10 (Figure 3); this may related to a higher rate of fertilizer application when set up, as well.

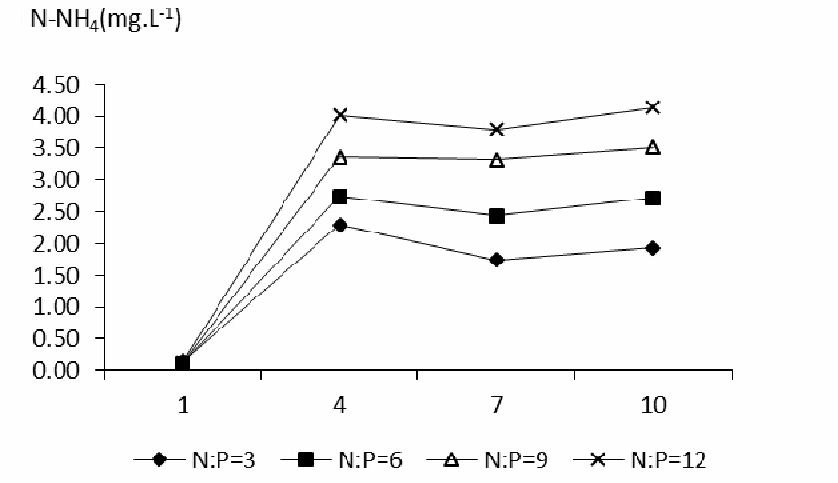

Figure 3. Variation of NH_4^+ in the experiment.

Nitrate is an appropriate nutrient source that can be suitably absorbed and is non-toxic to phytoplankton; $N\text{-}NO_3^-$ commonly exists in seawater in the range of 0.2-0.4 mg L^{-1} as indicated by Nguyen (1994). Average $N\text{-}NO_3^-$ concentrations in the treatments of N:P = 3, N:P = 6, N:P = 9 and N:P = 12 throughout the experiment were recorded as 0.6±0.3 mg L^{-1}, 0.81±0.5 mg L^{-1}, 1.01±0.6 mg L^{-1} and 1.21±0.7 mg L^{-1}, respectively. The treatment of N:P = 12 reached the highest value (i.e. 1.64 ± 0.03 mg.L^{-1}) compared to the others at day 10. At day 7, $N\text{-}NO_3^-$ declined in treatments 1, 2 and 3, but not in treatment 4 (N:P = 12) due to an increase in algae densities. At day 10, algae started to

collapse, and as the dead algae decomposed, they then released nitrogen into environment and, therefore, $N\text{-}NO_3^-$ increased again (Figure 4).

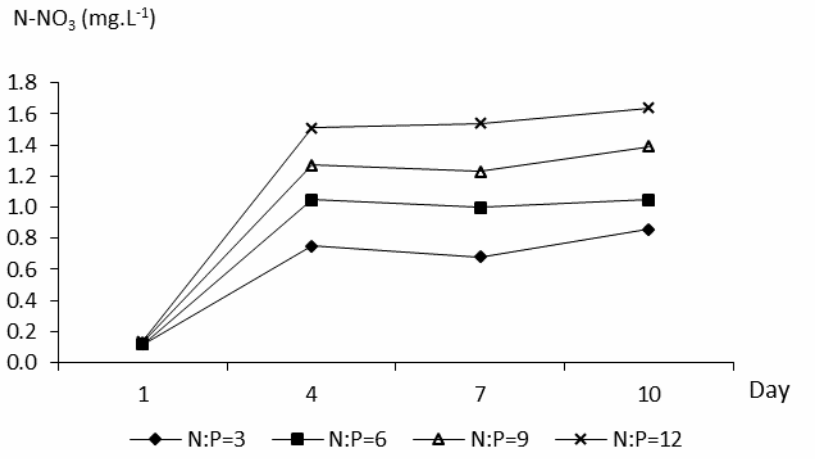

Figure 4. Variation of $N\text{-}NO_3^-$ in the experiment.

Variations of average PO_4^{3-} in treatments 1, 2, 3 and 4 throughout the experiment were 0.31±0.17 mg L^{-1}, 0.2±0.09 mg L^{-1}, 0.16±0.05 mg L^{-1} and 0.14±0.04 mg L^{-1}, respectively, of which treatment 1 (N:P=3) displayed its highest PO_4^{3-} concentration (i.e. 0.43±0.02 mg L^{-1}) at day 10 (Figure 5). There were similar patterns for all treatments, as PO_4^{3-} was low from the beginning (i.e. varied from 0.05 to 0.08 mg L^{-1}) and increased quickly at day 4 after fertilization, then declined slightly from day 4 to day 7, which coincided with the maximal growth of algae. However, algae collapsed starting from day 8 onwards, and dead algae that decomposed to release PO_4^{3-} into the environment, except for treatment 4 (N:P = 9), were recorded. At day 10, the PO_4^{3-} concentration remained rather high, as 0.43±0.02 mg L^{-1}, 0.26±0.04 mg L^{-1}, 0.16±0.04 mg L^{-1} and 0.16±0.04 mg L^{-1} in consecutive treatments of 1, 2, 3 and 4, respectively.

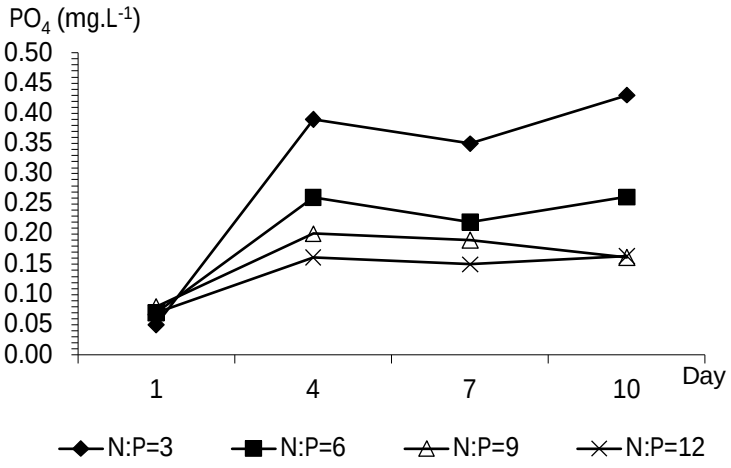

Figure 5. Variation of PO_4^{3-} in the experiment.

An average concentration of TN of treatments N:P = 3, N:P = 6, N:P = 9 and N:P = 12 were 5.24±2.76 mg L^{-1}, 6.03±3.52 mg L^{-1}, 5.84±2.99 mg L^{-1} and 8.04±4.63 mg L^{-1}, respectively throughout the experiment. The treatment N:P = 12 had the highest TN, attaining a level of 11.11±0.99 mg L^{-1} at day 10 (Figure 6). Initial TNs varied, ranging from 1.38±0.25 mg L^{-1}, 1.13±0.16 mg L^{-1}, 1.43±0.16 mg L^{-1} and 1.22±0.15 mg L^{-1} for treatments 1, 2, 3 and 4, respectively. The same tendency was observed as TNs increased after fertilization (day 4) and decreased at day 7, when algae developed tremendously. Algae were then to collapse from day 8 (except for treatment 1, N:P = 3),

and dead algae decomposed to release nitrogen into the environment to increase the TN levels for all treatments.

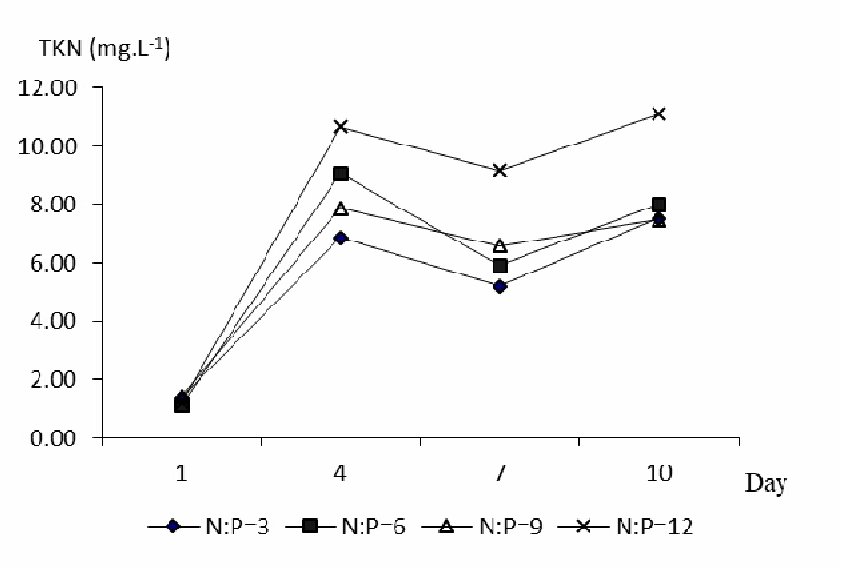

Figure 6. Variation of TN in the experiment.

Variations of average TP in the respective treatments were 0.73±0.45 mg L^{-1}, 0.45±0.19 mg L^{-1}, 0.35±0.16 mg L^{-1} and 0.33±0.12 mg L^{-1}, respectively throughout the experiment, in which treatment 1 (N:P = 3) displayed its highest level (i.e. 0.81±0.40 mg L^{-1}) at day 4 (Figure 7). TP varied from 0.13 to 0.18 mg L^{-1} for all treatments from the beginning of the experiment, and it then increased to 0.63±0.17 mg L^{-1}, 0.47±0.10 mg L^{-1}, and 0.45±0.15 mg L^{-1} for treatments 2, 3 and 4, respectively; however, the most TP was in treatment 1 (1.23±0.28 mg L^{-1}), which was almost two- to threefold compared to the others. The same patterns were observed as TP levelled down at day 7 due to algae development; however, TP in N:P = 3 remained higher than in the others. Then TP in treatments of N:P = 3 and N:P = 12 increased again afterwards, when algae collapsed, but TP in treatments of N:P = 6 and N:P = 9 continued going down at day 10.

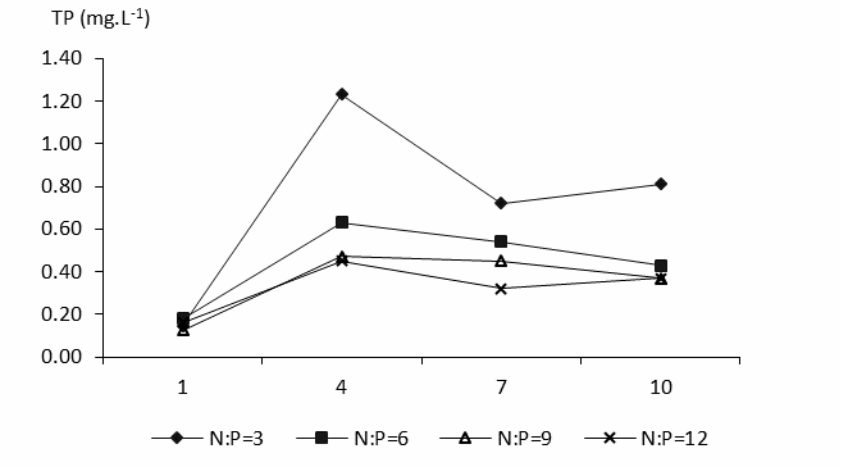

Figure 7. Variation of TP in the experiment.

The N:P of respective treatments of N:P = 3, N:P = 6, N:P = 9 and N:P = 12 varied in the ranges of 7.2-9.3, 6.3-18.6, 11.4-20.2 and 7.5-30, respectively, and increased towards the end of experiment. Through this, N:P = 3 displayed its limitation in nitrogen, as N:P=3 was always less than 16 (Redfield 1934), while N:P = 9 indicated less limitation of nitrogen than N:P = 6 and N:P = 12. However, N:P = 12 proved its limitation in phosphorous from days 4, 7 and 10, as N:P varied from 23.7, 28.7 and 30, and these were much higher than for treatments 2 and 3 (N:P = 6 and N:P = 9).

Phytoplankton quantifying. After fertilization, algae grew and reached their maximal chlorophyll *a* concentrations at day 7 of treatments N:P = 3, N:P = 6, N:P = 9 and N:P = 12 were 85.97±2.90 μg L^{-1}, 94.39±4.11 μg L^{-1}, 78.00±3.31 μg L^{-1} and 76.46±4.46 μg L^{-1}, respectively; of these, treatment N:P = 6 displayed its highest chlorophyll-*a* in correspondence to the maximum algae density (1.69±0.04 cells mL^{-1}) (Figures 8a, 8b and 9).

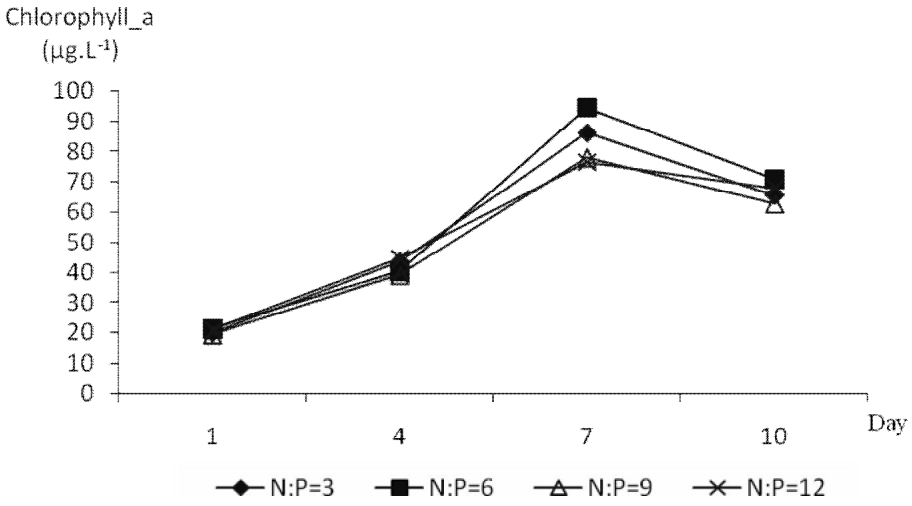

Figure 8a. Variation of chlorophyll *a* in the experiment.

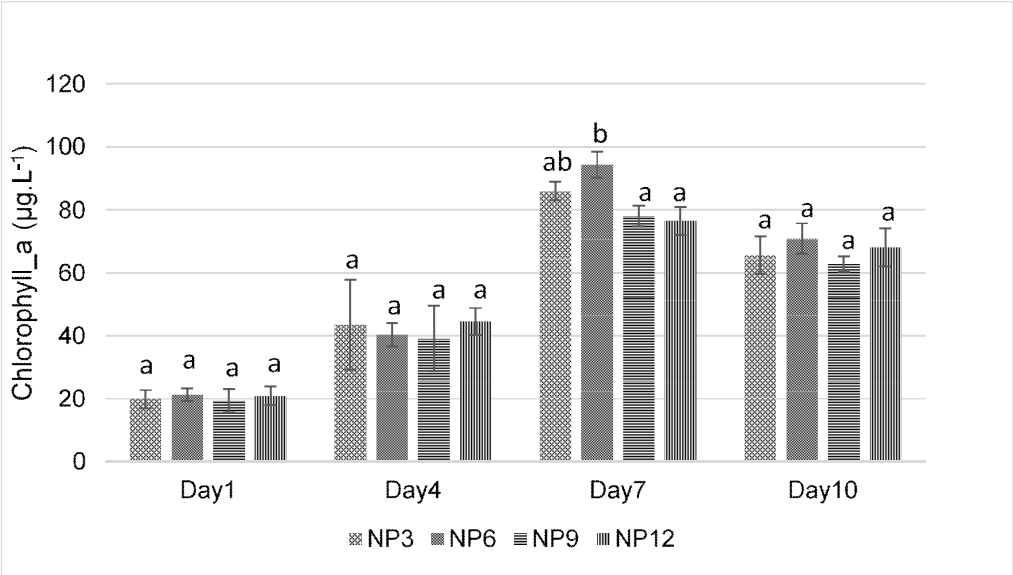

Figure 8b. Variation of chlorophyll *a* throughout the experiment.

However, N:P = 6 displayed a significant difference ($p < 0.05$) to N:P = 9 and N:P = 12, but not to N:P = 3. Moreover, the similar growth curves observed for all treatments (Figure 9) and their chlorophyll-*a* levels were all to decrease at day 10 (Figure 8a).

Algae increased and reached the highest densities in treatments N:P = 3, N:P = 6, N:P = 9 and N:P = 12 as 2.23±0.13 × 10^6 cells mL^{-1}, 2.43±0.07 × 10^6 cells mL^{-1}, 1.92±0.02 × 10^6 cells mL^{-1}, and 1.77±0.03 × 10^6 cells mL^{-1}, respectively (Figure 9) at day 7, except for N:P = 3, which occurred at day 8. Treatment N:P = 6 was the highest and most significant difference ($p < 0.05$) relative to the rest, followed by N:P = 9, which was also significantly different to N:P = 3 and N:P = 12; however the last two were not significantly different ($p > 0.05$) (Table 2). Remarkably, treatment N:P = 6 favoured the development of micro-algae rather than of filamentous algae; treatment N:P = 3 had a

similar growth curve, but the lowest algae density throughout the experiment compared to the others.

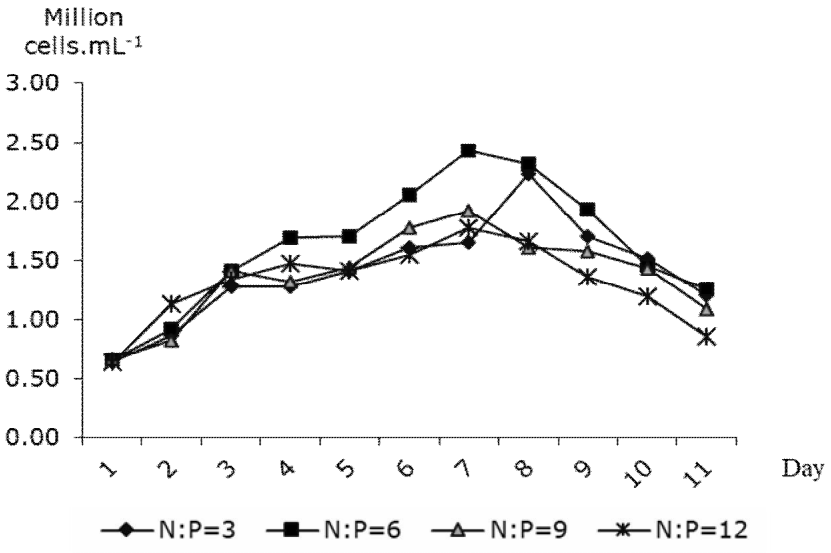

Figure 9. Variation of algae density in the experiment.

Phytoplankton composition. Six phyla of phytoplankton – Bacillariophyta, Cyanophyta, Chlorophyta, Chrysophyta, Pyrrophyta and Euglenophyta – were identified, representing proportions of 38, 13, 41, 2, 2 and 4%, respectively (Figure 10). Altogether, 52 species have been identified, of which Chlorophyta, Bacillariophyta, Cyanophyta, Euglenophyta, Pyrrophyta and Chrysophyta represented 21 species (41%), 20 (38%), 7 (13%), 2 (4%), 1 (2%) and 1 (2%), respectively.

In addition, the number of algal species in the four treatments (i.e. N:P = 3, N:P = 6, N:P = 9, N:P = 12) have been identified as 38, 32, 35 and 27, respectively (see Table 3), and it seems that higher N:P ratios have shaped the number of algal species – that is, the higher the N:P ratio, the lower the number of algal species. A high frequency of occurrence was observed with *Nitzschia acicularis*, *Thalassiosira weissflogii* and *Cyclotella meneghiniana* (Bacillariophyta); *Isochrysis galbana* (Chrysophyta); *Tetraselmis suecica*, *Tetraselmis chui* and *Entomoneis pulchra* (Chlorophyta); *Oscillatoria* sp., *Oscillatoria lanceiformis*, *Lyngbya birgei* and *Phormidium* sp. (Cyanophyta); and *Euglena acus* (Euglenophyta).

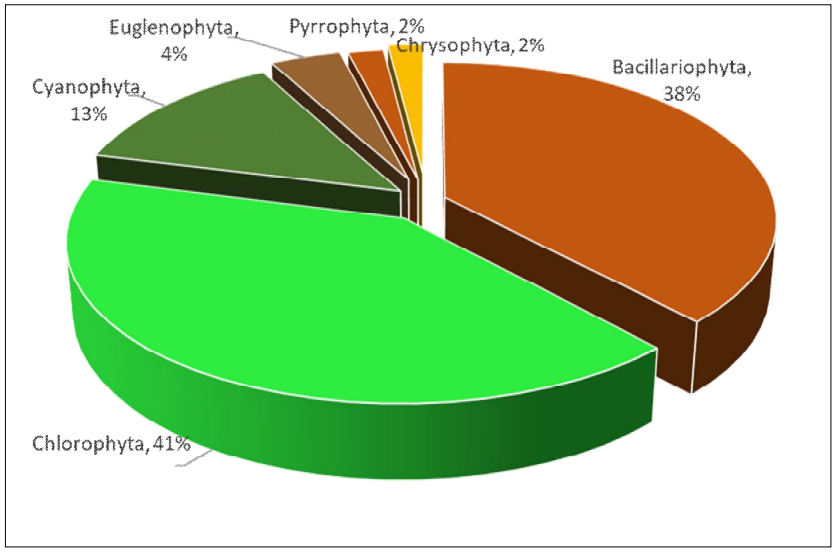

Figure 10. Algae composition of phytoplankton in the experiment.

Table 2

Algal development throughout the culture (n = 3)

Treatment	*Day 1*	*Day 2*	*Day 3*	*Day 4*	*Day 5*	*Day 6*	*Day 7*	*Day 8*	*Day 9*	*Day 10*	*Day 11*
NP3	0.64± 0.01^{ns}	0.87± 0.01^{ab}	1.27± 0.03^{b}	1.27± 0.06^{a}	1.41± 0.02^{a}	1.60± 0.05^{a}	1.65± 0.06^{a}	2.23± 0.13^{b}	1.93± 0.11^{c}	1.51± 0.05^{a}	1.20± 0.01^{ab}
NP6	0.66± 0.00^{ns}	0.92± 0.05^{b}	1.41± 0.01^{a}	1.69± 0.04^{c}	1.70± 0.02^{b}	2.06± 0.02^{c}	2.43± 0.07^{c}	2.32± 0.04^{b}	1.70± 0.14^{bc}	1.45± 0.02^{a}	1.25± 0.10^{b}
NP9	0.65± 0.01^{ns}	0.83± 0.02^{a}	1.41± 0.03^{a}	1.32± 0.02^{a}	1.77± 0.06^{b}	1.77± 0.06^{b}	1.92± 0.02^{b}	1.61± 0.15^{a}	1.58± 0.09^{ab}	1.43± 0.05^{a}	1.09± 0.04^{a}
NP12	0.64± 0.01^{ns}	1.13± 0.04^{c}	1.34± 0.08^{ab}	1.47± 0.05^{b}	1.54± 0.08^{a}	1.54± 0.08^{a}	1.77± 0.03^{a}	1.66± 0.07^{a}	1.34± 0.10^{a}	1.19± 0.03^{b}	0.86± 0.02^{c}

ns: non-significant difference. Means with different letters are significantly different at $p < 0.05$.

Table 3

Algae species observed in different C:N ratios

Phylum	*Treatment*							
	N:P = 3		*N:P = 6*		*N:P = 9*		*N:P = 12*	
	Species	*%*	*Species*	*%*	*Species*	*%*	*Species*	*%*
Bacillariophyta	16	42.1	15	46.9	15	42.9	14	51.9
Chlorophyta	15	39.5	12	37.5	12	34.3	5	18.5
Cyanophyta	4	10.5	4	12.5	6	17.1	6	22.2
Dinophyta	1	2.6	0	0.0	0	0.0	0	0.0
Euglenophyta	1	2.6	0	0.0	1	2.9	1	3.7
Chrysophyta	1	2.6	1	3.1	1	2.9	1	3.7
Total	38		32		35		27	

Discussion. Temperature and salinity are known to be critical factors that influence the development of algae as well as their composition in nature (Smith 2006). According to Renaud et al (2002), micro-algae are cultured outdoors in tropical zones, where daytime temperatures that vary between 25 and 35°C on a year-round basis represent a suitable range for growth. The same range of temperatures was recorded in the current study, which certainly favoured the development of algae. In addition, the salinity as recorded in our study was also suitable for marine algae, as they usually developed well in salinities ranging from 9 to 40 g L^{-1} (Sigaud & Aidar 1993). Transparency plays an important role among other factors for the growth and development of phytoplankton (Truong & Vu 2011). While lower transparency (i.e. in order to prevent light penetration into the water column) may cause a decline in algae photosynthesis, higher transparency may be linked to nutrient limitation (i.e. the creation of an oligotrophic environment). A fluctuation of transparency was observed in most of the water bodies, which was usually due to mineralized colloidal solids, suspended organic matter, micro-algae, wave action, tidal cycles and precipitation. However, the transparency that occurred in fertilizer ponds had a strong correlation to micro-algae development. In the current study, treatment N:P = 6 displayed the lowest transparency as higher algae density ($2.43 \pm 0.07 \times 10^6$ cells mL^{-1}) than the others. pH range for most cultured algal species is between 7 and 9, with the optimum range being 8.2-8.7, and at high algal density, pH could reach a level of 9 (Lavens & Sorgeloos 1996). pH in this study stayed in a suitable range, although higher pH levels were recorded in all treatments on day 4 and day 10, which coincided with the higher algae density period (see Figure 9).

It was observed that $N\text{-}NH_4^+$ and $P\text{-}PO_4^{3-}$ were parameters for assessment of potential productivity of algae in the fertilizer pond (Baert et al 1997; Boyd et al 2002; Smith 2006). Moreover, retarded growth of algae in the pond was observed, even though there were sufficient light conditions and suitable temperatures when a shortage of any nutrient element occurred (Wetzel 2001). $N\text{-}NH_4^+$ differentiation among treatments due to set-up, as well as the same patterns, remained to the end of the experiment (see Figure 3), although N:P = 6 was more favourable to algae development compared to the rest (see Figure 9). Similar variation was found for $N\text{-}NO_3^-$, but again, higher $N\text{-}NO_3^-$ in N:P = 9 and N:P = 12 did not enhance algae development. According to Mueller & Helsel (1999), water bodies in which the PO_4^{3-} level is less than 0.01 mg L^{-1} are considered oligotrophic; where it is 0.01-0.02 mg L^{-1}, mesoeutrophic; and over 0.02 mg L^{-1}, eutrophic. Additionally, the appearance of PO_4^{3-} in the water column depends on absorption and release processes in the bottom layer (Boyd 1998). Therefore, the PO_4^{3-} levels recorded in the current experiment would be considered eutrophic and thus accelerate algae development.

Results indicated that when N:P was lower than 16:1, the development of Cyanophyta would be favoured (Smith 1983, cited by Gross & Pfiester 1988). In aquaculture, and especially for fertilizer ponds, both N and P are very crucial elements, as in most cases nitrogen inputs are usually in the form of inorganic fertilizer (i.e. urea, DAP), manure, faeces and leftover food. The ratio of N:P is one of the key factors concerning algae development in the ponds (Bulgakov & Levich 1999; Geider & La Roche 2002; Smith et al 2006, cited by Anh et al 2009). In natural waters, there are common phytoplankton species that may occur year-round and that adapt to a wide range of N:P ratios (e.g. from 10:1 to 40:1) (Suttle & Harrison 1988; Wetzel 2001; Smith 2006). Redfield et al (1963) proposed that the suitable N:P atomic ratio for marine algae development is approximately 16:1, and that limitation of either N or P will lead to the negative effect of algae development. Similarly, Boyd & Daniels (1994) conducted a fertilization experiment in a shrimp pond and concluded that an N:P of less than 20 will accelerate algae development. Smith (2006) recorded the dependence of chlorophyll *a* on N and P in 92 different estuarine and coastal areas worldwide, and stated the importance of ratios of N and P (by moles) to the development of marine phytoplankton. Chlorophyll *a* levels in this experiment varied with the richness of algae per treatment in the fertilizer ponds. According to a study by Nguyen et al (2019), the highest algal density was obtained when N:P = 5, which ties closely to treatment N:P = 6 in this study. Moreover, both studies were conducted in the same field station, and the minor difference may be

because of different weather conditions; during the current experiment, a few days were recorded as being 'calm' (i.e. no wave action and less turbulence in the water column), and thus algae may not have received sufficient nutrients via turnover from water circulation.

In general, all algae developed according to the same pattern (i.e. highest densities of algae occurred at days 7-8 and then declined), but treatment N:P = 6 displayed maximal algae density and significant difference ($p < 0.05$) in comparison with the others.

A similar recording of phytoplankton composition appearing along the Vinh Chau coastline was found by Vu et al (2013), with Bacillariophyta being identified as the major group, followed by Cyanophyta and Euglenophyta; however, the third group in the current study is Chlophyta, instead of Euglenophyta. According to Anh et al (2009), 4 phyla – Bacillariophyta, Cyanophyta, Chlorophyta, and Dinophyta – were found in the solar saltworks of Baclieu when observing the algae composition under the two treatments of N:P = 5 and N:P = 10, and Chlorophyta was also the third group. According to Kummar et al (1974) (cited by Duong et al 2014), Cyanophyta, Bacillariophyta and Euglenophyta have a positive relationship to rich organic matter, while Chlorophyta was closely influenced by the high nitrogen level in the water environment. Rhee (1978) as well as Rhee & Gotham (1980) stated that the N:P ratio might control the development of Cyanophyta, as this influences the competition in terms of nutrient availability among micro-algae and cyanobacteria in the water body. When N:P is low (i.e. less than or equal to 5), the environment is suitable for nitrogen-fixing cyanobacteria; otherwise, Chlorophyta will be dominant when N:P is higher than 5. However, this was not the case of the dominant species at treatment N:P = 3 – for instance, an appearance of *Tetraselmis chui* (Chlorophyta) instead of Cyanophyta. With regard to algal species identification, Vu et al (2013) indicated that 125 species have been identified; Anh et al (2009) indicated that a total of 67 species have been found, and that the treatment of N:P = 10 presented 62 algal species, but only 52 algal species occurred in treatment N:P = 5, which contradicts what was found in this study (i.e. 38 species; see Table 3). As observed by Anh et al (2009) and Anh (2015), the proportions of algal species of Bacillariophyta, Cyanophyta, Chlorophyta and Dinophyta were 60-63%, 15-16%, 13-15% and 8-10%, respectively; again, differences in species occurred, as more Chlorophyta but less Bacillariophyta were found in the present study. Differences in algae composition as well as in species identified in the current study could possibly be due to the fact that 1) the initial wild algae entering the fertilizer pond were limited, 2) the current study was conducted on a short-term basis, 3) algae composition may be shaped by N:P ratios and 4) algae composition fluctuated by season, location … when compared to the survey of Vu et al (2013), as multi-sampling points in a wider habitat were sampled, but close to the observation of Anh et al (2009) in their study of a small-scale fertilization pond. A number of algae species have been identified and listed in previous studies: *Nitzschia acicularis, Thalassiosira weissflogii* and *Cyclotella meneghiniana* (Bacillariophyta); *Isochrysis galbana* (Chrysophyta); *Tetraselmis suecica*, *T. chui* and *Entomoneis pulchra* (Chlorophyta). It was indicated that *I. galbana* and *T. suecica* as a good food, as they promoted the survival and growth of *Artemia* (Coutteau & Sorgeloos 1992). In an experiment conducted with algae that was isolated from marine wild algae in Australia and that was to be used as food for *Artemia*, Thinh et al (1999) used 13 species as mono feed (treatment) for *Artemia*. The authors found that *Artemia* fed with *Cryptomonas* sp. displayed the best outcomes in terms of growth difference, while those fed with *Chaetoceros* sp., *Nephroselmis, Tetraselmis* sp. and *Nitzschia palacea* resulted in similar growth after 24 h as those fed with *I. galbana* (the control), which implies that micro-algae from both Bacillariophyta and Chlorophyta may promote the growth of *Artemia.* At treatment N:P = 6, most algal species belonged to Chlorophyta and Bacillariophyta, in which *T. chui*, *T. weissflogii* and *I. galbana* are suitable species in term of appropriate dimension (i.e. less than 50 µm) (see Table 4) and nutritional level (Brown et al 1989) for *Artemia* feeding (Sorgeloos et al 1986). *T. chui* and *N. acicularis* are two dominant species at treatment N:P = 9. Possibly, at a higher N:P ratio, the growth of *Tetraselmis* was favoured, as was similarly found for *Tisochrysis lutea* and

Nannochloropsis occulata (Rasdi & Qin 2014). *Oscillatoria lanceiformis, Phormidium* sp. and *Lyngbya birgei*, which belong to Cyanophyta, were the dominant species in treatment N:P = 12. They live in stagnant waters and experience tremendous development when eutrophication occurs, consequently creating 'lab-lab' (a complex of phylamentous algae, organic matter, zoo plankton, worms and bacteria), as described by Nguyen (2011). 'Lab-lab' occurring in *Artemia* ponds have a negative effect on *Artemia* survival, growth, reproduction and cyst/biomass collecting (Nguyen & Nguyen 2019).

Table 4

Dimension of dominated species in the fertilizer ponds

Species	*Dimension*
Tetraselmis suecica	Length: 8.4 µm; width: 4.8 µm
Tetraselmis chui	Length:20 µm; width: 14 µm
Isochrysis galbana	Diameter: 4.4 µm
Thalassiosira sp.	Length: 8-15 µm; width: 6-20 µm;
Nitzschia acicularis	Length: 30-100 µm; width: 3-4 µm
Entomoneis pulchra	Length: 49 µm; width: 23 µm
Oscillatoria sp.	Length: 75 µm

In general, Bacillariophyta and Chlorophyta were the dominant species in all treatments. Cyanophyta usually occurred when eutrophication appeared, while Euglenophyta were recorded toward the end of the experiment, when nitrogen and phosphorus increased as a possible result of algae collapse. Bacillariophyta dominated in treatment N:P = 6 and represented suitable algae species for *Artemia* in terms of dimension and high nutritional level. As a result, N:P = 6 is considered to be an appropriate treatment for application to a fertilizer pond in an *Artemia* culture system.

Conclusions and Recommendations. A combination of fish meal (2^{nd} grade) and inorganic fertilizer at different N:P ratios were introduced to fertilizer ponds with seawater to obtain 52 algal species, which belong to the 6 phyla of Bacillariophyta, Chlorophyta, Cyanophyta, Dinophyta, Euglenophyta and Pyrophyta, within which the dominant species were identified as 21, 20, 7, 2, 1 and 1 species, respectively.

After a week, treatment N:P = 6 maximised micro-algae development (i.e. 2,430 × 10^3 cells mL^{-1}), which are appropriate for *Artemia* feeding. Dominant species with suitable dimension and nutritional content as food for *Artemia* included *Tetraselmis chui*, *Tetraselmis suecica, Isochrysis galbana, Nitzschia acicularis* and *Thalassiosira weissflogii*. Therefore, the application of fish meal (2^{nd} grade) at the rate of 30 g m^{-3}, combined with inorganic fertilizer (i.e. DAP and urea at the rate of 6 g m^{-3}), into fertilizer ponds has been recommended for *Artemia* culture systems. Additionally, fine tuning of the fish meal as well as fertilization frequency when applied on a larger scale of *Artemia* culture should also be considered.

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Authors:
Nguyen Van Hoa, Department of Coastal Aquaculture, College of Aquaculture and Fisheries, Cantho University, Campus II, 3/2 Street, Nink Kieu District, Can Tho City, Viet Nam, e-mail: nvhoa@ctu.edu.vn
Dao To Nhi, Department of Coastal Aquaculture, College of Aquaculture and Fisheries, Cantho University, Campus II, 3/2 Street, Nink Kieu District, Can Tho City, Viet Nam, e-mail: nhim0614022@gstudent.ctu.edu.vn

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