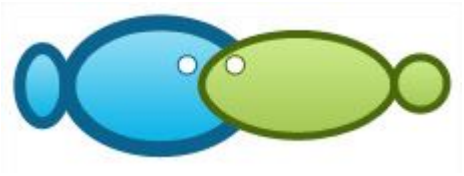

Determination of appropriate fertilisation frequencies for optimising wild algae development

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Abstract. This experiment was conducted to determine the appropriate fertilisation frequency for optimal wild microalgae growth in fertilising ponds. These wild microalgae serve as a natural feed for *Artemia* in culture ponds. The experiment was conducted in earthen ponds (100 m^2 each) and consisted of four treatments (T) with different fertilisation frequencies. T1: applying traditional fertiliser once per week (Trad_1perWeek); T2: applying formula fertiliser once per week (Formula_1perWeek); T3: applying formula fertiliser twice per week (Formula_2perWeek); T4: applying formula fertiliser seven times per week (Formula_Daily). Urea fertiliser (N) and DAP (P) were used as the main fertilisers and the same fertiliser dosage (i.e., a combination of 39.6 g mL^{-1} urea and 1 mg L^{-1} DAP per week) was applied for T2, T3 and T4, while T1 applied 3 g mL^{-1} urea and 1 mg L^{-1} DAP per week. At day 10, the highest algae density (2.81 x 10^6 cells mL^{-1}) was obtained in T4 (Formula_Daily) and was significantly different ($p < 0.05$) compared to the remaining treatments. After T4, the highest algae densities were obtained in T2 (Formula_1perWeek), T3 (Formula_2perWeek) and T1 (Trad_1perWeek) with densities of 1.89 x 10^6, 1.71 x 10^6 and 1.56 x 10^6 cells mL^{-1}, respectively. Therefore, the daily fertilisation for green pond in *Artemia* culture system is recommended.

Key Words: *Artemia*, fertiliser, fertilisation frequency, wild algae.

Introduction. *Artemia* is an excellent live food for aquaculture, especially for the early stages of shrimp fish larvae (Sorgeloos et al 1986). *Artemia* are distributed across a wide salinity range (i.e., 3 to 250 ‰) but do not occur in the marine environment. They are non-obligate filter feeders (Lavens & Sorgeloos 1996) and can filter most suspended particles in the water column (e.g., organic matter, bacteria, microalgae, etc.) with particle dimensions smaller than 50 µm (Van Stappen 1996). In nature, the growth of *Artemia* populations is decided by natural food such as microalgae (Zmora et al 2002). However, the appearance of filamentous algae may negatively affect *Artemia* feeding intake due to their larger particles. In *Artemia* pond culture, microalgae are developed in green ponds through the use of fertilisation (Nguyen & Nguyen 2019) and then supplied to *Artemia* ponds as feed (Nguyen et al 2020). Algae development in fertiliser ponds depends on several factors, including nutrients, light intensity, pH, temperature and salinity (Knud-Hansen 1998; Nguyen & Nguyen 2019). Moreover, their proximate composition can shift if these factors fluctuate (Chen 2012). To date, green ponds have been arbitrarily managed via the application of manure and fertiliser at predetermined and fixed fertilisation rates (Nguyen & Nguyen 2019). Wild marine algae usually develop and reach their maximal density within a week (Hoa & Nhi 2020). While this development depends on several factors, nutrient availability manipulation throughout the culture must be considered to maximise green water development. In general, different fertilization protocols have been applied in green-pond management in *Artemia* farming (Nguyen & Nguyen 2019) and conventional fertilization protocols are performed once per week, while Knud-Hansen (1998) indicated algae should be “fed” as frequently as they need to be in order to maintain optimally high net productivities; therefore, daily fertilisation has been noted to increase algal production due to sustained NP availability

(Milstein et al 1995), which in turn could help significantly to maintain steady *Artemia* production.

Material and Method. The experiment was conducted during dry season from February to March, 2020 in an earthen pond at Vinhchau Experimental Station, Cantho University. Natural seawater from the East Sea and wild algae were moved into a fertiliser pond through a supplying canal. Urea (46% nitrogen) and diammonium phosphate (DAP, total nitrogen 18%, total P_2O_5 46%) were used as fertiliser. Fertilisation doses for T1 were applied as a combination of urea and DAP at a rate of 3 and 1 mg L^{-1} per week, respectively. T2, T3 and T4 were fertilised with a combination of 39.6 g mL^{-1} urea and 1 mg L^{-1} DAP per week. Although the fertiliser doses for T2, T3 and T4 were similar, the fertilisation frequencies were different. As such, T2, T3 and T4 were fertilised once, twice and seven times per $week^{-1}$, respectively.

Experimental design. There were four treatments in randomisation with one replicate each: T1 involved traditional fertilisation (Trad_1perWeek) once per week and T2 (Formula_1perWeek) involved fertilisation with formula once per week, while T3 (Formula_2perWeek) and T4 (Formula_Daily) were fertilised with formula two and seven times per week, respectively.

Parameters. pH and temperature were recorded at 8 am and 2 pm daily, respectively, while NH_4^+, NO_3^-, NO_2^- and PO_4^{3-}, were measured every three days using a spectrometer (Hanna, Model: HI83200-02, made in Rumani).

Qualitative sampling. Green water was sampled at three-day intervals using a phytoplankton net (mesh size, 25-30 µm) pushed in a zigzag pattern. After sampling, algae captured in the net were washed, collected into sampling bottles and stored in a 100-mL water sample. Algae samples were kept in bottles and preserved with 2-4% formaldehyde. The identification of algae was based on Shirota (1966) and adopted with AlgaeBase (https://www.algaebase.org/). Moreover, the appearance frequency of algae followed the scale of Scheffer & Robinson (1939). When algal species appeared to be > 60% of the specimen, they would be marked as 'a lot of/so many' with the sign (+++). When algae appeared as 30-60% of the specimen, they would be marked as 'many' with the sign (++). When algae were < 30% of the specimen, they would be marked as 'seldom' with the sign (+).

Quantitative sampling. Water sampling was conducted at 8 am daily. Sampling occurred at five sampling points: four in the pond corners and one in the middle of the pond. A 2-litre sample was collected at each point, followed by a 1-litre subsample that was taken after mixing well. Samples were preserved in 2-4% formaldehyde and analysed within a month.

Algae were counted daily using a microscope with a Bürker chamber for microalgae with a size of less than 50 µm. The counting protocol and determination of algal density were in accordance with Coutteau (1996):

$$\text{Number of cells } (mL^{-1}) = [(n1+n2)/160]*10^6*d$$

where: n1 and n2 are the number of cells in the upper and lower chambers, respectively, and d is the dilution factor.

When algae larger than 50 µm, Sedgewick-Rafter were used for counting (Boyd & Tucker 1992):

$$X(\text{cell/L}) = \frac{T \cdot 1000 * Vcd * 1000}{A \cdot N * Vmt}$$

where: T is the counted individual, A is the counted area, N is the number of counting area, Vcd is the condensed volume (mL) and Vmt is the sampling volume (L).

Data analyses. Microsoft Excel 2007 spreadsheet software was used to calculate means and standard deviations. The Tukey HSD test was applied in SPSS 16 to statistically compare treatments by one-way ANOVA at a significance level of $p < 0.05$.

Results and Discussion

Environmental parameters

pH. pH varied from 9.0 to 10 (Figure 1) at 7 am and from 9.1 to 10.6 (Figure 2) at 2 pm. Average pH increased from day 1 to day 5 and was then stable before gradually declining towards the end of the experiment. It is possible that CO_2 was taken up sufficiently due to photosynthesis by algae and unbalanced the CO_2 released by respiration and water intake. Consequently, algae absorbed CO_2 via HCO_3^- conversion and released surplus O_2, which increased pH - especially at 12 pm with maximal light intensity. Thus, a large fluctuation in pH between day time and night time in algae culture has commonly been observed (Hoa & Nhi 2020). Since optimal pH values for marine algae have been commonly reported as pH 7-9 (Coutteau 1996; Hinga 2002; Andersen 2005), the pH values recorded in this experiment remained in the suboptimal range for marine algae development. From day 6 onwards, pH tended to decrease in response to declining algae density. However, the pH values displayed in T4 (i.e., daily fertilisation) increased continuously from day 10 onwards since algae growth continued to increase.

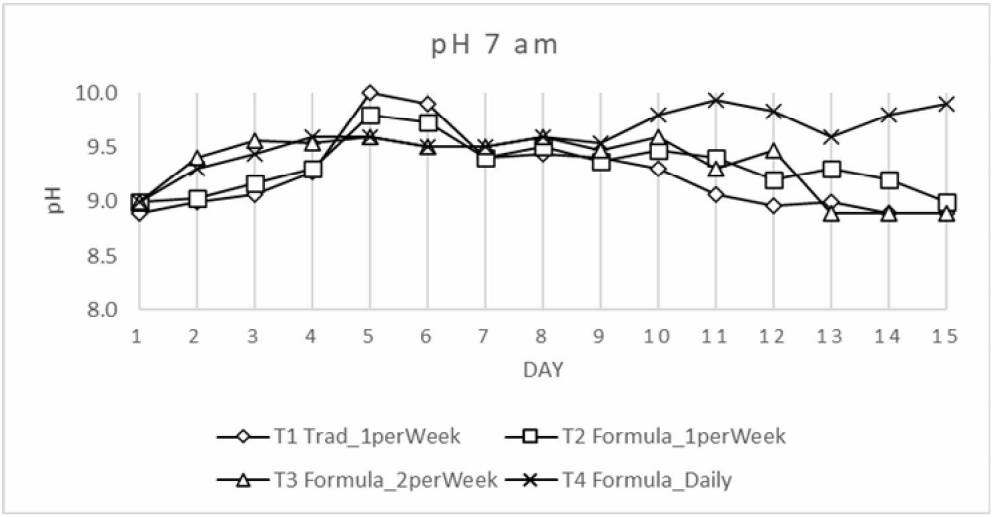

Figure 1. pH variation at 7 am.

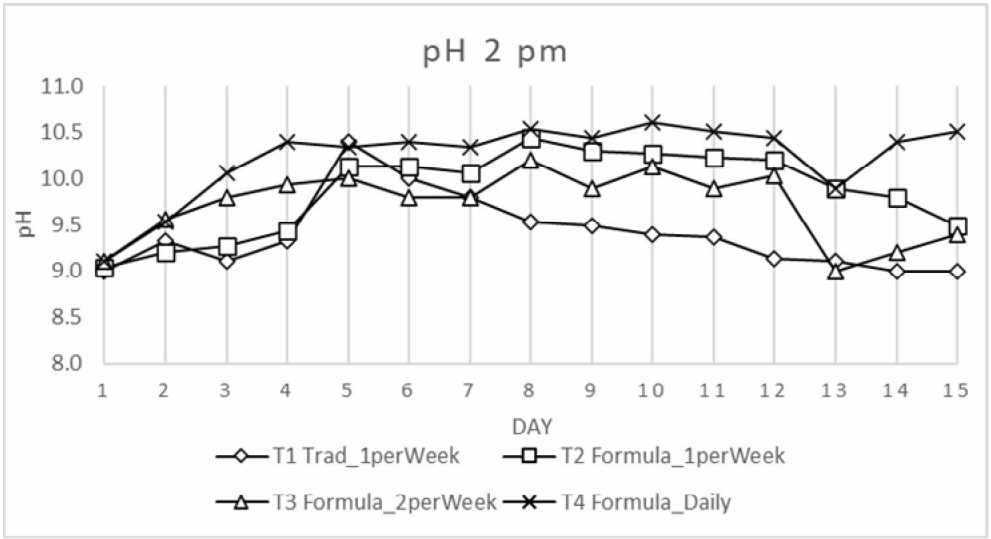

Figure 2. pH variation at 2 pm.

pH levels increase as NO_3-N is used for algae growth (Goldman et al 1971), while pH levels higher than 8 may favour Cyanophyta dominance. A similar observation was reported for the maximum growth of *Microcystis aeruginosa* (Gerloff et al 1952), while Eberly (1967) reported *Oscillatoria agardhii* reaching the exponential phase earliest with a maximum pH value of 10. Moreover, several species can tolerate a wider range of pH. For example, *Dunaliella tertiolecta* (6.0-9.3), *Nitzschia closterium* (5.3-9.8), *Nitzschia* sp. (6.7-9.5) can tolerate wide pH fluctuations; however, their maximum cell concentrations were reached at pH values of 8.3, 6.3 and 7.6, respectively (Hinga 2002).

Temperature. Temperature ranged from 25 to 29°C (Figure 3, Figure 4). Within the first week, temperatures differed between days 3 and 4, with a similar pattern towards the end. Afternoon temperatures ranged from 30 to 32.5°C and then remained stable (at 32°C) until the end of the experiment. While this temperature range is slightly higher than the optimal temperature, it remains within the threshold for algae development (Coutteau 1996). Higher temperatures correspond to greater light intensity, which enhances algae development. According to Coutteau (1996), the optimal temperature for algae development is 20-24°C (species dependent), while algae growth slows at < 16°C and some algae species may be subjected to mortality at > 35°C. Thus, the water temperatures recorded in the current experiment (25.0-33.2°C) are suitable for algae growth.

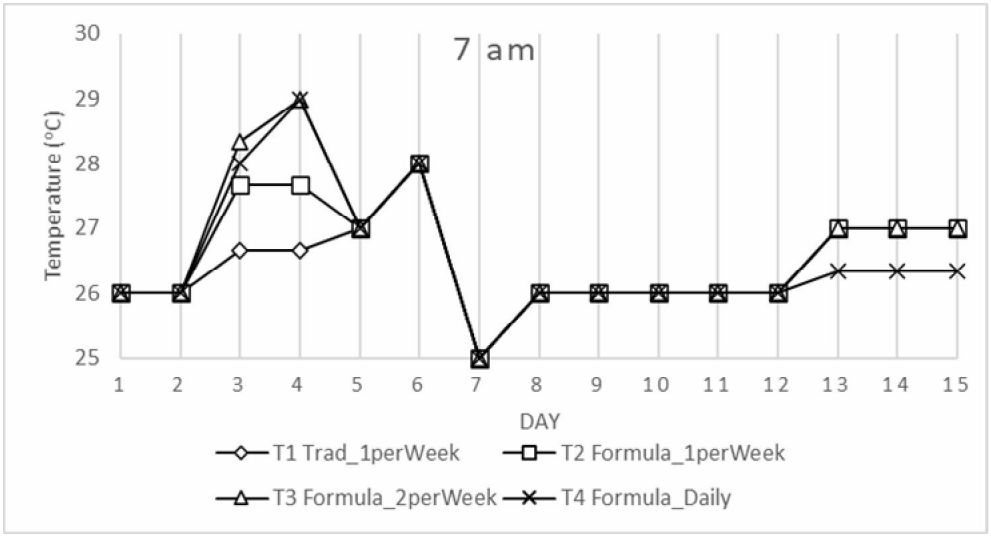

Figure 3. Temperature variation at 7 am.

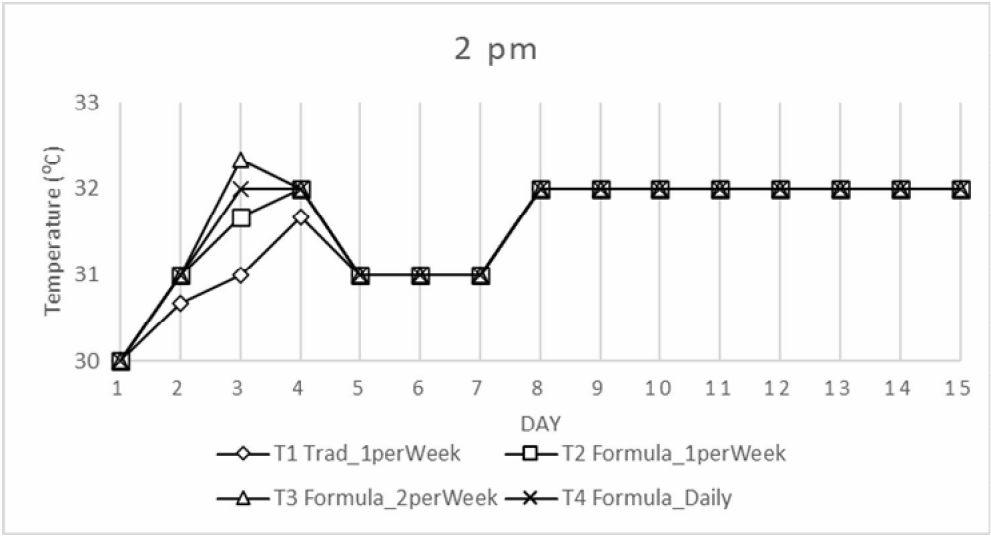

Figure 4. Temperature variation at 2 pm.

Salinity. In all treatments, salinity generally displayed similar patterns and fluctuated within the range of 25-30 ‰. Initial salinity was 25 ‰ and increased gradually due to evaporation. Salinity increased by approximately 1 ‰ per day. Such an increase in the salinity gradient may not negatively affect algae growth (Figure 5). According to Coutteau (1996), an ideal salinity for marine algae development is 20-24 ‰. Moreover, marine algae could stand larger fluctuations in salinity.

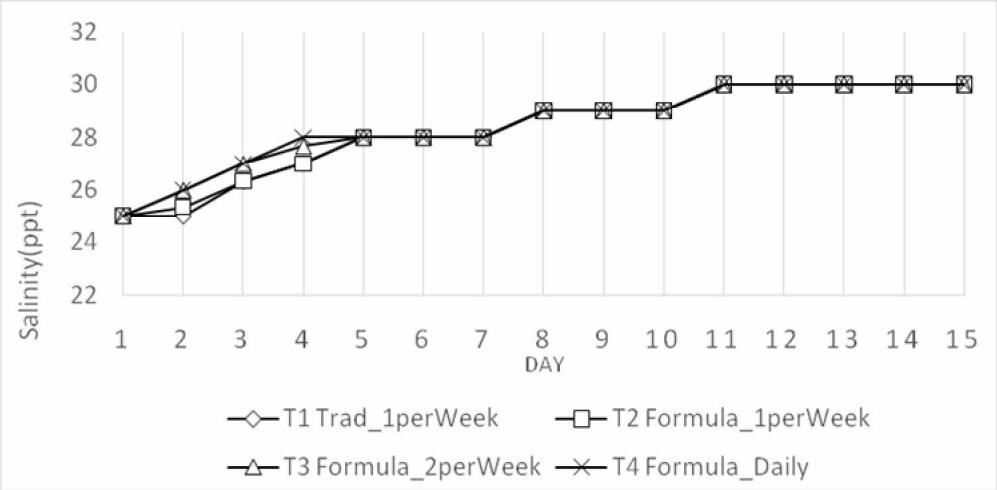

Figure 5. Salinity variation.

Turbidity. Figure 6 shows turbidity declining over the first four days, followed by stabilisation towards the end of the experiment, except for the shift between T2 and T3 in the last three days of the experiment. Turbidity is closely related to the growth of algae. These patterns coincide with the algae densities shown in Figure 12, through which lower turbidity is linked to higher algae densities and vice versa.

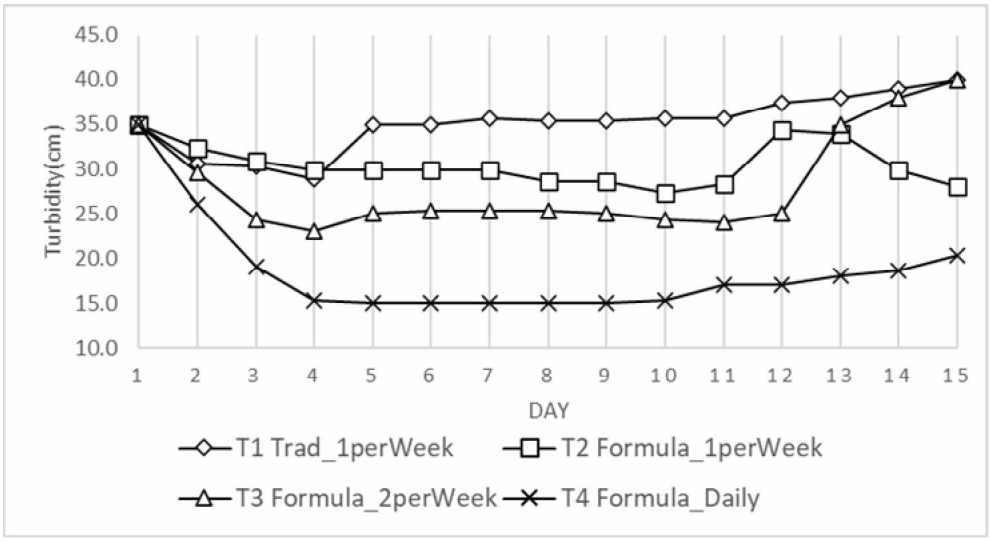

Figure 6. Turbidity fluctuation.

Ammonia (NH_4^+/NH_3). N-NH_4^+ is a crucial nutrient for algae development and has an inverse correlation with algae density (i.e., lower N-NH_4^+ at higher algae density) (Hussain et al 2014; Raven & Mario 2016). In the current study, N-NH_4^+ increased from day 1 to day 4 (Figure 7), at which point the highest N-NH_4^+ concentration was observed for T2 (i.e., fertilisation once per week) at 6.85 mg L^{-1}. Thereafter, N-NH_4^+ declined towards the end of the experiment for all treatments. From day 10 onwards, algae density declined in all treatments.

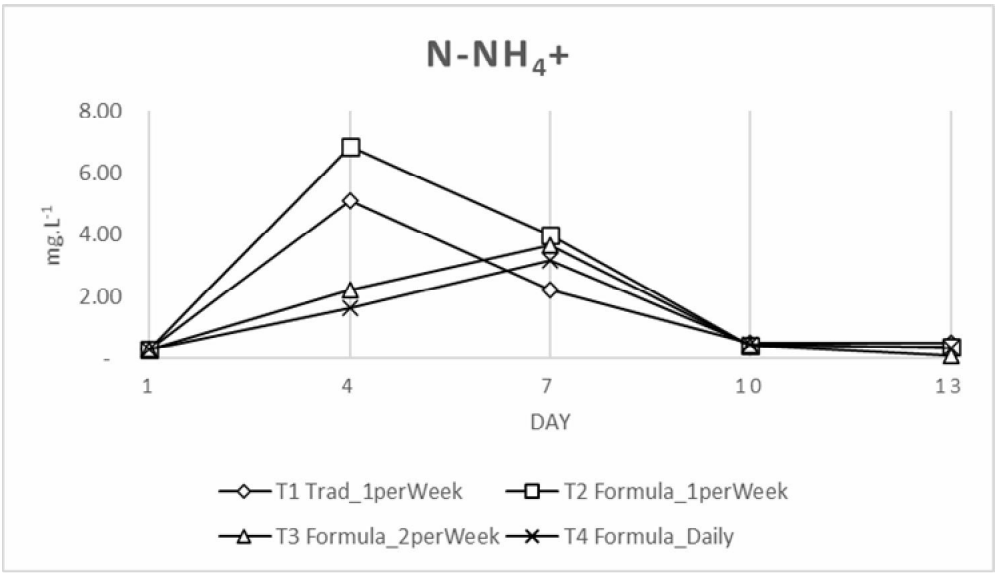

Figure 7. NH_4 variation per treatment.

NH_3 varied in accordance with the growth of algae. Figure 8 displays the highest NH_3 over the first 4 days, which declined towards the end of the experiment. T2 displayed its highest NH_3 value on day 2 (0.17 mg L^{-1}), which was higher compared to the other treatments. In contrast to $N\text{-}NH_4^+$, free NH_3 inhibited algal photosynthesis (Azov & Goldman 1982) and was higher at higher temperatures and pH values, which significantly reduced photosynthesis.

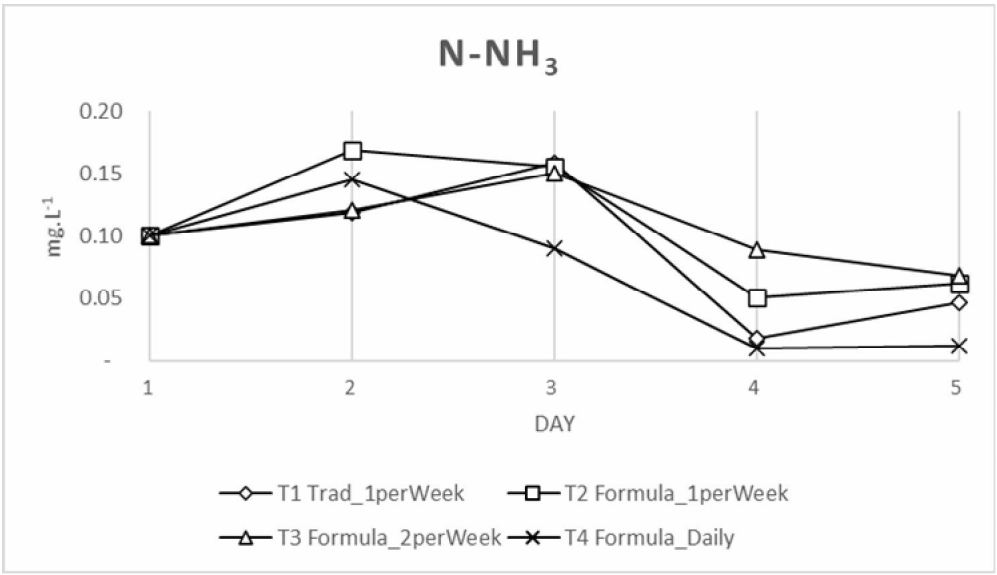

Figure 8. Variation of NH_3 per treatment.

Nitrite (NO_2^-). Nitrite was converted from NH_4^+/NH_3 by bacteria present in fertiliser ponds or from water intake. Figure 9 displays the NO_2^- variation for all treatments and a similar pattern of increase was observed from day 1 towards the end of the experiment. NO_2^- in T1 was highest (0.5 mg L^{-1}) at the end of the experiment; however, this difference was not significant ($p > 0.05$). Simultaneously, T3 was lowest (0.11 mg L^{-1}). NO_2^- fluctuated in contrast to algal growth when algae density peaked at day 7 and NO_2^- declined. A great effort of bacteria to convert NO_2^- to NO_3^- may have occurred in the aerated pond (Voss et al 2013); thus, initial NO_2^- was low for all treatments and dead algae later caused a drastic increase in NO_2^-. Moreover, an adapted species (e.g., *Prochlorococcus* ecotypes) could assimilate nitrite and nitrate (Martiny et al 2009) and thus slow down NO_2^-.

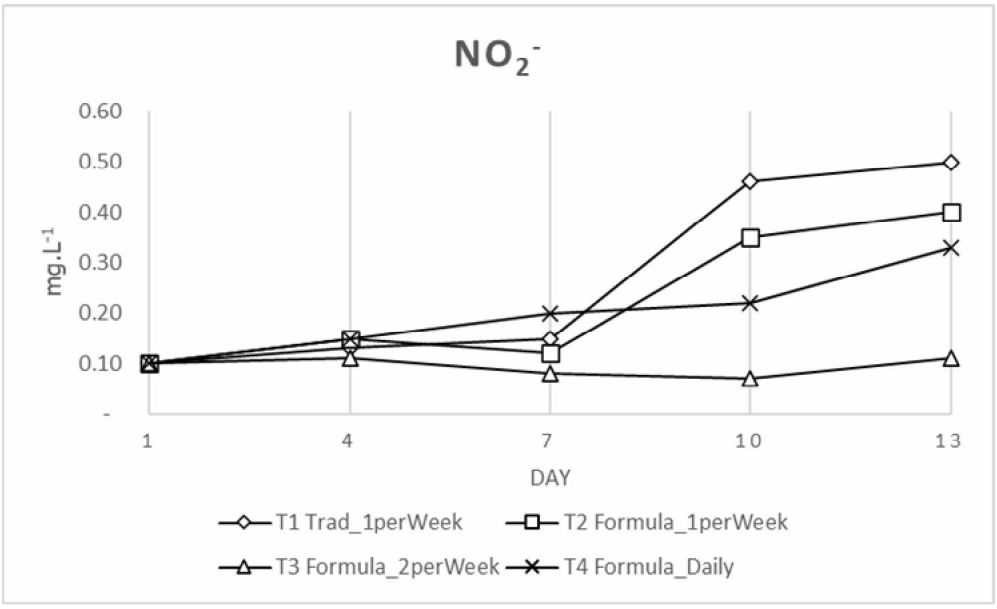

Figure 9. NO_2^- variation.

Nitrate ($N\text{-}NO_3^-$). Figure 10 displays the $N\text{-}NO_3^-$ variation, which ranged from 0.23 to 3.56 mg L^{-1}. While initial $N\text{-}NO_3^-$ was quite low (0.23 mg L^{-1}), it increased from day 2 until the end of the experiment. $N\text{-}NO_3^-$ fluctuations for all treatments were similar; however, the $N\text{-}NO_3^-$ of T2 and T3 increased considerably at day 7 (3.56 and 3.19 mg L^{-1}, respectively). T2 potentially had a higher fertiliser dose, while T3 received either a higher fertiliser dose or fertilisation frequency (i.e., twice per week versus once per week), which may have helped to stabilise development over T1 (i.e., lower fertiliser and application once per week). Different algae development, as defined by fertiliser doses and frequencies, may address the recorded variation in $N\text{-}NO_3^-$.

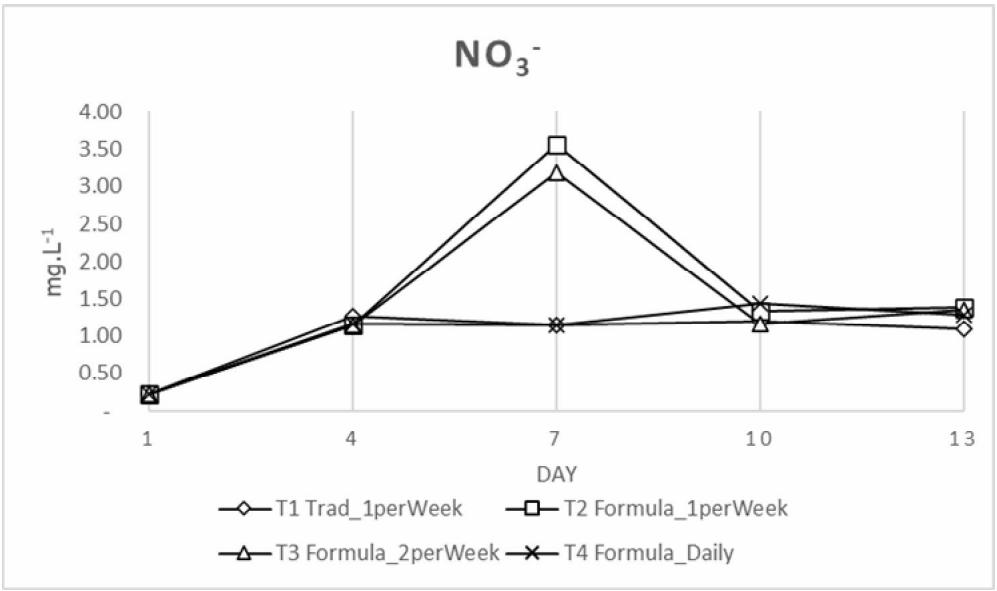

Figure 10. NO_3^- variation.

Phosphorus (PO_4^{3-}). Phosphorus is an essential element for algae metabolism, growth and development (Dyhrman 2016). Notably, surplus phosphorus with available nitrogen may cause eutrophication (i.e., algal blooms) (Drizo 2019). Notably, 1 g of phosphorus in a water medium is sufficient for algae to produce approximately 100 g of biomass (Drizo 2019). In practice, it was recorded that eutrophication occurred at 0.1-0.2 mg L^{-1} PO_4-P in water with current and 0.005-0.01 mg $L^{-1}PO_4$-P in stagnant water. In polluted water, PO_4-P may as be high as 0.5-10 mg L^{-1}. In natural water bodies, PO_4-P is present in the

form of $H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-} and algae usually absorb phosphorus as PO_4^{3-} (Vu et al 2013). In seawater-based high-pH algae ponds, the precipitation of phosphate salts can occur during the day (Larsdotter et al 2007). Initial pH in the current study was > 9 and soon reached > 10 from day 5. Algae development then led to the rapid decline of PO_4^{3-} from day 10 onwards.

Figure 11 shows the variation of PO_4^{3-} per treatment, which ranged from 2.5 to 3.55 mg L^{-1}. Notably, PO_4^{3-} concentration in T2 was usually higher than in the other treatments. However, the PO_4^{3-} concentration of T1 was lowest for the first two days and then stabilised before declining from day 4 onwards. The amount of fertiliser applied to T1 (control) was lower than for the treatments and rapidly declined when algae developed.

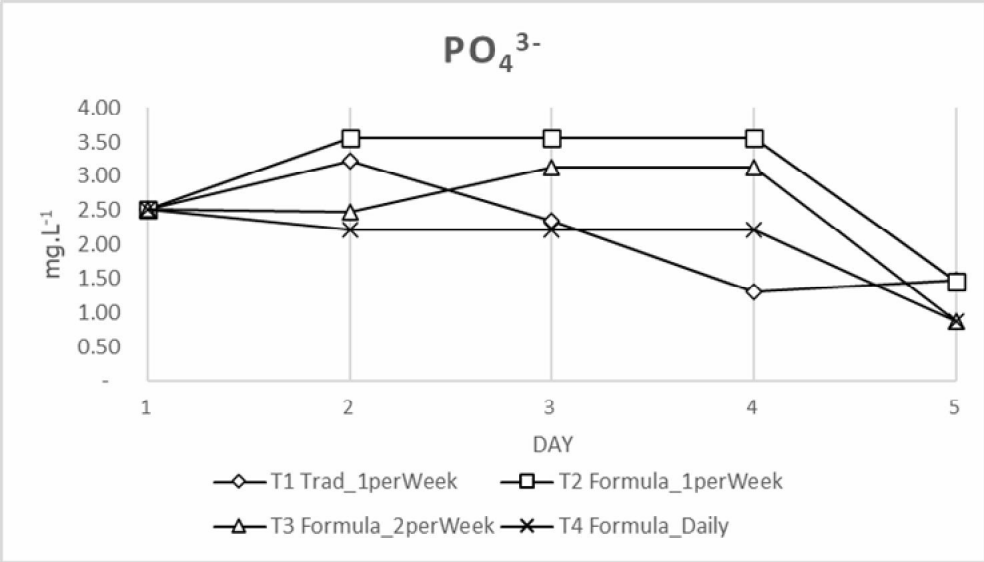

Figure 11. PO_4^{3-} variation.

Algae density and composition

Density. Factors that influence algae composition and density in water bodies include temperature, salinity, light, nutrients and filter feeders, among others (Wetzel 2001; Winder et al 2009; Asem et al 2012). However, nutrients are the key factors that influence the presence and reproduction of algae (Vu et al 2013; Deyab & El-katony 2015). *Artemia* can tolerate a wide range of salinity and primarily occur in high-salinity water (Van Stappen et al 2001; Nguyen et al 2007).

Figure 12 presents the growth of algae in different experimental treatments. The initial algae density for all treatments was 593,750 cells mL^{-1} and all treatments reached maximal densities at day 7. T4 displayed its highest algae density (2.81×10^6 cells mL^{-1}) at day 7, which was significantly different ($p < 0.05$) from the other treatments. The algae densities of T1, T2 and T3 were 1.56×10^6, 1.89×10^6 and 1.71×10^6 cells mL^{-1}, respectively. Algae densities across all treatments were stable from day 7 to day 10 and then declined thereafter. Except for T1, all treatments displayed higher algae densities when a higher dose of fertiliser was obtained. Notably, in T2 and T3, higher algae densities were recorded with greater fertilisation frequency (Table 1). The algae density of T4 was 60% higher when compared to T2 and T3 on day 4 and day 7, and threefold on day 13. Therefore, the advantages of daily fertilisation for green ponds in *Artemia* farming should be considered.

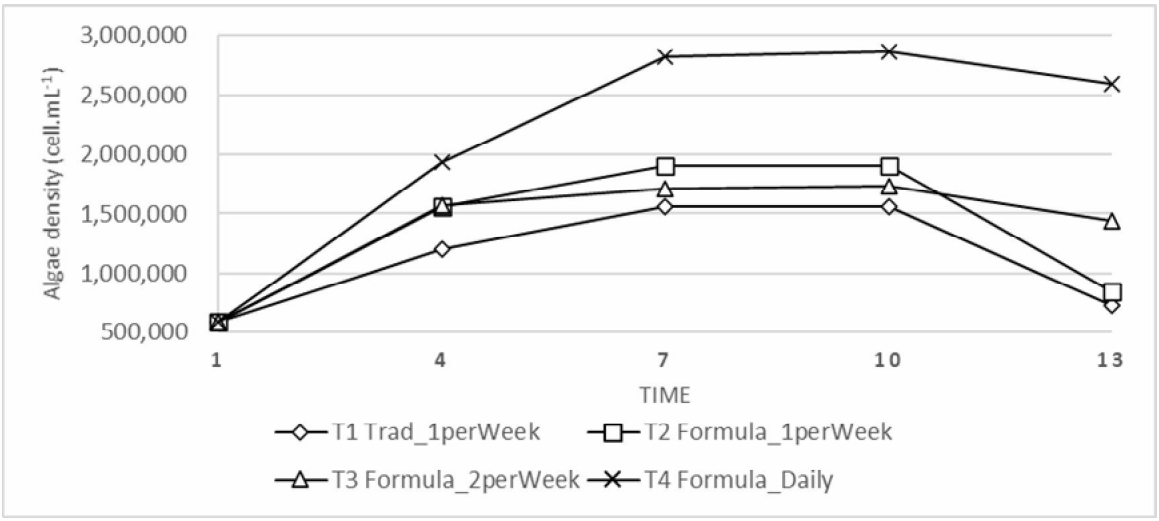

Figure 12. Algae variation.

Table 1

Variation of algae density (cells mL^{-1}) throughout the experiment

Treatment	*Day 1*	*Day 4*	*Day 7*	*Day 10*	*Day 13*
T1	593,750	1,206,250 ± 97,026[a]	1,562,500 ± 310,556[a]	1,560,417 ± 52,042[a]	725,000 ± 27,243[a]
T2	593,750	1,556,250 ± 21,651[ab]	1,897,917 ± 134,096[a]	1,897,917 ± 129,954[b]	852,083 ± 83,229[a]
T3	593,750	1,566,667 ± 127,527[ab]	1,710,417 ± 69,128[a]	1,729,167 ± 53,156[ab]	1,439,583 ± 78,146[b]
T4	593,750	1,935,417 ± 254,209[b]	2,814,583 ± 123,480[b]	2,860,417 ± 157,288[c]	2,587,500 ± 141,283[c]

Values displayed as average and standard deviation in columns with the same letter are not significantly different ($p > 0.05$).

Successful *Artemia* cyst production in solar saltworks depends on microalgae since *Artemia* feed on it in the culture system. Moreover, algae composition serves an important role in cyst production and quality, especially with regard to highly unsaturated fatty acids (HUFAs) (Lavens & Sorgeloos 1996; Baert et al 1996; Nguyen et al 2007). Moreover, it was observed that algae composition in fertiliser ponds relied on the natural algae population from water intake, while the dominant species occasionally changed as a function of physical factors and nutrient availability (Shaari et al 2011). The same authors also found that dominant species were less common in fertiliser ponds since they could not likely tolerate the physicochemical factors of such harsh environments, thereby resulting in less diversification.

Algae composition. Qualitative samples (Table 2) indicated species belong to Bacillariophyta are dominant as there were 9 species identified, however the microalga *Nannochloropsis* (Chlorophyta) with length of 3-5 micrometer overwhelmed in density. After fertilization this species dominated and outcompeted quickly other species.

Table 2

Algae composition fluctuation throughout the experiment

Species	*Day 4*				*Day 7*				*Day 10*				*Day 13*			
	T1	*T2*	*T3*	*T4*	*T1*	*T2*	*T3*	*T4*	*T1*	*T2*	*T3*	*T4*	*T1*	*T2*	*T3*	*T4*
Cyanophyta																
Phormidium sp.	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Chlorophyta																
Nannochloropsis sp.	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Tetraselmis sp.	+	+	+	+	+	+	+	+		+	+	+		+	+	
Bacillariophyta																
Thallassiosira sp.	+	+	+	+												
Navicula sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pleurosigma sp.	+	+	+	+												
Nitzschia sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Chaetoceros sp.	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Surirella sp.	+	+	+	+												
Gyrosigma sp.	+	+	+	+												
Cyclotella sp.	+	+	+	+	+	+	+	+								
Amphora sp.	+	+	+	+												
Euglenophyta																
Euglena sp.	+	+	+	+												
Dinophyta																
Peridinium sp.	+	+	+	+												

Note: (+++) algae appeared as > 60% of the specimen, marked as 'a lot of/so many'; (++) algae appeared as 30–60% of the specimen, marked as 'many'; (+) algae appeared as < 30% of the specimen, marked as 'seldom' (Scheffer & Robinson 1939).

Conclusions and recommendations. Wild algae development is heavily reliant on fertilisation frequency since the same dose of fertiliser (i.e., a combination of 39.6 g mL^{-1} urea and 1 mg L^{-1} DAP) with daily application can enhance and stabilise algae density when compared to an application once or twice per week. Therefore, it is necessary to adopt a guideline for green pond management protocols in *Artemia* pond culture to reduce fertiliser cost when surplus algae develop. However, to improve green pond management in the field, these results need to be clarified at a larger scale of fertilisation.

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