

Communication

Enhancement of carotenoid and chlorophyll content of an edible freshwater alga (Kai: *Cladophora* sp.) by supplementary inorganic phosphate and investigation of its biomass production

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Abstract: Enhancement of the carotenoid and chlorophyll content of an edible freshwater alga (Kai: *Cladophora* sp.) by supplementary inorganic phosphate in canteen wastewater was investigated. The mass cultivation of the alga was conducted at ambient temperature and light intensity in diluted canteen wastewater enhanced with dipotassium hydrogen phosphate at 5-20 mg L⁻¹. An increase in total carotenoid and chlorophyll was observed. However, it had no effect on biomass production. As a result of the algal cultivation, there was a dramatic improvement in the quality of canteen wastewater.

Keywords: freshwater alga, *Cladophora*, Kai

INTRODUCTION

Algae are a significant source of human food, especially in Asia. In Thailand an edible freshwater alga, *Cladophora* (known locally as Kai), consists of two species, namely *C. glomerata* and a to-be-identified *Cladophora* sp. [1]. Kai is abundant in Nan and Mekong Rivers in the northern part of Thailand. The local people around these rivers collect it for domestic consumption and it is sold in the local markets. Many reports on the nutritional value of *Cladophora* spp. show that they contain a significant amount of carotenoids that are nutritionally essential for humans and animals [1-5]. Carotenoids and chlorophylls, which are found in plants and algae, are extremely important in photosynthesis and growth [6-8]. They are also powerful antioxidants that are beneficial to human

health and are now used for supplementary food and animal feed. Previous studies have suggested that they can prevent or delay cancer and degenerative diseases in humans and animals by contributing to antioxidative defenses against metabolic oxidative by-products [9-11].

Mass cultivation of algae in canteen wastewater for use as feed for Mekong giant catfish [2] and *Tuoptim tilapia* [12] had been done. It was found that the algal biomass production strongly correlated with water quality and environmental factors, e.g. temperature, light intensity and nutrient concentration, results which were similarly obtained elsewhere [13-15]. However, several researches have indicated that phosphorus is a main factor related to growth and production of *Cladophora* [13-24]. Other researches have also shown that phosphorus is effective for algal carotenoid and chlorophyll production [25-30]. In contrast, stimulation of carotenoid and chlorophyll production with phosphorus starvation has been reported [31-35].

Culturing of algae in wastewater can also improve the water quality by decreasing BOD, COD and nutrients of the wastewater [2,12,36]. In this research, mass cultivation of *Cladophora* sp. (Kai) in canteen wastewater with different phosphate concentrations was carried out to investigate the effects of supplementary phosphorus on carotenoid and chlorophyll content of this alga, its biomass production and physico-chemical characteristics of the culture water.

MATERIALS AND METHODS

Culture Media Preparation

One thousand litres of canteen wastewater were collected from the canteen wastewater clarifier of Maejo University and left to settle in an open cement pond for 3 weeks to allow microorganisms to break down solid organic waste. The wastewater was then filtered through an 80- μm plankton net filter [36]. The filtrate was diluted to 10% and analysed for pH, dissolved oxygen (DO), biochemical oxygen demand (BOD), total hardness, ammonia-nitrogen ($\text{NH}_3\text{-N}$), nitrate-nitrogen (NO_3^- -N), nitrite-nitrogen (NO_2^- -N), total Kjeldal nitrogen (TKN) and orthophosphate-phosphorus (PO_4^{3-} -P) by following the methods of APHA et al [37].

Culture Condition

Cladophora sp. (Kai) was obtained from the Algae and Water Quality Research Unit, Chiang Mai University. The alga was cultured for 12 weeks at ambient air temperature (21-28°C) and light intensity (12,333-39,267 lux) by attachment on plastic nets (60 g m^{-2}) in cement raceway ponds (size $1.2 \times 2.3 \times 0.5 \text{ m}$) each containing diluted canteen wastewater 20 cm deep (552 L per experiment) with continuous pump-driven circulation of 0.15 m s^{-1} [2,4]. A complete randomised design (CRD) was carried out with addition of dipotassium hydrogen orthophosphate (K_2HPO_4) at concentrations of 5, 10, 15 and 20 mg L⁻¹ (treatment 1, 2, 3 and 4 respectively) with diluted canteen wastewater without K_2HPO_4 as control. The experiment was done in triplicate.

Carotenoid and Chlorophyll Analysis

The alga at 12 weeks of culturing was harvested, washed with tap water, air dried and freeze-dried. The total carotenoid content was determined according to the method of Britton [38]. The freeze-dried sample (0.4 g) was homogenised with 20 mL of 95% ethanol in an extraction tube. Two

mL of 5% KOH were then added, air was driven out of the tube with nitrogen gas and the tube was stored in the dark for at least 2 hours. Ether (3×5 ml) was added to extract the carotenoid portion. The ether supernatant was separated and its absorbance was read at 400-700 nm with a spectrophotometer. The total amount of carotenoid was calculated according to the following equation: total carotenoid = [(A_{max}/0.25)×ether supernatant volume]/sample weight, where A_{max} = maximum absorbance.

Extraction of carotenoid components and chlorophylls was performed by a modified method of Yoshii et al. [3] and Dere et al. [39] as follows: the freeze-dried sample (0.5 g) was mixed with ice-cooled acetone (25 mL) in the dark, the mixture stored in the dark at -20°C for 18 hours and the supernatant filtered. Carotene, xanthophyll and chlorophylls a and b ($\mu\text{g g}^{-1}$) were determined spectrophotometrically at 470, 645 and 662 nm respectively by means of equations proposed by Lichtenthaler and Wellburn [40]:

$$\text{Chlorophyll a} = 11.75A_{662} - 2.35A_{645}$$

$$\text{Chlorophyll b} = 18.61A_{645} - 3.960A_{662}$$

$$\text{Carotene} = (1000A_{470} - 2.270C_a - 81.4 C_b) / 227 \quad (C_a = \text{chlorophyll a}, C_b = \text{chlorophyll b})$$

$$\text{Xanthophyll} = \text{total carotenoid} - \text{carotene}$$

Biomass Production

The growth in terms of biomass (g m^{-2} wet weight) and specific growth rate (SGR) ($\% \text{ d}^{-1}$) were measured every week following the method of Premila and Rao [41]. The SGR was calculated as SGR ($\% \text{ d}^{-1}$) = $\{\ln(m_1/m_0)/t\} \times 100$, where m_0 = initial weight, m_1 = final weight, and t = time of culture in days.

Water Quality Analysis

Water samples from all the experimental ponds were collected once a week and analysed [37] for the physico-chemical properties, viz. temperature, pH, DO, BOD, total hardness, NH₃-N, NO₃⁻-N, NO₂⁻-N, TKN and PO₄³⁻-P.

Statistical Analysis

The data were presented as mean value \pm standard deviation. Comparison of mean values was made by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT) at a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

Carotenoid and Chlorophyll Content

Table 1 lists the carotenoid and chlorophyll content of *Cladophora* sp. (Kai) culture at different phosphate concentrations. The highest values of these pigments were observed in treatment 4 with K₂HPO₄ at 20 mg L⁻¹ added to the culture medium, whereas the lowest values were observed in the control group with no addition of K₂HPO₄.

Table 1. Carotenoid and chlorophyll content of *Cladophora* sp. (Kai) cultured at different phosphate concentrations

Pigment	Amount of pigments ($\mu\text{g g}^{-1}$ dry weight)				
	Control	Treatment 1	Treatment 2	Treatment 3	Treatment 4
	$\text{K}_2\text{HPO}_4(\text{mg L}^{-1})=0$	$\text{K}_2\text{HPO}_4(\text{mg L}^{-1})=5$	$\text{K}_2\text{HPO}_4(\text{mg L}^{-1})=10$	$\text{K}_2\text{HPO}_4(\text{mg L}^{-1})=15$	$\text{K}_2\text{HPO}_4(\text{mg L}^{-1})=20$
Total carotenoid	889±157 ^a	1,084±253 ^a	1,130±147 ^a	1,151±151 ^a	1,729±212 ^b
Carotene	44±4 ^a	86±9 ^b	87±6 ^b	96±7 ^{bc}	103±9 ^c
Xanthophyll	779±42 ^a	997±254 ^a	1043±145 ^a	1055±154 ^a	1626±220 ^b
Chlorophyll a	148±13 ^a	270±30 ^b	309±20 ^{bc}	305±52 ^{bc}	348±17 ^c
Chlorophyll b	56±31 ^a	173±11 ^b	245±15 ^{bc}	200±49 ^c	249±16 ^c

Note: Each value is mean ± SD

Data in the same row with different superscripts are significantly different ($p<0.05$).

Carotenoids in green algae are produced in two different compartments and by two different pathways, i.e. the acetate-mevalonate pathway and the phosphoglyceraldehyde-pyruvate pathway, and they are further synthesised from isopentenyl diphosphate and its isomers [42-43]. In the present study it was observed that phosphate increased carotenoid and chlorophyll production in the alga, which agrees with the findings by Khuantrairong and Traichaiyaporn [4-5] that total carotenoid, β -carotene, lutein and zeaxanthin in *Cladophora* sp. positively correlate with phosphate level. Celekli et al. [29] reported that the phosphate supply increased biomass and carotenoid production in a blue-green alga (*Spirulina platensis*) while Latasa and Berdalet [25] deserved that the synthesis of pigments in a dinoflagellate *Heterocapsa* sp. stopped upon phosphorus limitation. In contrast, Buapet et al. [30] suggested that phosphorus enrichment had no significant effect on chlorophyll a production by *Ulva reticulata* seaweed. Subudhi and Singh [35] reported that a high concentration of phosphate-phosphorus reduced the chlorophyll content of *Azolla pinnata* blue-green alga. Enhancement of astaxanthin in a green alga (*Haematococcus pluvialis*) [31,34] and that of β -carotene, zeaxanthin and violaxanthin in a marine microalga (*Nannochloropsis gaditana*) [33] were observed upon phosphorus limitation. Leonardos and Geider [32] stated that the nitrate-to-phosphate supply ratio was related to carotenoid and chlorophyll-a production in the cryptophyte *Rhinomonas reticulata*.

Biomass Production

For 12-week cultures, the biomass production was similar among the treatments and ranged between 3,406-3,464 g m⁻² (wet weight) (Figure 1). The highest value, 4,167 g m⁻², was observed in treatment 3 after culturing for 10 weeks. However, statistical analysis indicated that *Cladophora* sp. (Kai) cultured at different phosphorus concentrations showed no significant difference in growth ($p>0.05$).

The specific growth rate (SGR) was similar among treatments and ranged between -2.7-38.9% d⁻¹. After one week of cultivation, the alga began to grow quickly and the highest SGR values

were observed (Figure 2). Statistical analysis showed that the biomass production rate was not significantly different among the treatments ($p>0.05$).

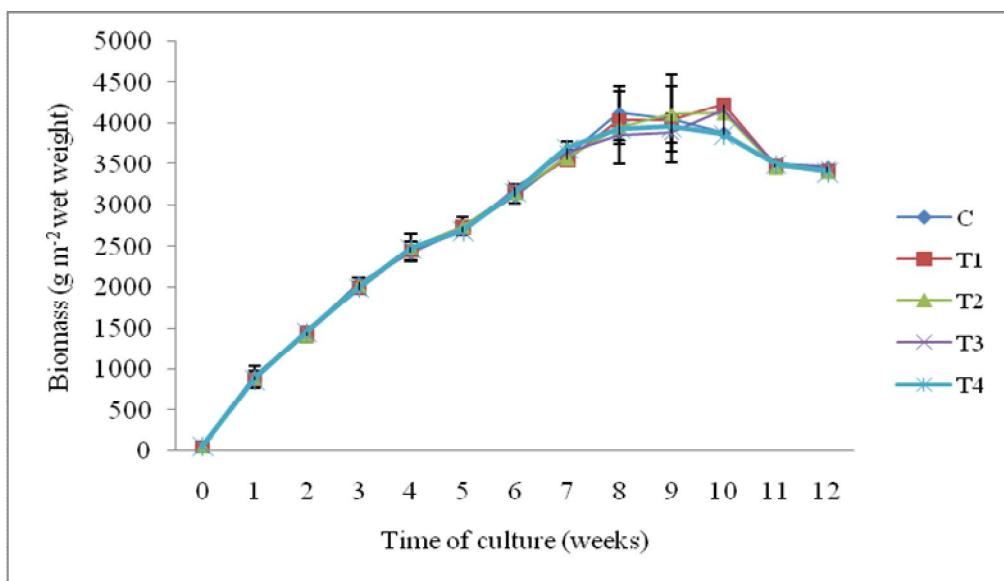


Figure 1. Biomass production of *Cladophora* sp. (Kai) cultured at different phosphorus concentrations (C = control group, T1 = treatment 1 (+5 mg L⁻¹ K₂HPO₄), T2 = treatment 2 (+10 mg L⁻¹ K₂HPO₄), T3 = treatment 3 (+15 mg L⁻¹ K₂HPO₄), and T4 = treatment 4 (+20 mg L⁻¹ K₂HPO₄))

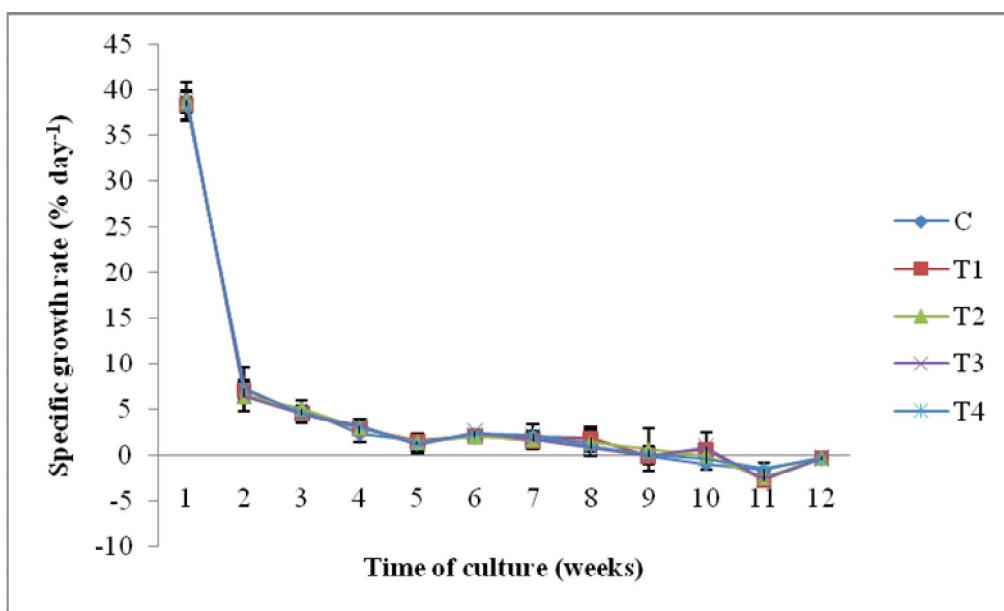


Figure 2. Specific growth rate (SGR) of *Cladophora* sp. (Kai) cultured at different phosphorus concentrations (C = control group, T1 = treatment 1 (+5 mg L⁻¹ K₂HPO₄), T2 = treatment 2 (+10 mg L⁻¹ K₂HPO₄), T3 = treatment 3 (+15 mg L⁻¹ K₂HPO₄), and T4 = treatment 4 (+20 mg L⁻¹ K₂HPO₄))

Thus, the biomass production and SGR of the alga cultured at different phosphorus concentrations showed no difference among all the treatments, indicating that the added phosphate had no effect on biomass production. Phosphorus is one of the main factors related to growth and production of freshwater *Cladophora* [13-24]. However, although Auer and Canale [24] observed that high dissolved phosphorus values in water induced an increase in stored phosphorus and growth rate of *Cladophora*, our results agreed with the findings that when phosphorus is above a certain concentration (0.01 mg L^{-1}), it has no effect on the growth and biomass production of *Cladophora* [13,18,44]. The biomass production of *Cladophora* also strongly depends on environmental conditions, especially temperature and light intensity. High biomass production was observed in winter season and under high light intensity [2, 5, 21].

Water Quality

For 12-week cultures, the physico-chemical properties of water of all treatments ranged as follows: temperature $19\text{-}28^\circ\text{C}$, pH 8.3-8.9, DO $8.47\text{-}11.75 \text{ mg L}^{-1}$, BOD $1.00\text{-}10.27 \text{ mg L}^{-1}$, total hardness $38.91\text{-}68.21 \text{ mg L}^{-1}$ (as CaCO_3), $\text{NH}_3\text{-N}$ $0.07\text{-}1.07 \text{ mg L}^{-1}$, $\text{NO}_3^-\text{-N}$ $0.19\text{-}1.58 \text{ mg L}^{-1}$, $\text{NO}_2^-\text{-N}$ $0.002\text{-}0.012 \text{ mg L}^{-1}$, TKN $0.19\text{-}4.16 \text{ mg L}^{-1}$ and $\text{PO}_4^{3-}\text{-P}$ $0.004\text{-}14.780 \text{ mg L}^{-1}$ (Figure 3). It was noted that the $\text{PO}_4^{3-}\text{-P}$ levels in all treatments were higher than the critical phosphorus level of 0.01 mg L^{-1} for *Cladophora* growth in the natural ecosystem [20].

High DO values occurred in all experiments as a result of aeration by the air pump. Algae cultivation in canteen wastewater with high nutrients has been observed to dramatically reduce nitrogen and phosphorous levels and at the same time produce a useful product for animal feed [36]. In this work, it was noted that culturing of *Cladophora* sp. (Kai) showed similar improvement in water quality with decreasing BOD and nutrient levels ($\text{NH}_3\text{-N}$, $\text{NO}_3^-\text{-N}$, TKN and $\text{PO}_4^{3-}\text{-P}$).

CONCLUSIONS

This study demonstrates that phosphate added to the culture of *Cladophora* sp. (Kai) enhances production of carotenoids and chlorophylls but has no effect on biomass production. In addition, cultivation of the alga in canteen wastewater improves the water quality by decreasing BOD and nutrients levels.

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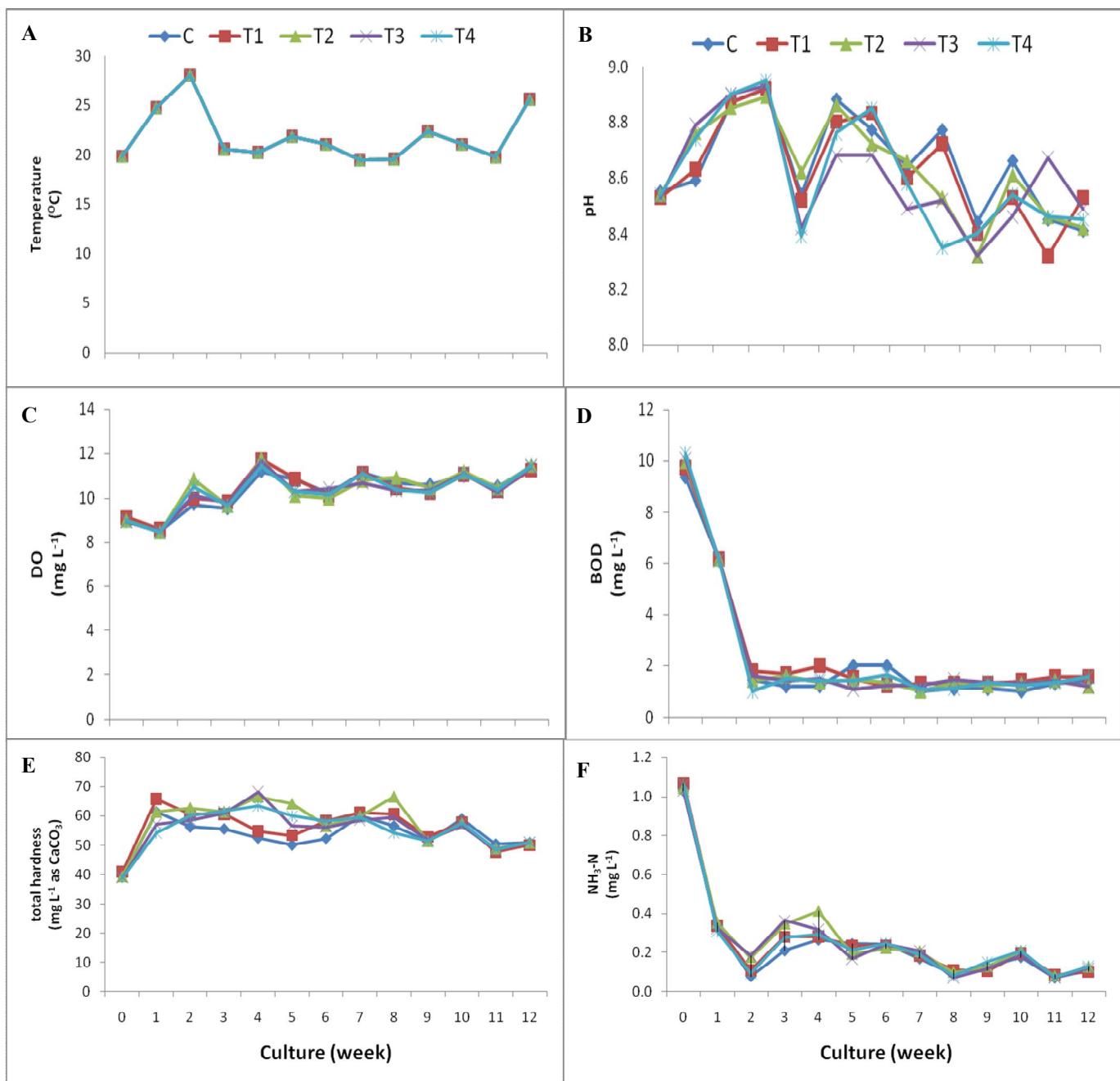


Figure 3. Water quality of *Cladophora* sp. (Kai) culture: water temperature (A), pH (B), DO (C), BOD (D), total hardness (E), and $\text{NH}_3\text{-N}$ (F) (C = control group, T1 = treatment 1, T2 = treatment 2, T3 = treatment 3, and T4 = treatment 4)

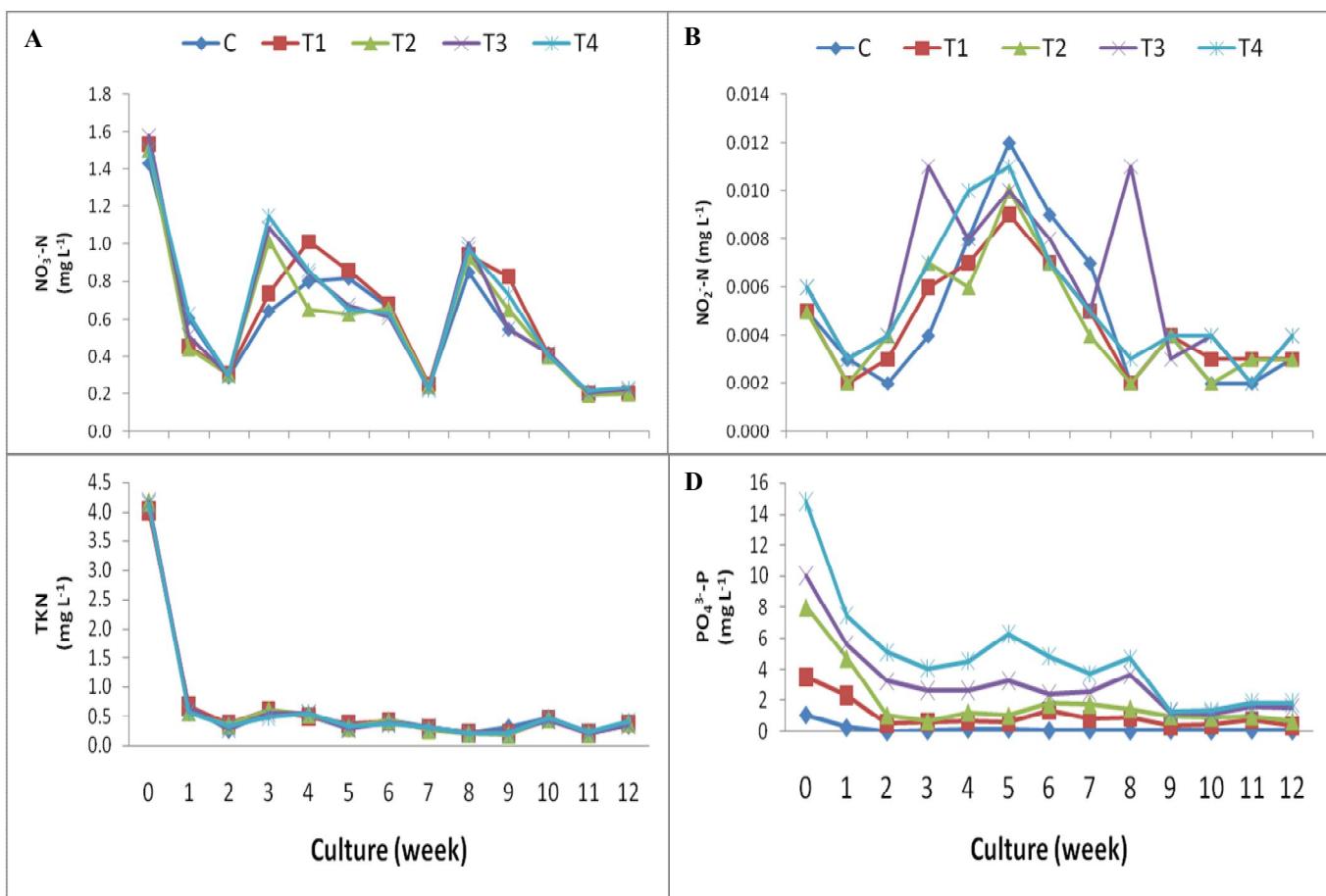


Figure 3 (Continued). Water quality of *Cladophora* sp. (Kai) culture: NO_3^- -N (A), NO_2^- -N (B), TKN (C), and PO_4^{3-} -P (D) (C = control group, T1 = treatment 1, T2 = treatment 2, T3 = treatment 3, and T4 = treatment 4)

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