Co-culture of microalgae-activated sludge for wastewater treatment and biomass production: Exploring their role under different inoculation ratios

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Abstract

In this study, mixed culture (microalgae:activated sludge) of a photobioreactor (PBR) were investigated at different inoculation ratios (1:0, 9:1, 3:1, 1:1, 0:1 wt/wt). This work was not only to determine the optimal ratio for pollutant remediation and biomass production but also to explore the role of microorganisms in the co-culture system. The results showed high total biomass concentrations were obtained from 1:0 and 3:1 ratio being values of 1.06, 1.12 g L^{-1}, respectively. Microalgae played a dominant role in nitrogen removal via biological assimilation while activated sludge was responsible for improving COD removal. Compared with the single culture of microalgae, the symbiosis between microalgae and bacteria occurred at 3:1 and 1:1 ratio facilitated a higher COD removal by 37.5-45.7 %. In general, combined assessment based on treatment performance and biomass productivity facilitated to select an optimal ratio of 3:1 for the operation of the co-culture PBR.

Keywords: Co-culture system; Activated sludge; Microalgae; Wastewater treatment; Biomass production.

1. Introduction

Domestic and industrial activities have discharged wastewater (WW) possessing concerned nitrogen and phosphorus level. Those nutrient compounds are the main cause of eutrophication in the receiving water reservoir because they diminish oxygen concentration for aquatic living and trigger algae bloom. Consequently, excessive nitrogen and phosphorus imbalance the function of ecosystem. It is a critical need to phase out nutrient elements in WW prior to discharging to avoid eutrophication and guarantee healthy water quality for community consumption. Generally, activated sludge processes (ASPs) are favorite ones which have been applied widely to target nutrient in the discharged WW stream (Mujtaba and

Lee, 2017). Such processes are sequencing batch, anaerobic, anoxic, oxic reactors and hybrid systems of those given single technology. However, ASPs are restrained by high energy consumption which is mandatory to provide oxygen for bacteria respiration and nutrient metabolism process. Energy for aeration accounts for 60% to 80% total used energy as a whole of wastewater treatment process using ASPs (Clarens et al., 2010). Another one-hybrid system (e.g., anaerobic-anoxic-oxic) is a viable option but goes along the high capital cost. It is essential to propose a robust single-stage process for dual purposes of nutrient remediation and energy-efficient. This process aims to apply for various types of wastewaters as municipal wastewater, winery wastewater, piggery and, fermentation wastewater (Godos et al., 2009; Mujtaba and Lee, 2017; Qi et al., 2018; Higgins et al., 2018a).

Microalgae have demonstrated their feasibility for wastewater remediation thanks to their low cost, easy operation and revenue-raising potential (e.g., bioenergy production and pigment extraction) (Rittmann, 2008; Mata et al., 2010). They are prominent candidate to develop the above-mentioned single-stage technology. Microalgae can assimilate high load of nutrient and start producing biomass in a short time (< 24 h) (Godos et al., 2009; Zhang et al., 2011). Concurrently, photosynthesis of microalgae generates oxygen given a good chance to provide oxygen for ASPs process (Mata et al., 2010). Therefore, microalgae can serve as an “aeration device” and ideally replace the mechanical aeration system and cut off energy cost for aeration in ASPs (Jia and Yuan, 2018). Based on this concept, microalgae and bacteria can mutually support each other. Studies on the symbiosis of microalgae–bacteria have been accelerating as a mean for domestic and industrial wastewater remediation (Lee and Lei, 2019). Given a single-stage technology, microalgae consumes nitrogen and phosphorus in WW for biomass production whilst bacteria metabolize organic matters in a greater extent.

As reported for the co-culture system, nutrient remediation and biomass growth are influenced by several factors, encompassing different inoculum ratio, operating condition, wastewater composition and reactor configuration (Zhu et al., 2019). The effects of wastewater matrix and inoculum ratio has been reported in several studies (Ji et al., 2018a) (Huo et al., 2020a). The former reported that 1:3 inoculation ratio (microalgae:bacteria wt/wt from now on) was an appropriate one for both nutrient remediation and biomass production amongst a range of ratio (1:0, 1:1, 1:2, 1:3, 2:1, 3:1 wt/wt) (Ji et al., 2018a). The latter study indicated that COD, total nitrogen (TN), and total phosphorus (TP) in vinegar production wastewater were removed more significantly by adding either 1% (v/v) or 10% (v/v) of *Bacillus firmus* and *Beijerinckia* species into the real WW-cultivated microalgae (Huo et al., 2020a). Compared to a single microalgae culture, COD, TN and TP assimilation efficiency in co-culture system increased by 22.1%, 20%, and 8.1%, respectively (Huo et al., 2020a). The ratio of mixed microalgae-bacteria could affect performance of nutrient remediation. However, the co-culture system of microalgae and bacteria still faces inherent obstacles in term of biomass harvest (Mallick, 2002). The traditional technologies for biomass harvest are coagulation and centrifugation/separation. The efficiency of those traditional technologies remains insignificant and operation cost is still high (Su et al., 2012). The drawback in settling and biomass harvest of microalgae could be tackled by adding activated sludge. Activated sludge performs better settling ability compared to microalgae and exhibits extensive COD removal than the available bacteria in wastewater (Gutzeit et al., 2005). Therefore, several studies have integrated microalgae and ASPs to be co-culture system based on the above-mentioned co-benefits for WW remediation (Su et al., 2012); Mujtaba and Lee, 2017; Zhu et al., 2019). For instance, the 5:1 ratio was found for sufficient nitrogen and phosphorus removal (91.0% and 93.5% respectively) (Su et al., 2012) . In turn, their findings indicated the inoculum ratio did not impact on COD removal (Su et al., 2012).

In another study, an optimal ratio primed to 2:1 to attain the highest efficiency of municipal wastewater treatment (Mujtaba and Lee, 2017). High removal of COD (82.7%), TN (75.5%) and TP (100%) could be obtained under an inoculum ratio of 1:1 (Zhu et al., 2019). It can be seen the optimal microalgae:sludge ratio of the previous studies varied due to the discrepancies of wastewater composition. For instance, Su et al. (2012) studied high loading concentration of COD (380 mg L^{-1}), TN (50 mg L^{-1}) and PO_4^{3-} (8 mg L^{-1}) while (Mujtaba and Lee, 2017) experimented low concentration level of COD (60 mg L^{-1}), NH_4^+-N (50 mg L^{-1}) and TP (1.3 mg L^{-1}). Also, COD: N ratio could affect bacteria and microalgae growth. A 4.3:1 ratio could enrich microalgae biomass and nutrient recovery (Zhu et al., 2019). It is important to note that the previous works did not explore thoroughly an optimal ratio for simultaneous nutrient remediation and biomass production. In addition, the role and contribution of microalgae and activated sludge in the co-culture system have not been addressed adequately. A minor change in microalgae:activated sludge ratio could influence the whole performance. Therefore, biomass fraction of both microalgae and bacteria needs to be quantified, and thus to elaborate their role on organic and nutrient remediation. Given the research gaps indicated above, this study was built to explore the following objectives: i) To determine optimum microalgae-activated sludge inoculum ratio (wt:wt) for simultaneous nutrient/organic matter remediation and biomass production; ii) To investigate the mechanism of nutrient remediation and (iii) to validate the role of microalgae and activated sludge incorporated in a symbiotic system.

2. Materials and methods

2.1. Microorganism and synthetic wastewater

The microalgae strain used in this study was taken from Aquaculture Research Institute 2-Ministry of Agriculture and Rural Development, Vietnam. This strain is *Chlorella* sp., which is capable of nitrogen and phosphorus uptake as nutrient sources. The strain was cultivated and maintained in a sterilized Bold´s Basal Medium (BBM). *Chlorella* sp. was incubated in a bubble column photobioreactor (PBR) (20 cm diameter, 60 cm height) under typical conditions: the light intensity of 100 μmol m^{-2} s^{-1} and at room temperature and air aeration, which has been fully described previously (Nguyen et al., 2016; Vo et al., 2018). The pre-cultured algae cells were taken during the log growth phase. Such action was firstly settled down for 12 h to remove the supernatants, followed by centrifuged at 3600 rpm for 10 min and washed twice with deionized water before it was used for inoculation in the following experiments.

Activated sludge was collected from the aerobic reactor in a local CASP wastewater treatment system. Mixed liquid suspended solids (MLSS) has a concentration of about 4000 mg L^{-1}. Prior to experiments, the activated sludge was also settled down for 3 h to remove the suspended solids, followed by centrifugation at 3600 rpm for 10 min. The settled solids washed twice with the distilled water before it was used as a source of bacterial consortium.

The synthetic wastewater was prepared with the particular components denoted in Table S-1. The main components are acetate (medium A), NH_4CL (medium B), KH_2P0_4 (medium C) used as the carbon and nutrient sources for cultivation. As prepared the synthetic wastewater contains COD 500 mg L^{-1}, NH_4^+-N 200 mg L^{-1}, TP 45 mg $L^{-1,}$ and is remained at pH 7.6. A low COD/N ratio remained about 2.5, which facilitates for microalgae biomass enrichment and nutrient recovery.

2.2. Experimental design

The stirred photobioreactors (PBRs) were used for all batch experiments. The transparent glass reactors have a working volume of 14 L and the dimension of length x diameter = 60 cm x 20 cm. Led lamps were rolled around the PBRs to provide a specific light intensity of

100 µmol m^{-2} s^{-1}. All batch reactors were operated under a cycle of 12 h light–12 h dark. Constant mixing was maintained using a stirrer (100 rpm) to avoid algae sedimentation. Detail schematic diagram of the PBRs system is illustrated in Fig. S-1.

Five reactors were prepared with different inoculum ratios: the pure culture of microalgae (1:0 wt/wt), co-culture of microalgae:activated sludge under the ratios of (9:1, 3:1, 1:1 wt/wt), and only activated sludge (0:1 wt/wt). The total initial biomass concentration has remained about 400 mg L^{-1} for all reactors. Detailed initial concentrations of microalgae and activated sludge were presented in Table 1.

Insert Table 1.

2.3. Analytical parameters

Prior to analyses, a 200 mL sample was filtered using a filter with a pore size of 0.45 µm (Fisher Whatman puradisc-25 mm). Such parameters as COD, TP, TKN, NH_4^+-N, NO_3^--N, NO_2^--N and TSS were analyzed according to standard methods (APHA, 2005). The dissolved oxygen (DO) concentration was measured using a DO meter and the pH was measured by a pH meter. Light intensity was directly measured using a submersible spherical light sensor (US-SQS/L, ULM-500, FA Walz, Germany).

2.4. Microbial biomass analyses

The total biomass in reactors was defined through measuring the dry weight. In detail, 10 mL of mixed liquid samples for all reactors were taken every day for analyses. All samples were filtered using a membrane with a pore size of 0.45 µm (Fisher Whatman puradisc-25 mm), followed by being dried at 105 °C for 2 hours and then weighed. Dry biomass was defined based on a change in weight between before and after filtered samples. For the co-culture system, the dry biomass includes microalgae and activated sludge features ($C = C_m + C_b$).

Where C is the total biomass concentration (g L^{-1}), C_m is the microalgae biomass concentration (g L^{-1}), C_b is the activated sludge concentration.

For the microalgae biomass (C_m), it was measured through C*hlorophyll-a* content extracted from the microalgae cell. *Chlorophyll-a* concentration was defined based on the previous method (Tang et al., 2018). Such concentration was then converted to dry weight through a standard curve with the equation: $y = 4216.4\,x - 302.43$. This equation performs the correlation between C*hlorophyll-a* concentration and the dry weight of microalgae. The standard curve was presented in Fig. S-2. Where y is the concentration of C*hlorophyll-a*, and x is the dry weight of microalgae.

Chlorophyll-a content was extracted using an acetone solution (Lee et al., 2015). Firstly, a 40 mL sample taken from either pure culture or co-culture systems was centrifuged at 4000 rpm for 10 minutes. After supernatants were discarded, the residual features were mixed with 90% acetone solution and 0.05 g $CaCO_3$ and then was sheared using a vortex mixer for 1 minute. Secondly, such suspension was stored at 4 °C for 24 h in darkness before it was centrifuged at 4000 rpm for 10 minutes for supernatant recovery. These supernatants were used to determine the *Chlorophyll-a* content. In detail, *Chlorophyll-a* concentration was measured using ultraviolet spectrophotometry under different wavelengths: 630, 645, 663, 750, 772, and 850 nm. 90%. Acetone solution was used as the blank. As reported the C*hlorophyll-a* concentration of microalgae in a co-culture system was defined as Eq. (1) (Lee et al., 2015):

$$C_m = \frac{[11.64(OD_{663} - OD_{750}) - 2.16(OD_{645} - OD_{750}) + 0.10(OD_{630} - OD_{750}) - 25.2(OD_{772} - OD_{850})]V_1}{V.\sigma} \qquad (1)$$

Where V is the sample volume (L), V_1 is the volume of acetone-based extract (mL), OD (Optical Density) is the absorbance of the light at a corresponding wavelength, and σ is the optical path of the cuvette (cm). Also, such parameters as total biomass productivity, specific growth rate, and specific uptake rate were expressed as Eq. (2), (3), (4).

Total biomass productivity (β, mg L^{-1} day^{-1}): $\beta = \frac{X - X_o}{\Delta t}$ (2)

Specific growth rate (μ, d^{-1}): $\mu = \frac{lnX - lnX_o}{\Delta t}$ (3)

Specific uptake rate (η, g $gbiomass^{-1}$ d^{-1}): $\eta = \frac{C_o - C}{(X - X_o)\Delta t}$ (4)

Where, X_0, X are total biomass at the initial and final time of the log phase; C_0 and C represent for the substrate concentration at the initial and final time of phase; Δt is the interval days e.g., from the initial time to a time which total biomass reach steady state.

2.5. Nitrogen mass balance

Removal mechanism of nitrogen was defined according to a mass balance. The contributions to nitrogen mass include TN uptake by biomass, TN stripping, TN-denitrification, and residual TN. Since all batch reactors were operated under the stirred condition and remained a pH of 7.5-9.0, TN stripping has a negligible contribution. Thus, total nitrogen mass balance can be defined as follows:

Initial TN = Residual TN + TN-denitrification + TN uptake by biomass (5)

Whilst TN uptake by biomass might be due to contributions of either biological assimilation of microalgae or bacteria, TN-denitrification is obtained from bacteria metabolism. The nitrogen content in biomass was referred from a past work that used synthetic wastewater for cultivation (Zhu et al., 2019). As reported the nitrogen content was 8.25% for the co-culture system. Such values were used to calculate the TN uptake by biomass.

2.6. Statistical analysis

Results were showed as the average value ± standard deviation. Parametric one-way analysis of variance (ANOVA) was used to examine significant differences among groups of samples using the IBM SPSS statistics software 20. $p < 0.05$ indicated significance at 95% confidence.

3. Results and discussion

3.1. Effect of inoculum ratios on biomass growth and variation of dissolved oxygen and pH

As denoted in Fig. 1, after 5 d total biomass concentration increased from 0.4 g L^{-1} to 1 g L^{-1} for either single microalgae culture or co-culture systems i.e., inoculum ratios of 1:0, 9:1 and 3:1 wt/wt (Fig. 1a, b, c). For 1:1 ratio, the total biomass concentration reached to 1 g L^{-1} after 10 d. On the other hand, the experiment with a 0:1 ratio resulted in a slight increase of the total biomass concentration to 0.6 g L^{-1}. The maximum total biomass concentrations were obtained at day 6^{th} yielding 1.12 g L^{-1} and 1.07 g L^{-1} for 1:0 and 3:1 ratios, respectively. However, this fact occurred a later stage i.e., on day 7^{th} for 9:1 ratio (1.1 g L^{-1}), on day 11^{th} for 1:1 ratio (1.08 g L^{-1}). Such findings indicate the initial inoculum ratio impacted on biomass growth in some certain extent. Higher initial fraction of microalgae in co-culture system could contribute to shorten acclimatization period.

The biomass fractions of microalgae and activated sludge also changed following the inoculum ratios (Fig. 1). Only the fractions of 9:1 ratio were unchanged, being around 90%, during experimental period (Fig. 1b). It is important to note that a major change of biomass fraction was observed between the initial and final stage of experiment for 3:1 and 1:1 ratios (Fig. 1c, d). Especially for the latter ratio, the fraction of activated sludge remained stable at 50% for the first 4 d, then it decreased gradually to 18% on the last day. The reason laid on the composition of feeding wastewater for microalgae and sludge which comprised a low COD/N ratio (2.5:1). This low COD/N ratio could augment biomass yield of microalgae rather than bacteria (Zhu et al., 2019). Microalgae could also assimilate organic carbon competitively with bacteria in photoheterotrophic condition (Guo and Tong, 2019). Thus, upon organic matter in feeding wastewater exhausted since day 4^{th}, bacteria growth depleted and their fraction in total biomass decreased (Fig. 4).

Insert Table 2.

Another point, it was found that the PBRs operated with higher initial activate sludge concentration could render to decrease the maximum microalgae biomass. This fact implies suspended activated sludge would interfere photosynthesis process of microalgae by reducing light intensity for microalgae cells. As a result, photosynthesis yield was diminished and biomass yield of microalgae dropped. Our calculation showed that the specific growth rates of microalgae decreased proportionally with the decrease of microalgae:sludge ratios (Table 2). In practice, high specific growth rates of microalgae in 1:0, 9:1 and 3:1 ratios offered benefit as this fact reduced retention time and reactor volume. To further define a proper inoculum ratio for biomass production, total biomass productivity of all ratios was calculated and compared (Fig. 1f). The result showed that all pure microalgae culture (ratio 1:0) or co-culture (ratios 9:1, 3:1, 1:1) possessed superior total biomass productivity than the single activated sludge culture (ratio 0:1) (One-way ANOVA, $p < 0.05$). Such findings reinforce the vital role of microalgae on the biomass production under the following typical conditions: feed wastewater having low COD/N (2.5:1), photoheterotrophic, stirred condition and light: dark cycle of 12:12. The ratio of 3:1 showed comparable total biomass productivity with 1:0 ratio ($p > 0.05$), being 136 ± 10 mg L^{-1} d^{-1} and 144 ± 10 mg L^{-1} d^{-1}, respectively. However, 3:1 ratio exhibited significant higher total biomass productivity than other ratios i.e., 9:1 (88 ± 20 mg L^{-1} d^{-1}) and 1:1 (58 ± 20 mg L^{-1} d^{-1}) ($p < 0.05$). The findings suggested that ratio 1:0 and ratio 3:1 were the optimal one for total biomass production. The pure microalgae culture (ratio 1:0) could produce high biomass when feeding wastewater for cultivation (Gao et al., 2014; Jaatinen et al., 2016; Wang et al., 2017). As previously reported microalgae biomass has been utilized for biofuel production (Rittmann, 2008; Mata et al., 2010). Therefore, for the co-culture system, the 3:1 ratio might be an alternative way to attain beneficial biomass production, with microalgae biomass attaining 0.95 g L^{-1}. To come up with the conclusion which inoculum ratios was optimal in this study, we further investigated performance of those ratios for pollutant remediation in section 2.2.1 and 3.2.2.

Insert Fig. 1

Regarding DO, it is an indicator for microalgae biomass growth (Morales et al., 2018; Kazbar et al., 2019) and thus it was recorded for all the studied ratios (Fig. 2a,b). For 0:1 ratio (sludge only), DO concentration always stayed below 0.5 mg L^{-1} since day 1 for the whole studied period (Fig. 2a). This happened because the stirred condition of the experiment favored anoxic condition and consequently dropped DO level significantly (Su et al., 2012). Such condition prefers to slow-growing microorganism, which resulted in longer acclimatization and consequently restrained microorganism growing (Nguyen et al., 2016). Meanwhile, the single microalgae system (ratio 1:0) performed differently and such DO concentration rose two-fold from 4 mg L^{-1} to 8 mg L^{-1}, indicating substantial microalgae growth. Notably, compared to 1:0 ratio, ratios in co-culture systems (i.e., 9:1, 3:1, and 1:1 wt/wt) exhibited lower DO concentration during the light phase. Furthermore, DO concentration is expected to be lower during the dark phase, being 0.5 mg L^{-1}, as observed in Fig. 2b. This meant that bacteria consortium in activated sludge consumed oxygen which released from photosynthesis of microalgae in both light and dark phases. Thus, co-culture system which having higher initial fraction of activated sludge (3:1 and 1:1 ratios) possessed lower DO level (Fig. 2a). These facts can be regarded as mutualism of microalgae and bacteria under the photoautotrophic condition (Guo and Tong, 2019). Given the aforementioned conditions, DO concentration started decreasing gradually from day 5 and this is attributed to the reduction of microalgae biomass (Fig. S-5b, d) during the death phase occurred. Meanwhile, DO of 1:1 ratio decreased to approximately 0 mg L^{-1} on day 3. This can probably be subjected to oxygen released from photosynthesis of microalgae being consumed quickly by aerobic bacteria (Muñoz and Guieysse, 2006). Then, DO increased gradually and remained unchanged from day 6 to 11. The result was consistent with the augment of microalgae fraction in those ratios (Fig. 1d).

Apart from biomass production and DO, pH variation of the microalgae: sludge ratios is presented in Fig. 2c. Since the initial stage, pH value of around 7.7 was set for all culture systems. For both 1:0 and 3:1 ratios, pH increased more than 1 unit in log phase, subsequently decreased slightly in death phase. pH increased due to the intensive CO_2 consumption from the medium by microalgae. If the released CO_2 from the respiration of bacteria is not sufficient for microalgae photosynthesis, the balance between CO_2 from the air and CO_2 uptake by microalgae tended to occur and render pH stability (Su et al., 2012). This fact was consistent with the case of 1:1 ratio which pH was stable from 7.6 to 8.0. The pH change might be attributed to either autotrophic microalgae and/or nitrifying bacteria existed in the reactor. As reported, photosynthesis of microalgae increased pH whilst the nitrification process of bacteria decreased pH (Gutzeit et al., 2005; Muñoz and Guieysse, 2006) . In a co-culture system, pH is a dependent factor on biomass growth of microalgae, alkalinity and DO concentration of the media themselves (Muñoz and Guieysse, 2006). Notably, pH in the activated system alone dropped from 7.7 to 6.5 at ratio of 0:1. This happened due to the occurrence of nitrification given it utilized oxygen, produced H^+ and thus reduced pH (Higgins et al., 2018b; Mujtaba et al., 2018).

Insert Fig. 2

3.2. Effect of inoculation ratio on the performance of PBRs

3.2.1. Nutrient removal

Fig. 3a shows that TN concentration of 1:0 ratio decreased 50-fold from 200 mg L^{-1} to 4 mg L^{-1}, meant 95% TN have been removed in 6 d of operation. For 0:1 ratio, TN concentration was removed slightly of 14 % in 12 d, which was due to a minor reduction of NH_4^+-N concentration (Fig. S6-a). As mentioned, DO concentration of 0:1 ratio stayed below 0.5 mg L^{-1}, which did not favor nitrification process. This further caused low NO_2^--N and NO_3^--N concentration in this ratio (Fig. S-6c, e). After 5 d, TN removal of the co-culture ratios differed i.e., 9:1 (67%), 3:1 (86%), and 1:1 (42%), and such results indicated a co-culture system having higher microalgae fraction provided sufficient TN removal. As a result (Fig. S7-a), under co-culture ratios of 1:0, 9:1, 3:1 wt/wt, such TN removal rates were significant higher compared to the other ratios (i.e., 1:1 and 0:1) (One-way ANOVA, $p < 0.05$). Notably, for the initial 4 d, although the TN removal rates obtained 40 mg L^{-1} d^{-1}, 36 mg L^{-1} d^{-1}, and 35 mg L^{-1} d^{-1} for the inoculum ratios of 1:0, and 9:1, 3:1 respectively, overall removal rates between these ratios had no significant difference based on statistical analysis ($p > 0.05$). As reported in literature, the following conditions were essential to obtain TN removal rate of 4.06 mg L^{-1} d^{-1}: alone *C. vulgaris* cultivation (similar to 1:0 ratio of this study) and low COD:N ratio of 0.125 (Gao et al., 2015). Likewise, 2:1 ratio of co-culture system improved TN removal rate to 19 mg L^{-1} d^{-1} (Mujtaba and Lee, 2017). Such findings indicated the pivotal role of microalgae for TN assimilation in most ratios (e.g., 1:0, 9:1, 3:1). However, to select a proper ratio, an evaluation for phosphorus removal needs to be considered, and such results are discussed later.

Insert Fig. 3

To obtain an insightful evaluation for optimal microalgae:sludge ratio and understand nitrogen transformation, concentrations of NH_4^+-N, NO_2^--N and NO_3^--N were measured for the whole course of experimental assay (Fig. S-6a, c, e). In general, concentrations of NO_2^--N and NO_3^--N were very low for the first 2 d. Later on, the concentrations started increasing gradually for all co-cultures and this might contribute to nitrification occurred. The maximum concentrations of NO_2^--N and NO_3^--N were obtained in 3:1 ratio of the co-culture system. For 0:1 ratio, low NH_4^+-N assimilation was denoted as well as the concentrations of NO_2^--N and NO_3^--N appeared very minor (Fig. S-6a) which was due to insufficient dissolved oxygen concentration (DO < 0.5 mg L^{-1}) (Fig. 2a, b). Through literature, it has been indicated that adequate nitrification requires DO level higher than 2.5 mg L^{-1} (Nguyen et al., 2016). In contrast, NH_4^+-N concentration of 1:0 ratio decreased eight-fold from 200 mg L^{-1} to 25 mg L^{-1} in 4 d (Fig. S-6a) indicated that assimilation was the main pathway for nitrogen remediation (Fig. 3b). The symbiosis of microalgae and bacteria occurred in the co-culture systems; thereby the released oxygen from photosynthesis of microalgae was consumed by bacteria towards the nitrification process. However, NO_3^--N concentration was not high, and this fact was caused by either NO_3^--N denitrification or NO_3^--N assimilation of microalgae. As reported microalgae also used NO_3^--N as a nutrient source for cell built-up upon exhausted NH_4^+-N source (Kim et al., 2010). The mechanism of nitrogen removal could be described using the results in Fig. 3b. Generally, assimilation process contributed dominantly for overall nitrogen removal. Table 3 exhibits the results of TN specific uptake rates for all microalgae:sludge ratios. Higher microalgae fraction in a co-culture system resulted in higher biological assimilation of nitrogen (Fig. 3b). Compared to activated sludge, microalgae played a dominant role in biological assimilation of nitrogen. It can be seen that, for ratio 1:0, TN denitrification and assimilation fractions were minor and marked 9.3% and 13.5%, respectively. It rendered low TN removal. Among the co-culture systems, inoculation ratios having higher sludge fraction provided better denitrification pattern. As observed in Fig. 2a, DO concentration in the light phase was over 4 mg L^{-1}, which hindered the denitrification process. It is anticipated that denitrification was possible to occur in the dark phase with DO lower than 0.5 mg L^{-1}. However, this fact was activated in a weak manner for 9:1 and 3:1 ratios. Given these conditions, visualization of the outcomes was made by microscopic images. Our results confirmed a spatially adjacent microcosmic structure forming in between activated sludge and microalgae cells (Fig. S-4a, b). Such structure supported bacteria to obtain adequate O_2 released by microalgae, it also potentially hinder bacteria from contacting the anoxic zone in the reactor and inhibited denitrification (Guo and Tong, 2014). As reported from past work (González-Fernández et al., 2011), while the substrate extent strongly influenced nitrogen formation, their study indicated denitrification was the main contribution in a co-culture system for pig slurry wastewater having high COD:N ratio of 7.7:1 (González-Fernández et al., 2011). Another one reported that with the COD:N ratio of 3.5:1, 80% nitrogen removal was obtained from nitrification-denitrification (Wang et al., 2015). Therefore, a low COD:N ratio (2.5:1) of feed wastewater used in this study might be a final explanation for a low denitrification. Overall findings indicate that the COD:N ratio and inoculum ratio posed certain effects on the nitrogen removal mechanism.

Insert Table 3

Apart from nitrogen, phosphorus also played a key role for microalgae growth as it was a vital element for cell metabolism. Polyphosphate-accumulating organisms (PAOs) are a vital group in activated sludge which functioned phosphorus removal (Gutzeit et al., 2005; ; Ji et al., 2018b; Huo et al., 2020b). The data showed that ratios of pure microalgae and co-culture systems remediated TP in a greater extent than single bacteria culture ($p < 0.05$). In general, TP was removed increasingly with the fraction of microalgae in co-culture systems (Fig. 3-c). For 0:1 ratio, TP was removed insignificantly and this fact can probably be attributed to the poor presence of PAOs in the experimented cultures (Church et al., 2017). In microalgae cultivation, TP could be assimilated to form algae biomass which was the key mechanism (Su et al., 2012). Therefore, in this study, the highest TP removal attributed to single microalgae culture (1:0 ratio) which remediated 98% TP after 9 d. Meanwhile, for the co-culture systems, the removal efficiencies of 9:1, 3:1 and 1:1 ratios were 98%, 93% and 62%, respectively. Such results indicated that bacteria did not contribute effectively in phosphorus removal rather than microalgae. Another study reported that pH and DO could also impact TP remediation. Phosphorus could be precipitated at pH beyond 8 and high DO level (Godos et al., 2009). In this study, pH was higher than 8 and DO exceeded 4 mg L^{-1} for 1:0, 9:1, and 3:1 ratios during the growth phase. This implied that phosphate precipitation joined to decrease phosphorus concentration; however, this mechanism appeared very minor in the co-culture system (Zhu et al., 2019). Fig. S7-b shows that TP removal rates ranged from 1.6 to 7.2 mg L^{-1} d^{-1} being higher than that of 1.3 mg L^{-1} d^{-1} of (Mujtaba and Lee, 2017) who co-cultured microalgae and activated sludge (1:1) with low-strength municipal wastewater (COD = 60 mg L^{-1}, TP = 1.3 mg L^{-1}). Higher TP removal rate in our study could be explained due to higher COD concentration of the feed wastewater (COD = 500 mg L^{-1} and TP = 45 mg L^{-1}). Although single microalgae culture has been noticed for high TP removal (Delgadillo-Mirquez et al., 2016), the obtained results suggested that 3:1 and 9:1 ratios of co-culture systems were highly feasible for total phosphorus remediation in practice.

3.2.2. Organic matter removal

Together with nitrogen and phosphorus, organic matter supply carbon source for cells to synthesize materials and store energy. Co-culture could also assimilate carbon from COD for those purposes. Fig. 4 indicates that COD removal by microalgae culture alone was much better than activated sludge culture. This is attributed to the experimental conditions given that PBRs were operated under photoheterotrophic mode which favored microalgae growth and COD uptake (Sforza et al., 2018). For 3:1 and 1:1 ratio, COD concentration decreased by 85 - 96% after 4 d and residual COD concentrations were 19 and 74 mg L^{-1}, respectively. Acetate substrate used in this study is a readily biodegradable compound; thus it could be a substrate for both aerobic sludge and microalgae in a co-culture system (Zhu et al., 2019). COD concentration of 3:1 ratio increased gradually after biomass growth curve reached death phase because of endogenous respiration of bacteria and microalgae. The highest COD removal rate belonged to 3:1 ratio possessing specific uptake rate of 132.7 mgCOD $gbiomass^{-1}$ d^{-1} (Fig. S7-c). This ratio is a proper one to promote cooperation between the microalgae and activated sludge. The ratios of 3:1 and 1:1 exhibited significantly higher COD removal rate than other ratios ($p < 0.05$) (Fig. S7-c), being 131 and 118 mg L^{-1} d^{-1}, respectively. However, those values were lower than COD removal rate in the results of Zhu et al., 2019 (930 mg L^{-1} d^{-1}). Compared to our work, the study of Zhu et al., 2019 used wastewater containing higher COD:N (4.3:1), 2% CO_2 aeration and 1:1 inoculum ratio. Such findings indicated COD removal rate depended on several factors such as COD loading, microalgae:activated sludge fraction and agitating condition of the co-culture system. Although aeration was not provided in the PBRs, COD was still eliminated sufficiently in 3:1 and 1:1 ratios. It potentially helps save operating cost. The ratio of 1:1 showed lower COD removal rate than that of 3:1 (Fig. S7-c). DO of the 1:1 ratio was low (< 1 mg L^{-1}) and it was probably inadequate for bacterial respiration to mineralize organic matters. Likewise, the 0:1 ratio was more severe as its DO concentration was lower than that of 1:1 ratio (DO < 0.5 mg L^{-1}) and thus resulted in low COD removal. DO concentration from 2 to 4 mg L^{-1} in the mixed liquor of the aeration tank of the activated sludge process was an important requirement to attain sufficient COD removal (Metcalf and Eddy, 2003). For 1:0 ratio, although COD was eliminated from 496 to 156 mg L^{-1} after 8 d, the COD removal efficiency was relatively low. That is, lacking of bacteria and microalgae joined insufficiently in COD removal. Overall findings reinforced the pivotal cooperation of microalgae and bacteria to increase COD remediation efficacy once the co-culture system was employed.

Insert Fig. 4

3.3. Role of microalgae and activated sludge under different co-culture systems

Insert Fig. 5

To explore the role of microalgae and activated sludge on the pollutant's removal, correlation analysis between residual pollutant concentration and biomass concentration in the reactor was conducted for different co-culture ratios (9:1, 3:1 and 1:1 wt/wt). Microalgae/activated sludge biomass concentration obtained in the log growth phase was plotted with the corresponded residual concentrations of TP, TN and COD so as to define the correlation factors through linear regression. As a result, for the TP removal (Fig. 5-a, b), for all ratios, R^2 values of 0.91-0.96 obtained from the microalgae scenario were higher compared to the activated sludge scenario ($R^2 = 0.54\text{-}0.93$), which signifies a stronger correlation between TP removal and microalgae biomass concentration. As a final point on the role of activated sludge, the low R^2 values of residual TP concentration and activated biomass concentration (0.64 and 0.67) were found in the co-culture condition of minimal activated sludge fraction (the ratio of 9:1). In contrast, for the ratio of 1:1, high activated sludge fraction, a higher R^2 value of residual COD concentration and activated sludge biomass was observed (Fig. 5-c, d), indicated the role of bacteria consortium in activated sludge on organic removal. For the ratio of 3:1, it is noted no significant difference in R^2 values was found between microalgae and activated sludge cases. Such results thus indicated a certain symbiosis between microalgae and activated sludge that occurred in the co-culture system operated under the ratio of 3:1. As reported microalgae has ability of high nitrogen uptake (Cai et al., 2013; Boonchai and Seo, 2015; Jia and Yuan, 2016);

thereby the high R^2 values (0.93-0.97) of residual TN concentration and microalgae biomass obtained is inevitable. As discussed in section 3.2.1, for the ratio of 1:1, although bacteria consortium in activated sludge contributed to nitrogen removal through assimilation and denitrification, such contribution was not major and thereby resulted in insufficient TN removal. These facts were consistent with a low R^2 value of residual TN concentration and activated sludge biomass concentration (0.51). To sum up, such ratios of 1:0, 9:1, 3:1 were considered for selection based on adequate nutrient removal obtained. Meanwhile, the co-culture system with ratios of 3:1 and 1:1 facilitated a certain symbiotic between microalgae and bacteria and thus improved significant COD removal. However, as a final point on biomass productivity, it is important to propose the inoculation ratio of 3:1 for the co-culture system to ensure both nitrogen and organic removal.

3.4. Implication works

In the current study, a summary of nutrient and organic removal rate, and biomass production were presented in comparison with the previous studies (Table S-2). Such factors as different feed wastewater compositions (COD:N ratio, N:P ratio), microalgae strain, light:dark cycle and mixing/aeration condition were summarized to give an evaluation on pollutant removal and biomass productivity. A series of inoculation ratios (i.e., 1:1, 2:1, 3:1, 5:1, 9:1 wt/wt of microalgae:activated sludge) was investigated into the co-culture systems. It was generally accepted that for the co-culture systems these factors had certain impacts on treatment performance and biomass production. For instance, whilst our works explored an optimal ratio of 3:1 for both nutrient/organic removal and biomass productivity, such a ratio of 5:1 was found in a past study (Su et al., 2012). This fact is attributed to that their co-culture system was operated under other typical conditions: wastewater-born microalgae, COD:N = 7.6:1, and N:P = 5.7:1, which is distinct for our work (*Chlorella* sp. microalgae, COD:N = 2.5:1 and, N:P = 4.4:1). In our study, under the optimal ratio of 3:1, biomass productivity achieved 144 mg L^{-1} d^{-1}, and such value is significantly higher compared to the past work (40 mg L^{-1} d^{-1}). A limited phosphorus concentration due to a high N:P ratio (22.1:1) in the co-culture system from a past work might be critical points on inhibited microalgae biomass growth whereas an operation of light-dark cycle (24h:0h) could cause an influence on bacteria growth (Mujtaba and Lee, 2017). Due to a low COD:N ratio (2.5:1) wastewater, low activated sludge biomass was always maintained in this study. This condition leads to be favorable for microalgae enrichment. Such findings, together with the results that with a past study (Zhu et al., 2019) indicates that COD:N ratio wastewater is a key factor to yield high microalgae biomass in a co-culture system. Finally, it is important to note that microalgae and activated sludge could assist together, leading to benefits associated with wastewater treatment.

Table S-2 highlights that choosing an optimal inoculation ratio of microalgae:activated sludge is strictly dependent on types of wastewater (e.g., different COD:N and N:P ratios). If the co-culture system is implemented in a continuous mode, it should be noted that influence of the factors such as wastewater compositions (COD:N and N:P ratios), microalgae/bacteria strain, light:dark cycle, light intensity, and mixing/aeration condition on pollutant removal and biomass production need to be considered for evaluation. Because of the complexity of wastewater composition, it is essential to adopt different operating strategies i.e., inoculation ratios and light:dark cycles for the co-culture system. To apply for wastewater possessing a low COD:N ratio (2.5:1), overall findings suggested that a typical inoculation ratio of 2:1-3:1 wt/wt (microalgae: activated sludge) and a light:dark cycle of 12:12 is a sound guideline for attaining sufficient treatment and high biomass production. This fact probably brings practical application for the municipal wastewater

(COD:N ratio of 1.5-5.0), winery wastewater (COD:N ratio of 2.1), etc. It is noted that choosing design and operating parameters need to be considered if a large-scale system is implemented in reality. For a continuous mode, controlling biomass retention time (BRT) plays a pivotal role in retaining the biomass concentration in a reactor. Based on the results of the maximum specific growth rate, this works can provide an estimation on biomass retention time (BRT) which is calculated equal to inverse of maximum specific growth rate. To retain an inoculation ratio of 3:1 in continuous mode, it is suggested that the BRT of 5 d corresponding to maximum specific growth rate of 0.206 d^{-1} should be maintained in the photobioreactor by controlling excess biomass. Such biomass can probably be utilized for bioenergy production. Several studies reported that added-value products can be obtained concomitantly with wastewater treatment by microalgae-activated sludge symbiotic. Such products are biofuels, lipids obtained from harvesting biomass in the co-culture of microalgae-activated sludge (Leong et al., 2018; Choi et al., 2020).

4. Conclusion

This work investigated the influence of different microalgae-activated sludge ratios on nutrients and organic matter removal and biomass production. This study confirmed a certain symbiosis between microalgae and activated sludge in the co-culture PBRs of 3:1 and 1:1 ratio. However, the 3:1 ratio of microalgae:activated sludge was proposed to attain simultaneously organic/nutrient removal and biomass production for low COD:N wastewater. This condition performed sufficient removal of TN (86%), TP (79%) and COD (99%) and total biomass concentration of 1.12 g L^{-1}. Microalgae played a pivotal role in nutrient assimilation while activated sludge contributed to TN assimilation, denitrification and COD removal.

Appendix A. Supplementary data

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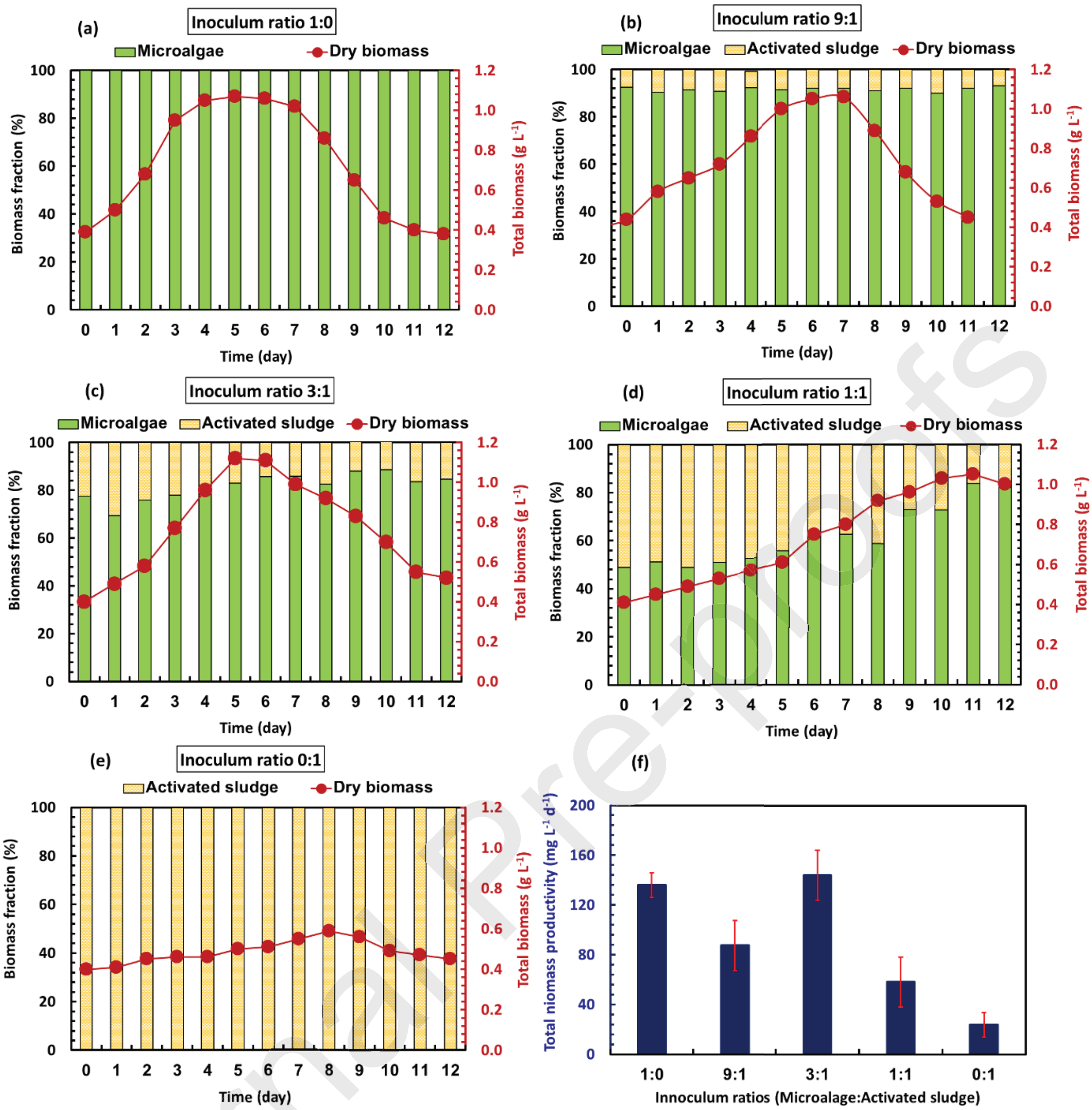
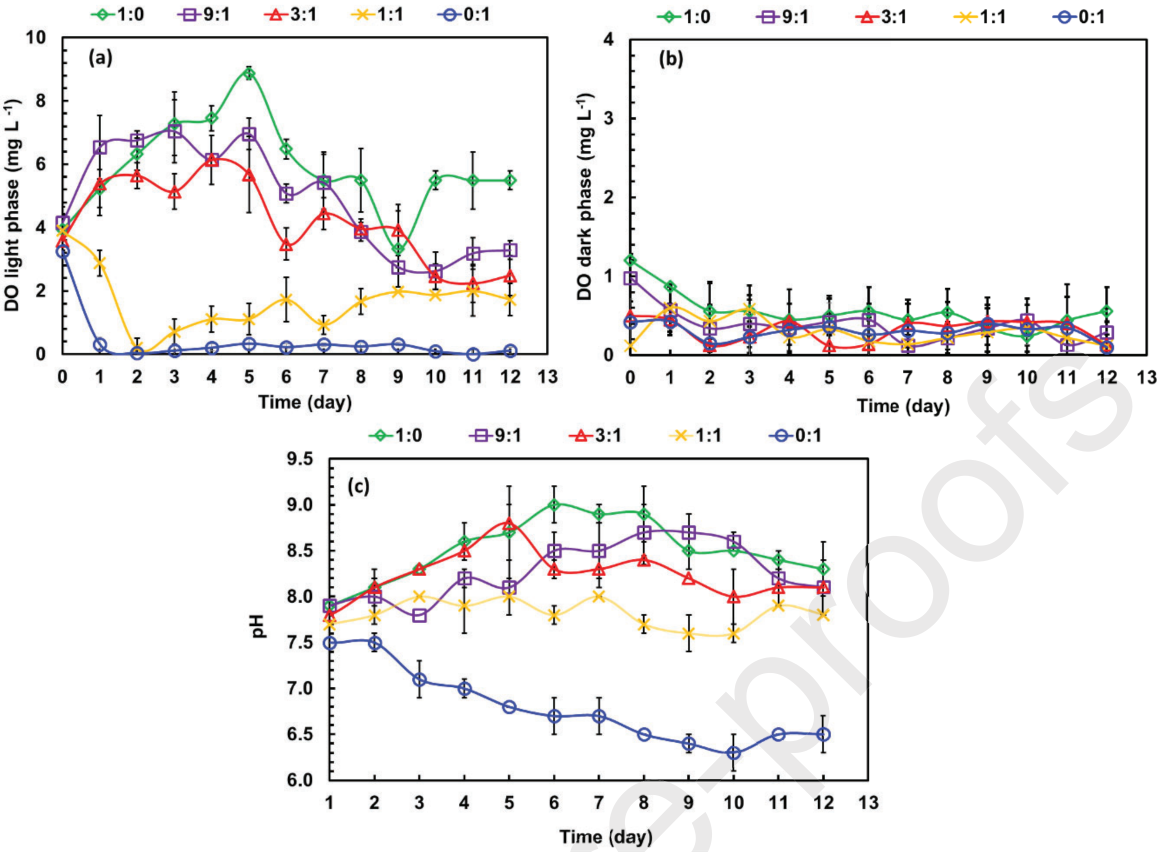


Figure 1. Total biomass, biomass fraction of microalgae and activated sludge under different inoculation ratios (microalgae: bacteria wt/wt): (a) 1:0, (b) 9:1, (c) 3:1, (d) 1:1, (e) 0:1 and (f) Total biomass productivity under ratio conditions



714
715
716 **Figure 2.** DO concentration and pH change at different microalgae-activated sludge
717 inoculation ratios: (a) DO in the light phase, (b) DO in the dark phase, and (c) pH

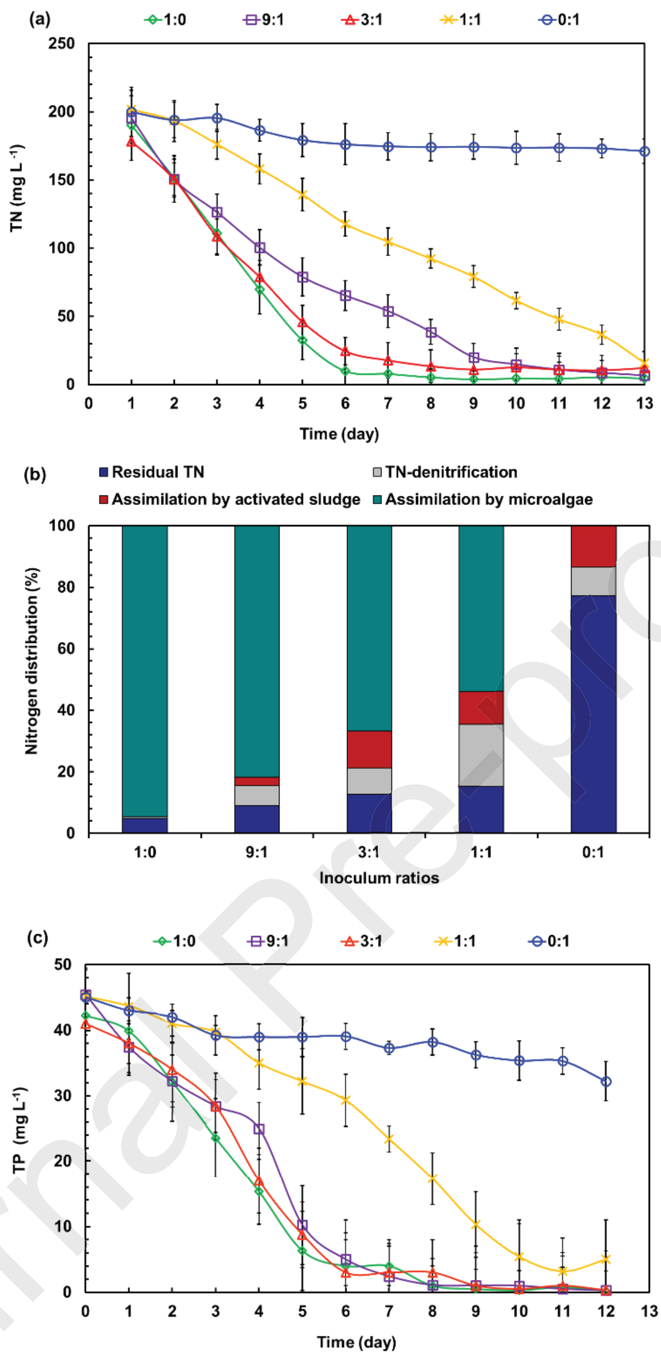


Figure 3. Nutrient removal in the PBRs operated under different inoculum ratios. (a) TN concentration as a function of time, (b) Mechanisms of nitrogen removal, (c) TP concentration as a function of time

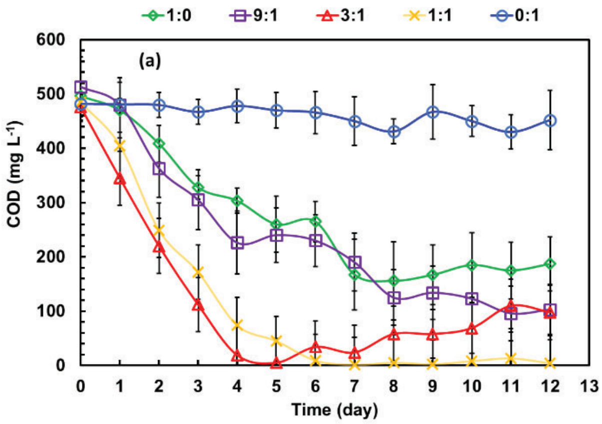


Figure 4. COD removal in the PBRs operated under different inoculum ratios: COD concentration as a function of time

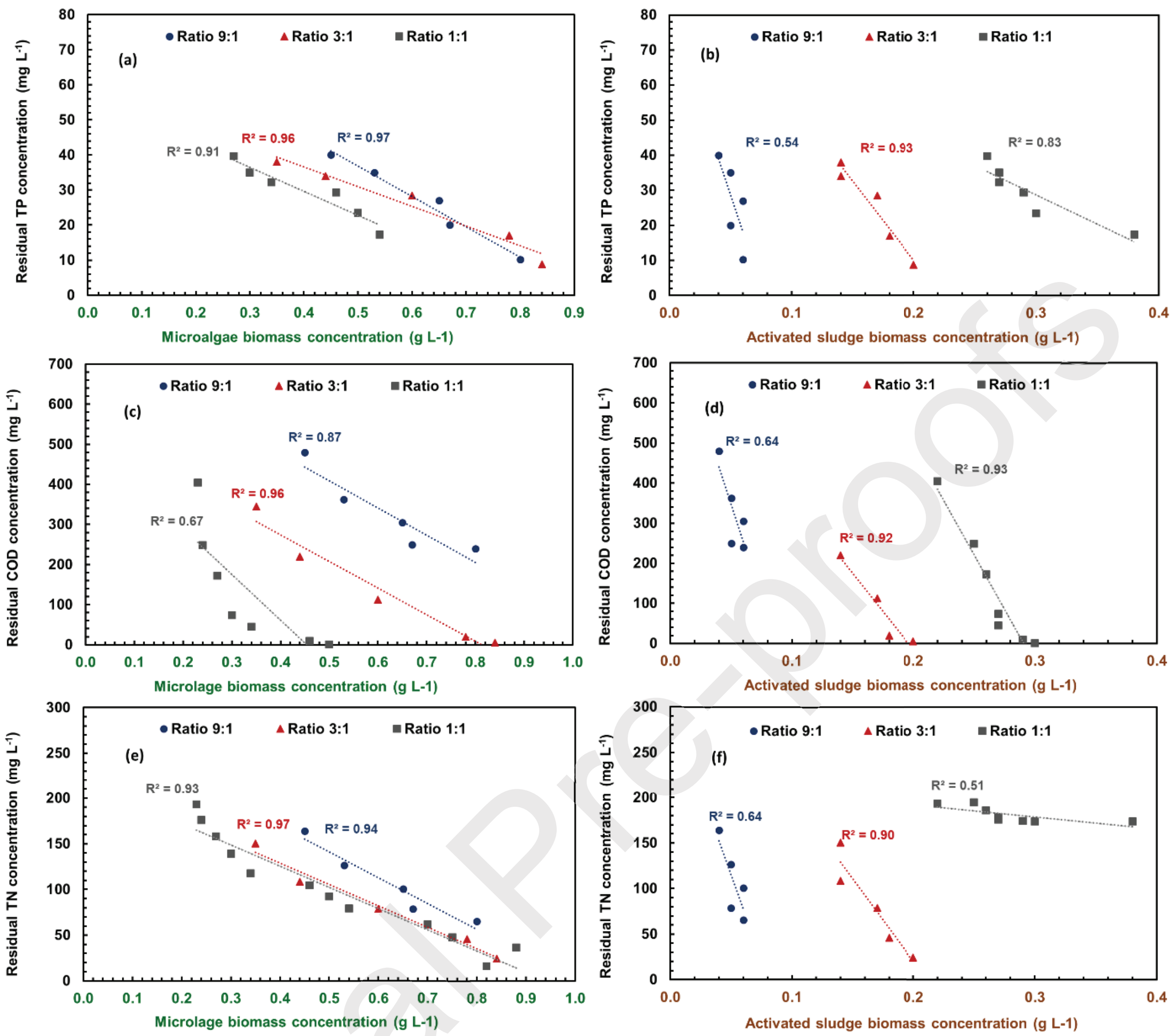


Figure 5. Correlation analysis between residual pollutant concentration and biomass concentration under different co-culture system. Residual TP, COD, TN concentration was plotted with microalgae biomass (a, c, d). Residual TP, COD, TN concentration was plotted with activated sludge biomass (b, e, f)

Table 1. Initial concentration of microalgae and bacteria under different inoculation ratios

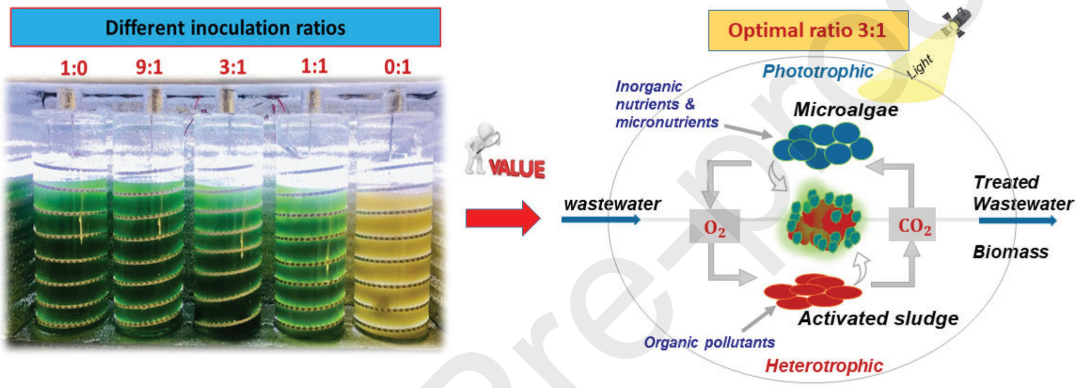
Microalgae:activated sludge inoculation ratios (wt/wt)	**1:0**	**9:1**	**3:1**	**1:1**	**0:1**
Initial conc. of microalgae (mg L^{-1})	400	360	300	200	0
Initial conc. of activated sludge (mg L^{-1})	0	40	100	200	400
Initial conc. of inoculum biomass (mg L^{-1})	400	400	400	400	400

Table 2. Specific growth rate of microalgae and activated sludge under different inoculum ratios

Specific growth rate (d^{-1})	Inoculum ratios				
	1:0	**9:1**	**3:1**	**1:1**	**0:1**
Microalgae	0.254	0.191	0.187	0.131	-
Activated sludge	-	0.101	0.084	0.078	0.049
Microalgae and activated sludge	-	0.210	0.206	0.092	-

Table 3. Specific uptake rate for different inoculum ratios

Specific uptake rate (mg gbiomass^{-1} day^{-1})	Inoculum ratios				
	1:0	9:1	3:1	1:1	0:1
TN specific uptake rate	53.0	31.3	43.3	17.4	11.1
TP specific uptake rate	10.6	7.7	7.6	6.0	4.7
COD specific uptake rate	61.8	63.3	132.7	68.8	23.6



Highlights

- Microalgae:activated sludge ratio (3:1) was the optimum co-culture operation.
- Microalgae played a vital role in biomass production and nutrient removal.
- Under the optimum ratio, COD removal was obtained 98% in 4 days.
- Biological assimilation majorly contributed to nutrient removal in the co-culture PBR.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: