

Article

Real-Time Behavior of a Microalgae–Bacteria Consortium Treating Wastewater in a Sequencing Batch Reactor in Response to Feeding Time and Agitation Mode

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Abstract: A study of a microalgae–bacteria treatment system was conducted in a sequencing batch reactor (SBR) by combining a precultured native algae *Nannochloropsis gaditana* L2 with spontaneous municipal wastewater microorganisms. Two types of agitation, air mixing (AI) and mechanical mixing (MIX), were assessed at continuous illumination (L) and photoperiod cycle light/dark (L/D). The obtained consortium, via native microalgae addition, has a better operational efficiency compared to spontaneous control. This allows the removal of 78% and 53% of total Kjeldhal nitrogen (TKN) and chemical oxygen demand (COD), respectively. Under the (L/D) photoperiod, the optimal removal rate (90% of TKN and 75% of COD) was obtained by the consortium at 4 days of hydraulic retention time (HRT) using the AI mode. Moreover, during feeding during dark (D/L) photoperiod, the highest removal rate (83% TKN and 82% COD) was recorded at 4 days HRT using the AI mode. These results bring, at the scale of a bioreactor, new data regarding the mode of aeration and the feeding time. They prove the concept of such a technology, increasing the attraction of microalgae-based wastewater treatment.

Keywords: microalgae; wastewater treatment; SBR; feeding time; hydraulic retention time; agitation type; photoperiod application

1. Introduction

Conventional wastewater treatment plants are still facing several techno-economic difficulties induced by their high operational energy cost and inefficient nutrient removals [1]. Generally, 1 kWh of electricity is required to supply the oxygen needed for 1 kg of biochemical oxygen demand (BOD) for aerobic wastewater treatment plants (WWTP) [2,3]. In fact, aeration is a costly process, presenting about 45–75% of the total energy consumption in WWTPs [4,5]. Consequently, oxygen produced from the photosynthetic reaction by microalgae can be used as a source for aerobic microbes and reduces aeration costs accordingly [6,7].

The development of cost-effective and efficient technologies for single-step wastewater (WW) treatment is in great demand since it can be applied as an alternative system for conventional biological WW treatment [8–10].

Therefore, microalgae as photosynthetic microorganisms produce oxygen and ensure the removal of contaminants (nitrogen, phosphorus, and carbon) from wastewater. Meanwhile, the microalgae biomass can also be used for the production of high-value chemicals [11,12].

Recently, the symbiotic process of microalgae and bacteria has been significantly used for the treatment of municipal and industrial wastewater [10,13]. The microalgae–bacteria consortia maintain cost-effective aeration, sequester the greenhouse gas CO₂, and limit the risk of pollutant volatilization [14–16]. The interaction between microalgae and bacteria ensures efficient removal of organic and inorganic carbon, nitrogen, phosphorus, heavy metals, and other pollutants.

Due to this interaction, the organic matter mineralization by aerobic bacteria produces the inorganic carbon needed by the microalgae. In turn, the O₂ required for bacterial degradation is produced photosynthetically by the microalgae [14,15].

Indeed, abiotic factors are considered as the main physical parameters affecting the efficiency of the microalgae–bacteria's interaction [16]. The main factors are the aeration, temperature, and photoperiod (light–dark cycle). It was reported that the latter influences the average of biomass productivity, which can be increased by $21.6 \pm 2.1\%$ at the artificial light/dark cycle [17]. The few studies available, focused on the illumination cycle [14,15], have proved that the dark phase promotes the recovery of nitrifiers. Moreover, a study reported that the influence of light activity could inhibit ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). These bacteria could be recovered in dark conditions in 10 days. This leads us to say that the dark phase improves the proper functioning of nitrifiers [18]. Accordingly, this photoperiod cycle enhances the “biological nitrification–denitrification” process [19], where the oxidation of NH₄⁺ into NO₃[−] (nitrification) by AOB and NOB is followed by the biological reduction of NO₃[−] into N₂ (denitrification). Hence, this process confirms that nitrification and denitrification require aerobic and anaerobic conditions [10].

Commonly, high rate algal ponds (HRAP) [20] are usually easy to design, construct, and operate. However, this technology presents significant limitations such as water evaporation, large space requirements, contamination of algal cultures, and lack of control over operating parameters [21]. Nowadays, photobioreactors (PBRs) are used as another technology for microalgae cultivation. In comparison with open ponds, PBRs are often characterized by improved photosynthetic efficiency and reduced footprint [19–21].

Among PBR types, the sequencing batch reactor (SBR) has been selected since it allows easy control of the treatment process, including a wide range of pollutants [22]. SBRs are industrial processing tanks for WWT, sewage treatment, and anaerobic digestion [23]. This technology allows the process adjustment of nitrification, denitrification, phosphorus removal, and the elimination of carbon compounds [24]. Moreover, SBR content is intensively mixed and aerated in order to prevent sedimentation, along with providing oxygen for organisms [25]. This process leads to greater flexibility and low-cost investment [26]. SBRs are believed to achieve high effluent quality in a very short aeration time. It also allows the reduction of up to 60% of the operating cost when compared with the conventional activated sludge process [27].

Therefore, the purpose of the present study is to evaluate the influence of new parameters linked to wastewater treatment by microalgae-based consortia in SBRs. Indeed, we focused on the effect of the pure strain addition, the HRT, the agitation mode, and the feeding time related to photoperiod application (L/D, L, and D/L). The results were compared in terms of COD, TKN and total phosphorus (TP) removals, dissolved oxygen concentration (DO), pH, and population dynamics of microalgae bacteria. We also aim to approve real-time conditions during feeding at the dark phase and reduce energy consumption compared to the conventional process with activated sludge.

2. Materials and Methods

2.1. Composition of Raw Wastewater

The average quality of the influent municipal wastewater is reported in Table 1. The influent presettled wastewater was collected from the Trento Nord municipal WWTP (Italy), which has a capacity of around 100,000 population equivalent (PE) [28]. The data indicate that the WW used for the experiments has a high concentration of ammonia nitrogen and organic compositions. For this reason, it is expected to activate bacteria and microalgae development.

For all the experiments, the samples were analyzed in triplicate. The maximum standard deviation recorded was estimated at 6%.

Table 1. Composition of the raw wastewaters used as influents to the cultivation systems.

Parameters	(mg/L)
TKN	83.3 ± 27
TP	9.5 ± 3
sCOD *	137 ± 33
COD	498 ± 182
TSS	361 ± 91

* sCOD: The soluble chemical oxygen demand.

2.2. Enrichment by the Algal Strain *Nannochloropsis Gaditana L2*

This microalga [29] was cultivated in a 500-mL Erlenmeyer flask with 250 mL of effective volume under sterile conditions. The cells were continuously air bubbled and illuminated by using LEDs ($200 \mu\text{E m}^{-2} \text{s}^{-1}$) and. The obtained microalgal biomass in the exponential growth phase was used as inoculum for the PBR. The microalgae growth media composition is as follows: CaCl_2 (25 mg/L), NaCl (25 mg/L), NaNO_3 (250 mg/L), MgSO_4 (75 mg/L), KH_2PO_4 (105 mg/L), K_2HPO_4 (75 mg/L), and 3 mL of trace metal solution composed of FeCl_3 (0.194 g/L), MnCl_2 (0.082 g/L), CoCl_2 (0.16 g/L), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.008 g/L), and ZnCl_2 (0.005 g/L) [30]. All the media and apparatus used for microalgae cultivation were autoclaved at 121 °C for 20 min prior to use.

2.3. Bioreactor Set-up and Operations

A bench-scale SBR system consists of an influent tank, an SBR reactor, and an effluent tank. The SBR has a cylindrical form; the volume of the reactor vessel was 2 L, with a working volume of 1.5 L used for different HRTs of 8, 6, and 4 days. The reactor operation was performed from November 2018 to February 2019 in Trento (Italy).

Considering the above parameters, the microalgal-bacteria WWT process is clearly demonstrated in Figure 1. The SBR was connected to the WW tank and it is periodically charged with fresh municipal wastewater after the primary clarifier.

The reactor was primarily inoculated with pure microalgae at 8 days HRT under both types of agitation, AI/MIX, separately. Each agitation mode starts with an acclimation period of one week by using only continuous illumination. The complete process was operated for 121 days with three experimental phases, as indicated in Figure 2.

Each experimental phase involves the variation of photoperiod, the HRT, and the agitation mode. The experiment cycle consists of 5 min feeding period, light phase (18 h), dark phase (6 h), a settling period, and 5 min of discharging period. In the third phase, the effect of feeding at dark phase was investigated at 4 days HRT with the two types of mixing.

The fresh wastewater was pumped continuously through a peristaltic pump (Kronos Seko, Italy). As demonstrated in Figure S1, the SBR was illuminated in the light phase with cool-white lamp (8 LEDs × 0.5 W, 0.18 m high, 0.065 m wide, Orion-Italy), arranged on one side of the reactor.

The two different types of mixing were performed by a magnetic mixer set at 200 rpm (AGE, Velp, Italy) and an air diffuser in order to maintain the biomass in suspension during the process. The volumetric exchange ratio has been changed during the experiments, which lead to the creation of different HRTs of 8, 6, and 4 days. The effluent discharged by the SBR was regularly sampled and used for water quality analysis.

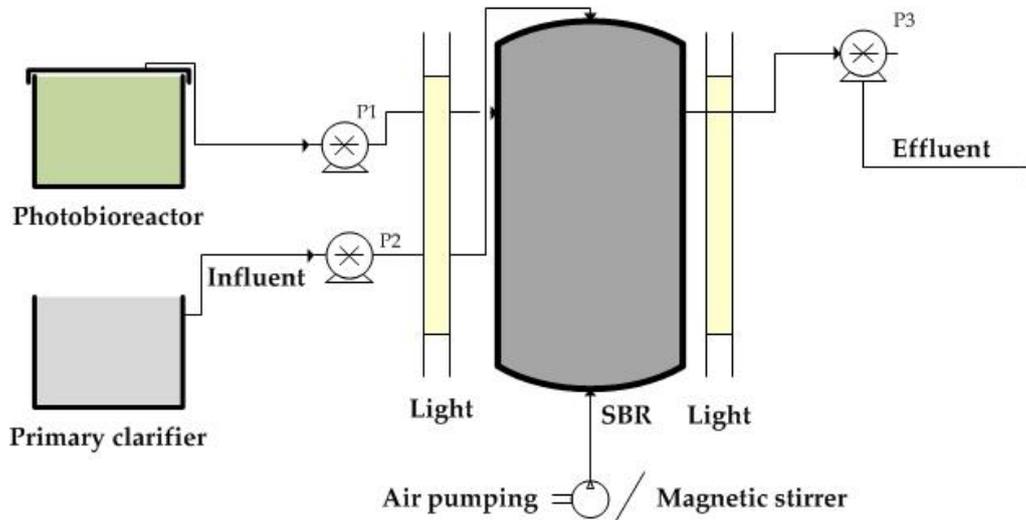


Figure 1. Schematic illustration of an integrated process treatment photobioreactor. (P1, P2, P3; pump).

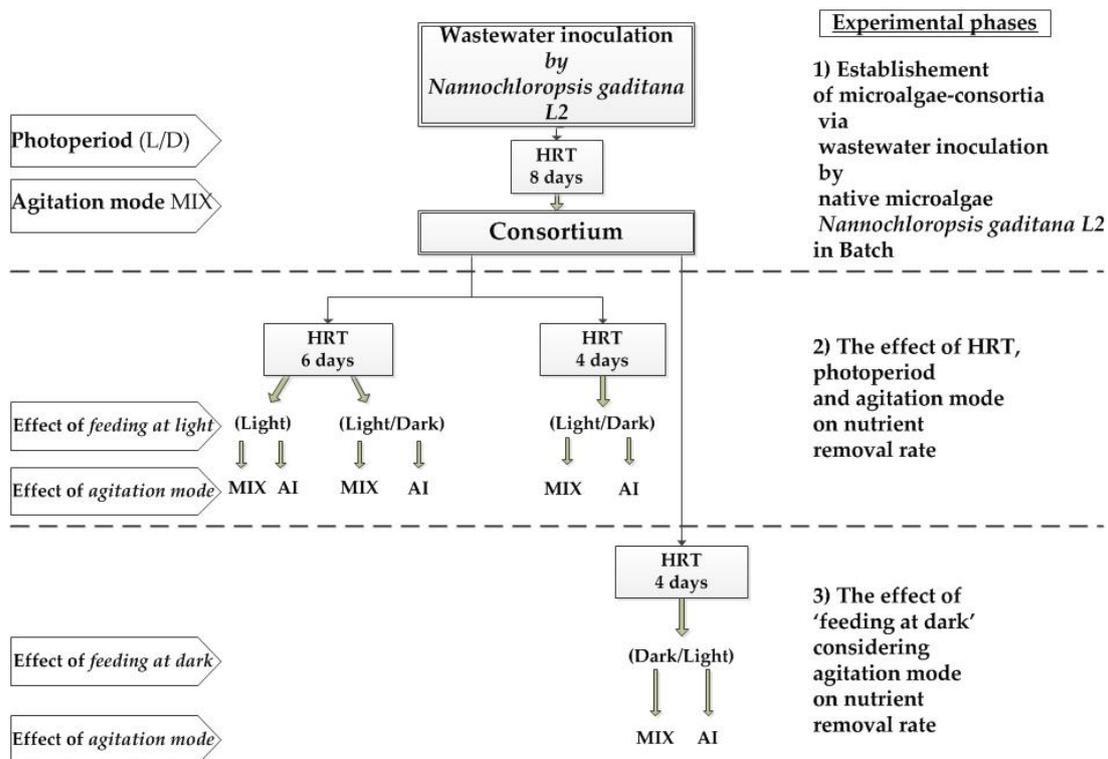


Figure 2. Diagram of experiments carried out in this work. Three experimental phases related to the evaluation of the main studied parameters in sequencing batch reactor (SBR) treating wastewater (WW) by microalgae-based consortium: HRT, agitation mode, and the effect of feeding time.

2.4. Consortia Establishment

The consortium, as depicted in the first phase (Figure 2), was obtained after one month of operation. During this phase, a fresh WW was inoculated with a native microalgae *Nannochloropsis gaditana* L2, under L/D at 8 days HRT in batches.

Accordingly, it was reused as inoculum for the second and the third experimental phases, as depicted in Figure 2. The consortia obtained after each experimental phase were assessed by a microscopic observation, as demonstrated in Figure S2.

2.5. Chemical Analysis and Data Processing

It is important to note that each chemical analysis was performed (in triplicate) during the SBR process. Samples were collected for the influent and effluent in each cycle (Figure 2) and were filtered, then finally analyzed.

The concentration of ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), orthophosphate (PO_4^{3-}), and total solid suspended (TSS) were analyzed in the influent and effluent wastewater according to Standard Methods (APHA 2012). The soluble chemical oxygen demand (sCOD) was measured following the filtration of the sample using a 0.45- μm membrane. The TSS was measured to determine the concentration of the microalgal-bacterial consortium in the PBR.

The pH, and DO contents were periodically monitored every 60 minutes *in situ*. They were measured with a pH 3310 meter coupled, respectively, with the electrodes Sentix[®]ORP and Sentix[®]41, along with a Multi3410 and FDO[®]925 sensor (all from WTW, Weilheim, Germany), respectively.

In fact, both parameters were recorded continuously during the whole process. However, the pH in the reactor was not controlled during all phases. The AI/MIX and lightning were also controlled by Timers (TR 612 top 2, Theben, Haigerloch, Germany) to control on/off pumps, lamp, and mixer.

The obtained consortia were examined for a microalgal-bacterial growth using an optical microscope (Nikon Optiphot EFD-3 Microscope (Nikon, Tokyo, Japan)).

The samples were analyzed in triplicate. The maximum standard deviation rate was estimated at 6%.

3. Results

As described in the diagram (Figure 2), the results were detailed for each experiment phase, considering nutrient removal rate and microscopic observation.

3.1. Establishment of the Consortium by *Nannochloropsis Gaditana* L2

The nutrient removal rate was assessed in the first experimental phase, as depicted in Figure 2. During this initial stage, the bacterial mechanism was not established. The cultivation of *Nannochloropsis gaditana* L2 in raw nonsterile WW recorded removal rates for the COD, TKN, and TP with an average of 53%, 78%, and 47%, respectively. These efficient removal rates were observed with the MIX mode and under L/D phases during 8 working days, as indicated in Table 2. Regarding the AI process, the results have been discarded due to its inconsistency, with only 25% of TP removal (data not shown).

Table 2. Characteristic of influent (T0)/effluent (TF) inoculated with native microalgae using mechanical mixing (MIX) under light/dark (L/D) conditions.

Parameters (mg/L)	Batch 1		Batch 2		Batch 3		Removal Rate % *
	T0	TF	T0	TF	T0	TF	
TKN	60.5	19.2	69	11.7	72.1	12.8	78.84
TP	7.2	3.8	4.9	3.9	8.4	3.8	47.22
COD	395	206	263	183	481	156	53.67
sCOD	181	92	113	78	157	83	54.14

* The total average removal rate of the consortium after one working month.

3.2. The Effect of Hydraulic Retention Time and Agitation Mode

As indicated in the second phase in the diagram in Figure 2, the SBR bioreactor process was evaluated considering three main parameters: HRT, photoperiod application, and agitation mode (AI/MIX).

The results in Table 3 show that the best conditions of SBR operation were observed at 4 days HRT under the L/D photoperiod with the AI process. AI process with continuous illumination (L) and 4 days HRT was not considered due to a high level of dissolved oxygen (DO; data not shown).

During L/D phases, under the AI mode and when the HRT was reduced from 6 to 4 days, the TKN and COD removal rate recorded an improvement, reaching 90% and 75%, respectively, as depicted in Table 3. However, at 4 days HRT, the TP removal rate under the AI mode was lower (29%) than the results using the MIX mode. This could be explained by the phosphorus precipitation or bad sedimentation, with reference to the low sCOD rate (<23%) during the AI mode [31].

In contrast, better sedimentation (sCOD %) was observed during MIX process growth for L and L/D photoperiod, considering 6- and 4-day HRTs.

Table 3. Analyses of the sequencing batch reactor effluents using a two-agitation mode (AI/MIX) for 6 and 4 days HRT under different photoperiods.

Parameters	HRT 6				HRT 4	
	(L)		(L/D)		(L/D)	
	MIX	AI	MIX	AI	MIX	AI
TKN%	55.2	92	80.6	74.5	68.3	91
TP%	59.67	24.63	45.8	38	47.2	29.5
sCOD%	57.69	22	56.9	10.31	49.2	22.5
COD%	78.44	74.78	60.5	61.4	47.8	75.9

3.3. The Effect of Feeding at Dark on the SBR Process

During the third phase, Figure 2, it can be seen that the agitation process affects the DO profile shown in Figure 3. The DO rate during the AI process was standing around 8.01 mg/L along the overall process. Moreover, the pH profile was visibly constant at around 7.5. This range of pH complies consistently with the condition of medium broth [15]. In our case, the pH did not exceed the range of 9, which is the limit for microalgae media cultivation [32].

However, the MIX process began with a lower level (O_2) = 2 mg/L and it was increased only during the second day of the treatment, reaching 10 mg/L. This positive variation occurred when the microalgae fitted into its atmosphere and started producing oxygen, as similarly reported by Osundeko et al. [33].

Furthermore, it was observed that the DO rate for MIX mode was decreased during the night period, as shown between 24–30 h. This is explained by the fact that the DO, which is produced by microalgae, was consumed by bacteria during the dark phase.

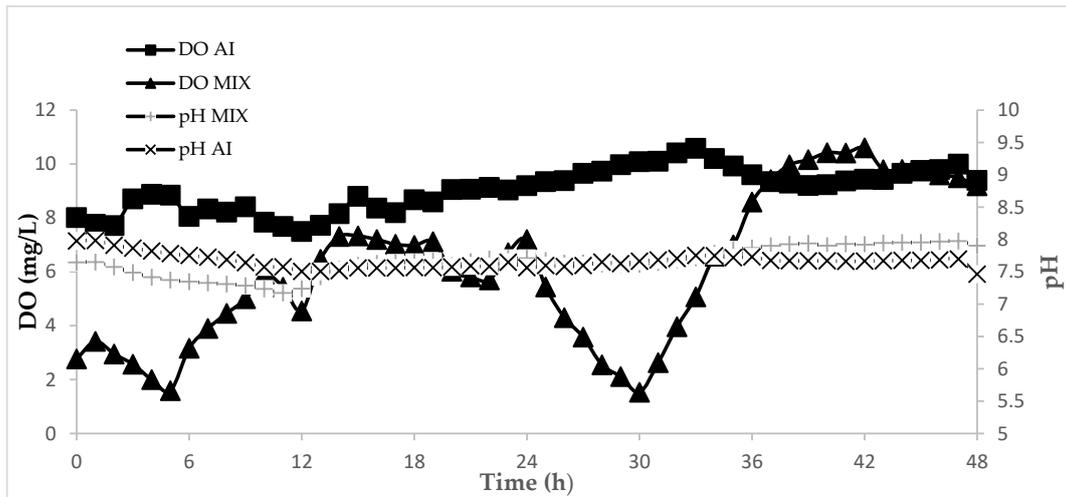
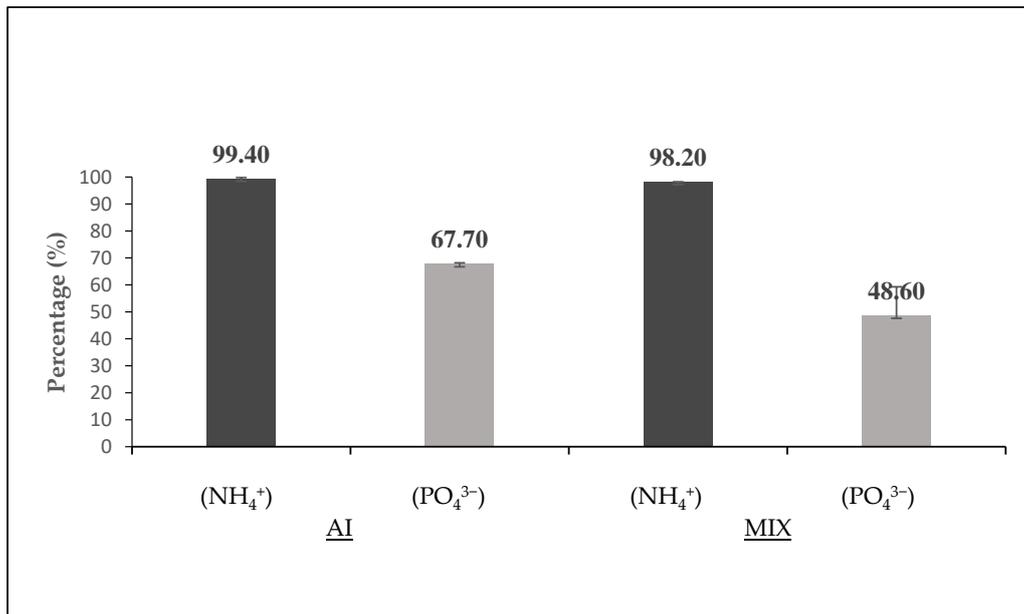


Figure 3. Dissolved oxygen profile for AI (air mixing)/MIX (mechanical mixing) for 48 h throughout D/L photoperiod with feeding at dark phase.

As shown in Figure 4a, nutrient removal rates were significantly observed for both processes. The ammonium elimination was successfully achieved at 98%, while the phosphorus removal rate had recorded only 49% for the MIX process.

Under D/L phases, at 4 days HRT, and during the AI process, the TKN, TP, and COD removal rates reached the valuable range of 80%, 75%, and 80%, respectively, as shown in Figure 4b.



(a)

Figure 4. Cont.

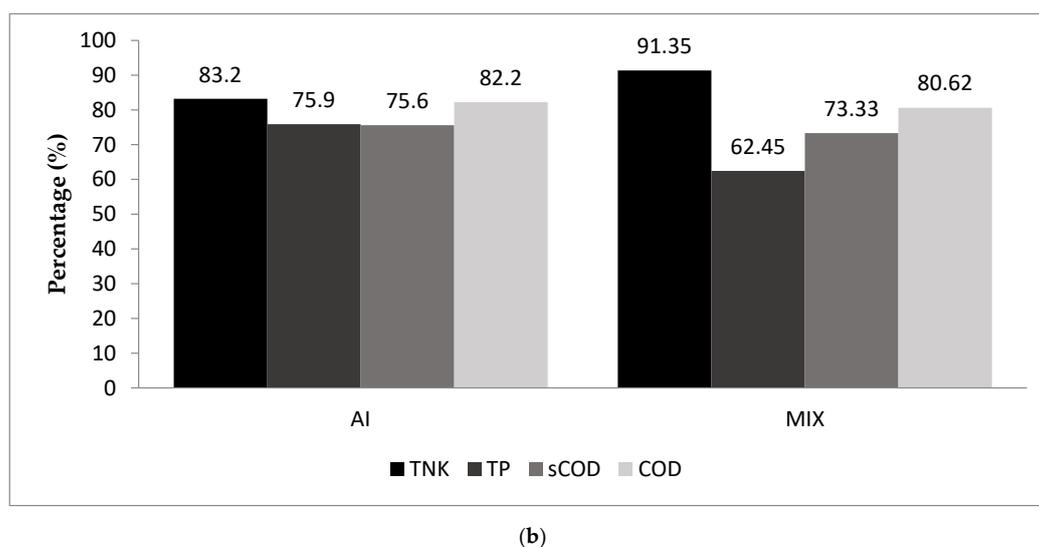


Figure 4. Removal rate for AI and MIX processes, with feeding at night. (a) (NH_4^+) and (PO_4^{3-}) removal for AI/MIX. (b) TNK, TP, sCOD, and COD removal for 48 h under AI/MIX.

3.4. The Interaction Associated with the Addition of *Nannochloropsis Gaditana* L2

3.4.1. TKN and COD Removal Rates

The effect of the inoculation of the WW with *Nannochloropsis gaditana* L2 was further studied. As illustrated in Table 4, the removal rates of TKN and COD were significantly improved to 86% and 60%, respectively, using the “L2 + WW + consortium” combination. The consortium is formed by intrinsic microalgae–bacteria issued from raw WW.

It is worth noting that the addition of native microalgae doubled the removal rate of TKN while working with sterilized WW compared to raw WW. Regarding the cycle duration, the process revealed that the addition of *Nannochloropsis gaditana* L2 can also enhance the treatment process period from 7 to 3 days, as depicted in Table 4.

Table 4. *Nannochloropsis gaditana* L2 improvement in TKN and COD removal rates.

	Time (Day)	Removal Rate (%)	
		TKN	COD
WW	7	16.3	25
L2 + sterilized WW	7	30	39
L2 + WW + consortium	3	86	60

3.4.2. Microscopic Observation

After 30 days, the algal-bacterial consortia was monitored under a microscope and their morphological features are presented in Figure S2. *Nannochloropsis gaditana* L2 was found along with surrounding small bacteria in the samples. It was aggregated within a clumping transparent sheath, followed by the formation of a clump of algal-bacterial consortia.

After each experimental phase, the microscopic analysis of consortiums showed a variety of microalgae as a major component of a microbial biomass complex, such as *Chlorella*, *Scenedesmus*, and *Tetrademus* [34,35], as observed in Figure S2.

In conclusion, the *Nannochloropsis gaditana* L2 was gradually covered by different bacterial flocs that were developed during the WWT process (initial phase).

4. Discussion

This work is based on wastewater treatment by microalgae–bacteria consortia in a SBR-type bioreactor. Parameters such as HRT, agitation mode and feeding time were assessed to reach optimal results for a green, scalable algae-applied WWT bioprocess.

The establishment of the consortium during the first experimental phase showed significant nutrient removal during the 8 working days, considering the MIX mode. In contrast, these removal rates were reached in 12 days by using the normal activated sludge, which shows the efficiency of the microalgae-based WW process [36]. The operation modes were selected to create a favorable environment, where new organisms such as microalgae and bacteria could be developed. In turn, the inconsistency of the AI process results, during the establishment of the consortium, is explained by the chemical precipitation of phosphorus following the formulas of precipitates, as reported by Larsdotter et al. [37]. This precipitate is usually comprised of calcium phosphates such as hydroxyapatite, magnesium hydroxide, and calcite. During the AI process, $(\text{CO}_3)_2^-$ is provided by air supply. In fact, Ca^{2+} is steadily consumed as calcite, and then, this results in a partial shortage of Ca^{2+} to formulate the precipitation of calcium phosphate.

According to morphological examination under a microscope, the consortium had faster development of microorganisms compared to the control (WW without microalgae addition). The same result obtained by microalgae inoculation in 30 days was observed only after three (3) months by the control treatment (data not shown). Following the increased density of the flocs during the experiment, the algal-bacterial biomass was sufficiently settled, along with producing a very clear effluent. This suggests that harvesting algal-bacterial biomass may not induce high dosages of flocculants, as reported by Munoz et al. [38].

During the second experimental phase, the study was extended to monitor different parameters such as hydraulic retention time, agitation mode, and photoperiods (L and L/D). Accordingly, values related to TKN, TP, sCOD, and COD showed that the used bioprocess worked efficiently in treating municipal WW during the L/D photoperiod. In fact, the addition of a precultured algae strain and the establishment of a microalgae–bacteria consortium represent a feasible approach of enhancing WWT at lower costs. Indeed, the AI process allows the discard of extensive use of turbines [7]. Regarding the continuous illumination (L) at 4 days HRT, a high DO level could generate photo-oxidative damage on microalgal cells and decrease treatment efficiency [39]. Furthermore, continuous illumination was discarded because of the high energy consumption. It is also important to underline that the agitation mode affects the treatment process. The AI mode showed the best TKN removal rate. In conjunction, the ammonium is nitrified by autotrophic bacteria (nitrification) and converted to organic nitrogen (assimilation). This WWT process shows that bacteria can easily reduce COD and produce the appropriate inorganic carbon for the photosynthetic microalgae process [40].

Thus, the aeration and photoperiod conditions are the key to optimize the nutrient uptake for the production of microalgae–bacteria consortia. It is also relevant to note that these conditions affect the microalgae–bacteria symbiosis in terms of metabolic plasticity and robustness.

With reference to the third experimental phase, the photoperiod was further assessed during the feeding at the dark phase for both agitation modes, AI/MIX. Moreover, both AI and MIX modes created different DO profiles, allowing a symbiosis between bacteria and microalgae. This symbiosis enhanced the removal rate by the nutrient exchange between microorganisms. It is worth noting that heterotrophic bacteria have a better growth rate (around three times higher) than autotrophic nitrifying bacteria. During the dark phase and under the MIX mode, the heterotrophic bacteria process will be developed, considering the limited DO. However, the autotrophic bacteria process is improved during the AI mode in the presence of oxygen. According to the latter metabolism, the inorganic carbon needed for the microalgae will be produced. Consequently, the AI agitation mode might enhance the microalgae multiplication, as reported [41]. On this account, the high performance of the photobioreactors under D/L was ensured by the bacterial degradation (nitrification and denitrification) [10]. It was also followed by the synergistic cooperation between the algae and bacteria from their organic and inorganic nutrient

exchange through photosynthesis and respiration [38]. Therefore, this study shows the necessity of considering dark phase feeding as an important factor.

Furthermore, microscopic observation showed the variety of algae in our SBR that can efficiently remove nutrients under sufficient lighting and warm temperatures.

In addition, we highlight that the use of *Nannochloropsis gaditana* L2 coupled to the consortium will definitely enhance the treatment in terms of duration and removal rate. These improvements are most likely due to the contribution of *Nannochloropsis gaditana* L2 in TKN removal. In fact, algae reduce the nitrogenous substances by their biological metabolism in addition to the bacterial effects.

To summarize, the assessment of real-time condition processes stimulates good results for the treatment duration. Moreover, the selected parameters for WWT gave meaningful results for nutrient removal rates, noting that our collected data is more significant than what was reported in other studies [31].

5. Conclusions

Based on microalgae–bacteria’s interaction in bioreactors, this study addresses additional data such as the feeding time and agitation mode in WWT. The overall results show that in SBR bioreactors, moderate agitation by bubbling air is an economical, feasible, and efficient solution. The photosynthetic activity of microalgae produces the main dissolved oxygen. Once this activity is coupled with moderate air injection, the need to bring high aeration by turbines used for bacterial secondary ponds can be discarded. Moreover, the addition of precultured microalgae strains lead to the creation of a stable consortium for the WWT at 4 days HRT during feeding at the dark phase (D/L), with air bubbling injection. Hence, operating the SBR bioreactor type in optimal conditions, as per our study, presents an important background to develop further applications of a green-algae-applied WW-treatment system.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4441/12/7/1893/s1>, Figure S1: Photobioreactor (PBR) used for lab-scale microalgae–bacteria consortium culturing. Figure S2: Microscopic observation of the consortium development. (A) Inoculum picture showing only *Nannochloropsis gaditana* (500×). (B) Consortium resulted after 1 month of experimenting, at 8 days HRT (125×). (C) Consortium, aged 2 months, resulting from the 6- and 4-day HRT (400×). (D) The final consortium after 4 months of experiments, where different strains of microalgae, bacteria (protozoa) have been developed (400×).

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