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THE USE OF A SELECTED FAST-SEDIMENTATION GREEN MICROALGAE FOR WASTEWATER TREATMENT

Ana Valentina Ardelean [1,2,*,a], Cristina Moisescu[1,a], Ana-Maria Moroşanu[1], Mihaela Marilena Stancu[1], Ioan Ardelean[1], Calina Petruţa Cornea[2]

[1]Department of Microbiology, Institute of Biology Bucharest of Romanian Academy, 296 Splaiul Independentei, Bucharest 060031, P.O. Box 56-53, Romania
[2]Faculty of Biotechnology, University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Mărăsti Blvd, District 1, Bucharest, Romania

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Abstract
The use of microalgae in biotechnological processes, such as bioenergy production and wastewater treatment have been and continue to be of great interest. However, the commercialization of their potential is hindered due to various technical challenges, with biomass harvesting being the largest and most expensive energy consumer among them. Therefore, a major challenge is in finding an efficient harvesting method with high economic feasibility. In this study, a rapid-sedimentation freshwater green microalga was used for batch treatment of artificial wastewater. This strain, in addition to its ability to efficiently remove and use N, P as a source of nutrients, has the advantage of a fast-sedimentation innate feature that allows for a rapid biomass settling (less than 10 minutes) without the addition of any flocculant. This green microalgal strain grows in the form of macrocolonies that significantly favor harvesting by rapid natural gravitational sedimentation. Thus, as this microalga will not require centrifugal harvesting, which is expensive and energy-consuming, its use at both bench and pilot scale could be a promising, cost-effective, and environmentally friendly approach for biotechnological applications.

Keywords: fast-sedimentation, green microalgae, nutrient removal, wastewater

1. INTRODUCTION

Currently, the drinking water supply is under increasing stress due to rapid urbanization and the pollution of water resources through the discharge of untreated wastewater. In this sense, new approaches are needed for the management and recovery of water and nutrients from wastewater. One of the internationally attractive technologies is the use of microalgae for the biological recovery of nutrients. In recent years, the potential of microalgae for wastewater treatment has shown increased scientific and industrial interest stimulating research in this field. Existing wastewater treatment technologies allow the recovery of phosphorus, for example, but with low efficiency, while other nutrients such as nitrogen and potassium cannot be recovered. Microalgae, on the other hand, can efficiently absorb not only phosphorus but also nitrogen and other microelements, transforming them into biomass rich in lipids or other compounds with commercial value (Ardelean et al., 2019).

*Corresponding author, E-mail address: ana.i_ardelean@yahoo.com
[a] The first two authors have contributed equally to this paper

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One of the biggest impediments to using microalgae for wastewater treatment is the high cost of the biomass separation step, the most energy-consuming step (Wan et al., 2015). A solution to reduce these costs would be the introduction of a microalgae preconcentration stage (Vandamme et al., 2013; Ummalyma et al., 2017) such as flocculation or gravity sedimentation. Flocculation involves the formation of aggregates between the microalgal cells and the added flocculant, the formed flocs allowing separation (recovery) by simple gravitational sedimentation. Gravitational sedimentation is the simplest and most economical alternative for separating the solid phase from a liquid suspension, being frequently applied in the field of water treatment (Ahmad, 2005). Different synthetic or natural flocculants have been tested to increase the efficiency of the flocculation process and the sedimentation speed (Matter et al., 2019). While natural flocculants have high production costs, synthetic ones are not biodegradable and consequently polluting (Lee et al., 2014).
In the literature, there are numerous studies on the co-cultivation of bacteria and microalgae as a bioflocculation method (Kim et al., 2011; Lee et al., 2008; Oh et al., 2001). Bacteria generally coexist with microalgae in natural environments, the relationships between them being species specific and most often with beneficial effects on both sides. The use of microalgae-bacteria aggregates is not only beneficial for biomass separation but is also expected to increase nutrient recovery efficiency (Lee et al., 2013; Magdouli et al., 2016). Also, co-cultivation of microalgae and bacteria could increase the removal of inorganic and organic pollutants and reduce the need for aeration (Subashchandrabose et al., 2011; Moisescu et al., 2018 Moisescu et al., 2019). To date, numerous studies use microalgae-bacteria consortia to treat different types of wastewaters (Su et al., 2011; Tricolici et al., 2014; Delgadillo-Mirquez et al., 2016; Pizzera et al., 2019; Ardelean et al., 2021).
In this work, two native self-sedimentation microalgal-bacterial communities were used to treat artificial wastewater under laboratory conditions. The microalgae present in this consortium were separated and purified, and the removal capacity of nitrogen and phosphorus of the two pure strains was compared with that of the initial consortia.

2. MATERIALS AND METHODS

2.1. Artificial wastewater

In this work, two recipes of artificial wastewater were used as nutrient media, one mimicking industrial wastewater and the second municipal wastewater. The industrial wastewater was prepared according to Tang et al. (2019) with small changes, and it contained (g/L): NH_4Cl 0.38, K_2HPO_4 0.08, KH_2PO_4 0.030, $NaHCO_3$ 0.248, $NaNO_3$ 0.85, pH 8.0. The municipal wastewater composition was formulated according to Takaya et al. (2003) respectively (g/L): $NaNO_3$ 0.85, peptone 0.6, bouillon extract 0.4, urea 0.1, NaCl 0.03, 0.01% KH_2PO_4 0.1, KCl 0.014, $MgSO_4 \cdot 7H_2O$ 0.02, $CaCl_2 \cdot 2H_2O$ 0.0185, pH 7.2. All media used in this study were sterilized (121ºC for 20 min) before use.

2.2. Microalgal strains

Two freshwater microalgae-bacteria consortia that exhibited native self-sedimentation characteristics were used in this study. These consortia were previously isolated (Ardelean et al., 2017; 2018) and based on preliminary screening (Ardelean et al., 2020) they were selected in this study to evaluate their nutrient removal potential. For these experiments, both the purified microalgae strains, namely axenic Ra and Rd, and the native microalgae-bacteria consortia, respectively Ra-N and Rd-N, were used.

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2.3. Nutrient removal

For nutrient removal experiments, all microalgal strains were initially cultivated in 150 mL BG11 medium (Zhang et al., 2012) dispensed in 250 mL flasks and incubated at 30℃ with agitation under a light intensity of 16500 lx and light:dark cycles of 12:12 h, for 7 days. The microalgal biomass was harvested by centrifugation (Thermo Scientific SL8R) at 7000 rpm for 5 min. The pelleted cells were washed with deionized water and centrifuged again at 7000 rpm for 5 min. The washed biomass was inoculated into 150 mL artificial wastewater dispensed in 250 mL flasks at an initial concentration of 0.1 g/L and incubated at 30℃, with agitation, under a light intensity of 16500 lx and light: dark cycles of 12:12 h. Incubation was carried out for 8 days in the experiments where industrial wastewater was used, respectively 3 days in the experiments in which municipal wastewater was used. For nutrients removal monitoring, every 24 h a sample was collected from each culture flask, centrifuged at 8000 rpm for 7 min, and the cell-free supernatant was used for the analysis of nitrate (NO_3), ammonium (NH_4), and phosphate (PO_4).

2.4. Sedimentation tests

The microalgae sedimentation tests were carried out using transparent Erlenmeyer flasks (250 mL) filled with 50 ml culture. The cultures were gently mixed for 1 min at room temperature and after that, an aliquot of the supernatant was withdrawn at a depth of 3 cm below the top of the microalgal dispersion every 1 min for 10 min. The biomass from each aliquot was harvested by centrifugation at 7000 rpm for 5 min, weighted, and the results were expressed as g/L biomass over time.

2.5. Analytical methods

Time-dependent consumption of NO_3, NH_4, and PO_4 was measured spectrophotometrically on a Specord® 210 Plus (Analytik Jena) using the Spectroquant® reagent test kits (MerckMillipore).
The removal efficiency percentage was calculated according to the following formula (Ansari et al., 2017):

$$\text{Percentage removal } \% = (IC - FC / IC) \times 100$$

where IC= initial concentration (mg/L) and FC= final concentration (mg/L).

2.6. Scanning Electron Microscopy (SEM)

For SEM imaging, the microalgal cultures were centrifuged at 7000 rpm for 10 min, washed with phosphate-buffered saline (PBS), and fixed overnight at 4°C with 2.5% glutaraldehyde in 0.1 M phosphate buffer. After fixation, cells were recovered by centrifugation for 10 min at 7000 rpm, dehydrated with an ascending series of ethanol solutions (10%, 30%, 50%, 70%, 90%, and 100%, 15 min each change), deposited on the surface of a glass slide and allowed to dry at room temperature. For visualization, the coverslips mounted on the SEM holder were coated with gold film using a JEOL JFC-1300 auto fine coater under vacuum. The samples were examined using a JEOL JSM-6610LV microscope.

2.7. Transmission Electron Microscopy (TEM)

For TEM studies, the microalgae biomass was centrifuged at 8000 rpm, for 7 min and the cells were pre-fixed overnight in 3% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4, at 4°C. After 6 successive washes with 0.05 M cacodilate buffer, the samples were postfixed with 4% OsO_4 for 2h at room temperature, followed by other washes, and then dehydrated in a graded series of ethanol (30, 50, 70, 90, and 100%). After treatment with propylene oxide (PO) and pre-embedment of samples (PO and Epon), the final embedment at 60° C for at least 24 hours followed. The ultrathin sectioning protocol was carried out according to Mirancea et al. (2007). Sectioning was performed on the

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Ultrotome III LKB ultramicrotome with a glass knife, and the ultrafine sections (70-90 nm thick) were double counterstained with Uranyless and lead citrate. All the prepared grid samples were analysed using a JEM-1400 (JEOL, Japan) operated at 80 kV accelerating voltage, and visualized with Quemesa CCD camera (Olympus Soft Imaging Solutions).

3. RESULTS AND DISCUSSIONS

3.1. Microalgae characterization

The two purified microalgae Ra and Rd were imaged by TEM and their ultrastructural characteristics are presented in Figure 1. Under microscopic observation, the purified Rd microalgae appear as big round cells with a diameter of approximately 6 µm, a surface area of 35 $\mu m^{2,}$ and a thick cell wall of 0.39 µm. The Ra strain showed smaller ellipsoidal cells of 4 µm length and 3 µm width, with an average surface area of 11 μm^2, and a thinner cell wall of 0.1 µm. Neither of the two microalgae shows any extension or flagella.

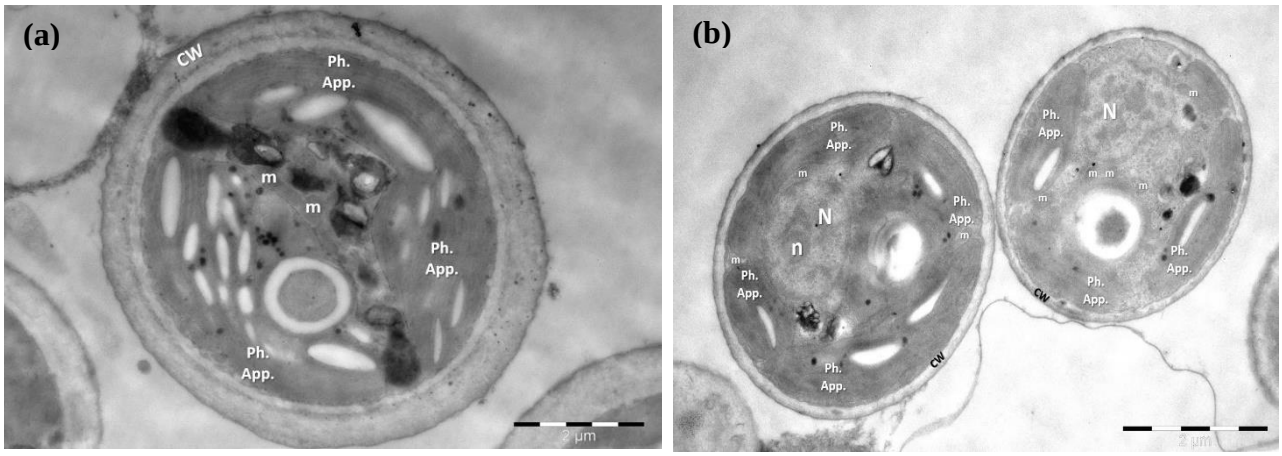

Figure 1. TEM images of a) Rd and b) Ra green microalgal strains (Ph. App. – photosynthetic apparatus; m – mitochondria; CW – cell wall; N – nucleus)

Other important characteristics that could be observed were a predominantly euchromatic nucleus along with a well-represented photosynthetic apparatus, mitochondria, and Golgi apparatus, indicating metabolically active cells.

In Figure 2, the SEM aspect of the Rd-N consortium is presented. As it can be observed, the cells are clumped together into big colonies of round or slightly ellipsoidal cells, with dimensions varying between 2 ~ 6 µm.

Another interesting feature that can be observed is the presence of an extracellular polymeric material (EPS) covering the cells, sometimes forming some extensions. This material is probably responsible for cells-clustering and the formation of macroscopic colonies ranging in size from 1-5 mm thus favouring gravitational sedimentation and contributing significantly to rapid biomass harvesting.

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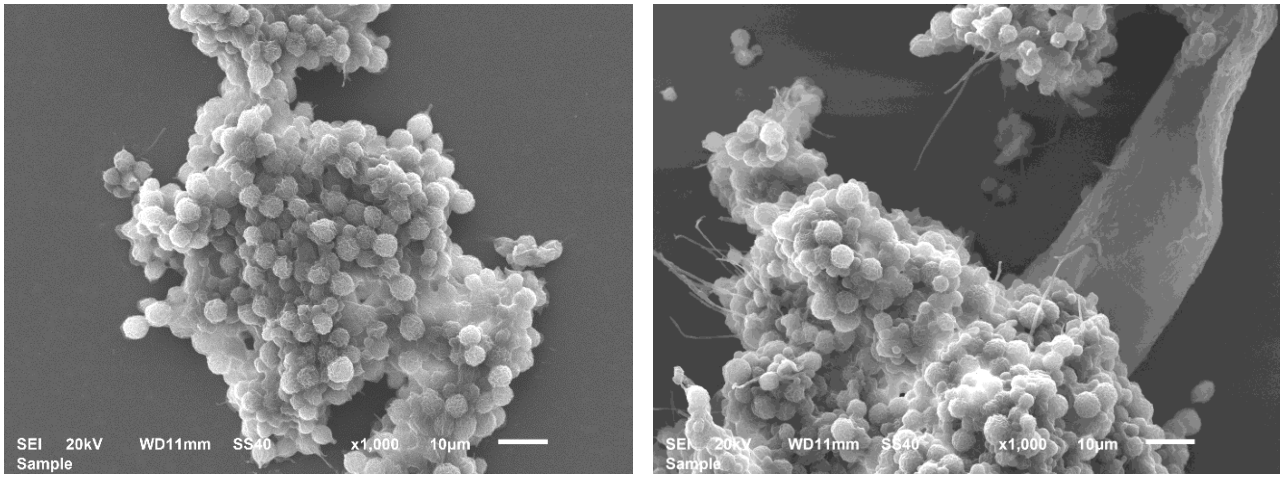

Figure 2. SEM images of the Rd-N consortium

3.2. Efficiency of sedimentation

The sedimentation efficiency of the four microalgae (i.e., Ra, Ra-N, Rd, and Rd-N) previously isolated and selected based on their ability to rapidly sediment, without the addition of any flocculant is depicted in Figure 3. If we compare the settling velocity of Ra and Rd axenic strains with the corresponding Ra-N and Rd-N consortia, the self-sedimentation efficiency was higher in consortia than that of axenic strains. This poorer microalgal harvesting performance could be ascribed to the growth behaviour of the axenic Ra and Rd strains as compared with the Ra-N and Rd-N consortia. The growth profiles of the axenic strains were different, that is the axenic cultures showed a more homogeneous growth, with well-dispersed cells that do not form clumps like their native consortia and therefore make stable microalgal dispersions that cannot be efficiently separated by gravitational sedimentation (Fig. 3).

Regarding the sedimentation speed of the two axenic strains, the Ra settled slower probably due to the smaller cell size compared to Rd.

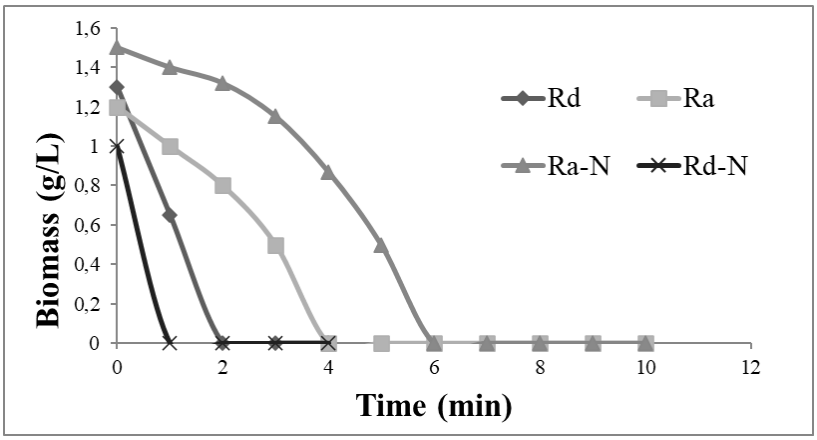

Figure 3. The dynamic of natural sedimentation efficiency measured for the four microalgal populations

A sequence of images is shown in Figure 4 in which the macro-colonies of the Rd-N consortia start settling only 32 seconds after mixing the sample, and at 2 minutes and 6 seconds the supernatant is

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already clarified, and the biomass is settled. Our results are comparable with other results reported in the literature. Jimoh (2021) used microalgae-cyanobacteria-bacterial flocs and obtained a sedimentation efficiency of 82% in 15 min without the addition of any flocculant. Instead, *Chlorella* sp. had the lowest sedimentation capacity, respectively less than 20% in 180 minutes.

Figure 4. The rapid natural gravitational sedimentation of green microalga Rd-N

Thus, since Rd-N showed the fastest sedimentation speed (i.e., 100% in less than 2 min), the use of this type of microalgae will not require harvesting by centrifugation, which is expensive and energy-consuming, making its use a promising, environmentally friendly, and economically viable alternative for biotechnological applications.

3.3. Nutrient removal potential

The potential of axenic and native green microalgal strains to treat artificial wastewaters and efficiently remove nitrogen (N) and phosphorous (P) was further analysed. Two different artificial wastewaters compositions were tested, one simulating an industrial wastewater (Tang et al., 2019) and the other a municipal wastewater (Takaya et al., 2003).

The experiment performed on artificial industrial wastewater (iWW) showed different behaviour of axenic strains versus consortia. As shown in Figure 5 and summarized in Table 1, the NH_4 removal efficiency by axenic Ra in the first 24 h (T1) was found to be 2% and reached 91% at the end of the experiment (Tf). For the native Ra-N consortium, the efficiency was 7% in the first 24 hours reaching up to 93% at Tf. The axenic Rd strain showed an NH_4 removal efficiency of 6% at T1 and 84% at Tf while the native Rd-N consortium removed 12% of NH_4 in the first 24 h and reached 100% at the end of the 8 days.

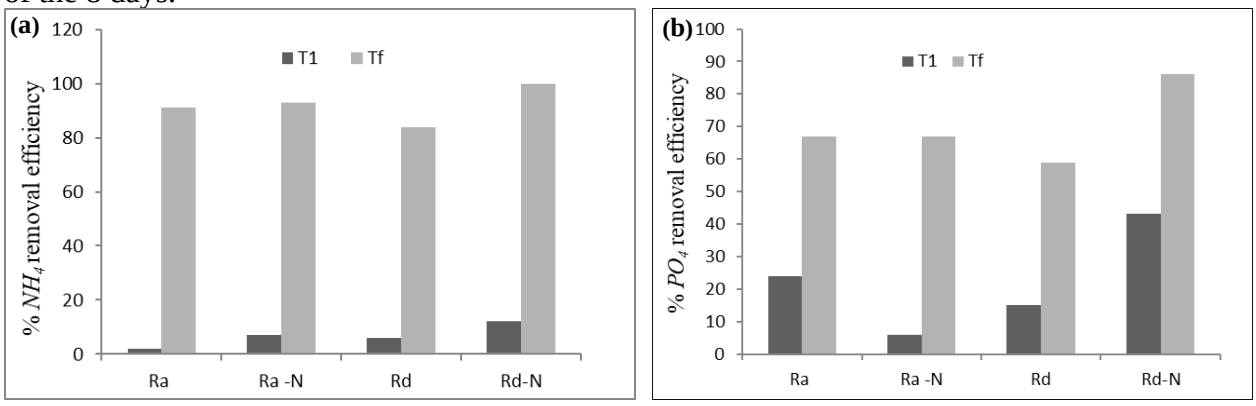

Figure 5. Nutrient removal efficiency over time of a) ammonium (NH_4) and b) phosphate (PO_4) by Ra, Ra-N, Rd, and Rd-N green microalgae

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Related to the PO_4 removal, the axenic Ra strain had an efficiency of 24% at T1 and scaled to 67% at Tf, whereas the Ra-N despite starting slower with only 6% in the first 24 h, at the end of the 8 days it also reached 67%. For the Rd strain, we recorded 15% at T1 and reached 59% at Tf while native Rd-N gave the best results starting from the first 24 hours when the PO_4 removal efficiency was of 43% and reaching 100% after 8 days of incubation.

Table 1. The removal efficiencies of NH_4 and PO_4 from artificial industrial wastewater (iWW) and municipal wastewater(mWW)

	iWW			**mWW**		
	NO_3	**NH_4**	**PO_4**	**NO_3**	**NH_4**	**PO_4**
Ra	0	91	67	3	90	83
Ra-N	0	93	67	7	0	80
Rd	0	84	59	-	-	-
Rd-N	0	100	86	5	100	92

NO_3 concentrations were also determined, but the results indicated that when both NO_3 and NH_4 are present in the culture media, the tested microorganisms prefer NH_4 as a nitrogen source.
For the second set of experiments, an artificial wastewater with a composition similar to a municipal wastewater (mWW) was used. This mWW had a richer composition and due to the urea, meat extract, and peptone from its composition, it was more suitable for the development of the bacteria in the consortia. For these experiments, we selected only the strains that gave the best results in the iWW first set of experiments. Thus, we used only the Ra, Ra-N, and Rd-N strains (Fig. 6).

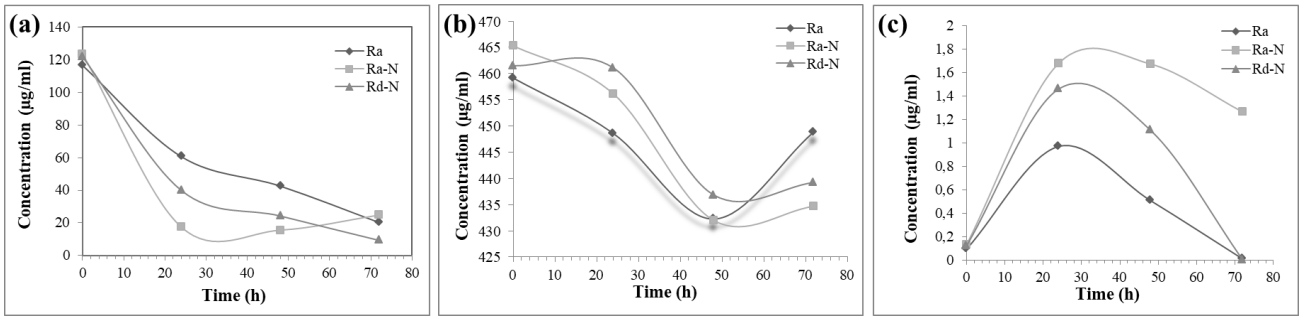

Figure 6. The removal of a) phosphate (PO_4) b) nitrate (NO_3) and c) ammonium (NH_4) by Ra, Ra-N, and Rd-N

When we used this type of wastewater (i.e., mWW), the nutrient removal time was much shorter, respectively 72 hours. The removal efficiencies by Rd-N were found to be 100% for NH_4, 5% for NO_3, and 92% for PO_4 in only 3 days. In the case of Ra strain, the removal efficiency was 90% for NH_4, 3% for NO_3, and 83% for PO_4. When Ra-N was cultivated in mWW, the nutrients removal it wasn't as good as for the other two microalgae tested, the removal percentage was 0% for NH_4, 7% for NO_3, and 80% for PO_4. It is possible that the Ra-N consortium needs more time to have an efficiency similar to that of the other two microalgae tested.

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For all three strains tested, at the beginning of the experiment, there was an increase in the NH_4 concentration which is probably a consequence of meat extract and peptone degradation, and nitrogen transformations, but after 24 h its concentration started to decrease.
Similar to the first set of experiments on iWW, low removal efficiencies of NO_3 compared to PO_4 and NH_4 were recorded. This can probably be explained by a lower requirement of this macronutrient by these types of microalgae. It is also known that green microalgae prefer NH_4 as a source of inorganic nitrogen (Jia and Yuan, 2016) while cyanobacteria show high NO_3 removal rates (Sutherland et al., 2017).
It seems that Rd-N consortium had the best efficiency in removing nutrients for both NH_4 (100%) and PO_4 (86-92%), in both experimental variants. Also, this strain has the best sedimentation speed among all four strains. This good performance of Rd-N was likely achieved by the synergy between microalgae and bacteria.
The results obtained by us in these experiments are similar to other data reported in the literature. Tricolici et al. (2014) used a microalgal–bacterial consortium for wastewater treatment and recorded a decrease of 65% of NH_4 and 42% of PO_4 after 48 hours of treatment. Guldhe et al., (2017) obtained a removal efficiency of 75.56% for NH_4 and 73.35% for PO_4 after 7 days using *C. sorokiniana* cultivated in aquaculture wastewater. Kiran et al., (2014) used *Chlorella* sp. IM-01 in their study for treating sewage wastewater. After 4 days, they removed 95.9 % of NH_4 and 33.12% of PO_4 when green microalgae were grown in the inlet water, and 95.6% of NH_4 and 82.5% of PO_4 in outlet water. On the 10th day the results for the inlet water were 96.83% NH_4, 82.38% PO_4 and for outlet water 98.35% NH_4 and 89.39% PO_4.
In addition to the ability to use wastewater as a source of nutrients, these microalgae also have the advantage of settling rapidly (in less than 10 minutes) thus allowing rapid settling of biomass without the addition of flocculant, which would involve additional costs in the case of industrial applications (Lv et al., 2018, Jimoh, 2021).

4. CONCLUSIONS

We have selected and isolated green microalgae with rapid natural gravitational sedimentation, namely Rd-N (1 min), Rd (2 min), Ra (4 min), and Ra-N (6 min). The selected microalgal strains showed high removal efficiencies for NH_4 and PO_4 and low efficiencies in eliminating NO_3. When we used artificial mWW, the nutrient removal percentage was higher and in a much shorter time (72 hours). Rd-N consortium showed the best results in the removal of nutrients on both types of wastewaters used and the best sedimentation velocity.

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*Corresponding author, E-mail address: ana.i_ardelean@yahoo.com

[a] The first two authors have contributed equally to this paper

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*Corresponding author, E-mail address: ana.i_ardelean@yahoo.com

[a] The first two authors have contributed equally to this paper