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Project title: **Pilot Project for Environmentally Friendly Algal Biomass Production for Fish Nutrition - Algae4FishRo-Md**

Study on optimal nutritional solutions for algae cultivation

Introduction

Within the project "Pilot for the Production of Environmentally Friendly Algal Biomass for Fish Nutrition - Algae4FishRo-Md", Code: ROMD00586, financing contract no. 109279/27.05.2025, and as part of activity A1.1, corresponding to the work package "Recycling wastewater from RAS with algae and introducing algal biomass into the fish diet", laboratory experiments were carried out for the cultivation of *Spirulina platensis* and *Chlorella vulgaris* algae on media with the addition of wastewater in concentrations of 10%, 30%, 60%, 90% and 100%.

To study the algae growth process, the team of the Moldova State University conducted research on determining the accumulation of algal biomass, changes in growth rate, reproduction coefficient and pH values of nutrient media and the concentration of chlorophyll and CO₂ was carried out jointly with the project leader, the results of which were generalized and will be presented by the project leader.

Analysis of algae biomass, growth rate and reproduction coefficient of algae cultivated on wastewater is essential for understanding how these organisms develop under specific nutrient environment conditions. Algal biomass represents the result of algal cell multiplication and is a direct indicator of culture productivity. When cultivation conditions are optimal, including the amount and concentration of nutrient medium, algae develop well and accumulate biomass efficiently. Conversely, if the nutrient medium is insufficient or nutrient concentrations are not adequate, cell growth and multiplication processes are reduced and biomass accumulation decreases significantly.

Determining the growth rate provides information about the algae development rate and allows the identification of conditions that support maximum reproduction and, respectively, biomass accumulation. At the same time, analysis of the reproduction rate reflects the ability of algae to maintain stable populations and respond to environmental variations, an essential aspect for the success of long-term cultivation.

Therefore, monitoring these parameters not only allows the optimization of cultivation processes and the maximization of algal biomass production, but also provides fundamental

information on the efficiency of algae in wastewater treatment, contributing to the development of sustainable and environmentally friendly treatment systems.

The results obtained are presented in the following tables and in the attached figures.

Working method and equipment used

To determine the biomass of *Spirulina platensis* and *Chlorella vulgaris* algae, the spectrophotometric method was used, which provides for the exposure of the algal suspension in the spectrophotometer basin and the measurement of the optical density at a wavelength of 750 nm. For the active determination of biomass, the optical density values are multiplied by those of the coefficient calculated based on the calibration curve previously constructed for each culture and the results obtained can be expressed either in g/l or g/l absolutely dry biomass (BAU).

The reproduction rate (μ) of the algae was determined according to the criterion established by K. M. Khaylov using the formula:

$$\mu = \frac{\log 2N_t - \log 2N_0}{t}$$

Where:

μ - reproduction rate;

N_t - biomass values at the final analyzed time;

N_0 - biomass values at the initial analyzed time.

The reproduction coefficient of the algae (Cr) was established according to the formula proposed by M. G. Vladimirov and V.E. Semenenco using the formula:

$$Cr = (B_f / B_i)$$

Where:

Cr - reproduction coefficient;

B_f – biomass values specific to the analyzed period;

B_i - initial biomass values.

The experiments were carried out in Erlenmeyer flasks with a volume of 100 ml, the volume of the nutrient medium being 100 ml, and the amount of algal inoculum is mentioned in tables 5-6 specific for the first day of cultivation. Both species of algae were cultivated according to the periodic method. The temperature in the cultivation laboratory during the experiments was 21-25 °C, natural lighting, and the experimental samples were not periodically shaken. All experiments were carried out in three repetitions. The algae *Chlorella vulgaris* and *Spirulina*

platensis were cultivated on standard nutrient media (control) with the composition indicated in tab. 1-4 and on media supplemented with wastewater in concentrations of 10%, 30%, 60%, 90% and 100% with the chemical composition indicated in the water quality analysis reports completed and presented for this stage.

Tab. 1. Composition of the nutrient medium used for the cultivation of *Spirulina platensis*

Ingredients	Quantity, g/l
NaHCO ₃	8,5
K ₂ HPO ₄	1,0
NaNO ₃	6,0
NaCl	1,0
K ₂ SO ₄	-
MgSO ₄ ·7H ₂ O	0,2
CaCl ₂	0,04
FeSO ₄ ·7H ₂ O	0,01
EDTA	0,08
Microelements (solution)	1 ml/l

Tab. 2. Microelement solution used in the cultivation of *Spirulina platensis*

Ingredients	Quantity, g/l
H ₃ BO ₃	2,86
MnCl ₂ ·4H ₂ O	1,13
ZnSO ₄ ·7H ₂ O	0,222
NaMoO ₄ ·5H ₂ O	0,39
Co(NO ₃) ₂ ·6H ₂ O	0,049
CuSO ₄ ·5H ₂ O	0,079

Tab. 3. Composition of the nutrient medium used to cultivate *Chlorella vulgaris* algae

Ingredients	Quantity, g/l
KNO ₃	5
MgSO ₄ ·7H ₂ O	2,5
KH ₂ PO ₄	1,25
FeSO ₄ ·7H ₂ O	0,003
EDTA	0,037
Microelemente	1 ml/l

Tab. 4. Microelement solution used in the cultivation of *Chlorella vulgaris*

Ingredients	Quantity, g/l
H ₃ BO ₃	2,86
MnCl ₂ ·4H ₂ O	1,81

ZnSO ₄ *7H ₂ O	0,222
MoO ₃	17,64
NH ₄ VO ₃	22,96

Results:

The results of the initial water analysis are presented in Tables 5-8 and the figures 1-2.

Tab. 5. Results of monitoring the biomass of *Spirulina platensis* algae cultivated on wastewater from the recirculating system, g/l BAU

Analysis days	Concentrations experienced					
	10%	30%	60%	90%	100%	Control
1	0,225±0,006	0,225±0,006	0,225±0,006	0,225±0,006	0,225±0,006	0,225±0,006
2	0,343±0,013	0,367±0,048	0,288±0,008	0,243±0,002	0,250±0,010	0,300±0,006
3	0,431±0,005	0,442±0,010	0,383±0,016	0,208±0,013	0,208±0,234	0,398±0,014
4	0,514±0,010	0,523±0,004	0,435±0,009	0,276±0,014	0,277±0,010	0,499±0,008
5	0,587±0,005	0,594±0,006	0,542±0,013	0,348±0,082	0,345±0,026	0,566±0,006
6	0,970±0,024	0,899±0,023	0,781±0,041	0,440±0,008	0,556±0,031	0,793±0,002
7	0,970±0,023	0,899±0,023	0,662±0,023	0,447±0,004	0,438±0,065	0,899±0,023
8	1,018±0,024	0,899±0,047	0,734±0,023	0,497±0,008	0,431±0,068	0,932±0,071
9	1,065±0,041	0,946±0,062	0,757±0,047	0,478±0,012	0,442±0,080	0,970±0,047
10	1,041±0,023	0,994±0,041	0,852±0,041	0,551±0,010	0,639±0,108	0,973±0,063
11	1,041±0,023	0,899±0,062	0,876±0,023	0,473±0,085	0,509±0,059	1,089±0,024

*- BAU – absolutely dry biomass

Biomass monitoring was carried out over 11 days, a period in which the “exponential growth phase” of *Spirulina platensis* was manifested. Starting with the 12th day, the “growth stabilization phase” is attested, and subsequently, starting with the 15th day, the initiation of the “decline phase” of the culture is observed. It is important to analyze these growth parameters especially during the “lag” and “exponential” phases, because during this period the culture registers the highest cell multiplication rate, which ensures the obtaining of biomass. In the process of algae cultivation, an essential indicator of the efficiency of the culture is the rapid accumulation of a significant biomass in a short time interval.

As we can see, from the data presented in tab. 1 on the first day, the initial biomass was identical for all variants, 0.225±0.006 g/l, which reflects the uniformity of the initial inoculation conditions. During days 2–5, a progressive increase in biomass is observed, the most pronounced being in the variants with 10% and 30% residual water. On day 2, the values vary between 0.243±0.002 g/l (90%) and 0.367±0.048 g/l (30%), the control presenting 0.300±0.006 g/l. On day 5, the biomass reaches 0.587±0.005 g/l for 10%, 0.594±0.006 g/l for 30%, 0.542±0.013 g/l for 60%, 0.348±0.082 g/l for 90%, 0.345±0.026 g/l for 100%, and 0.566±0.006 g/l for the control. This indicates that lower concentrations of wastewater (10–30%) favor biomass accumulation at a faster rate compared to high concentrations (90–100%). On days 6–11, distinct trends are observed: the variant with 10% wastewater reaches a maximum biomass of

1.065±0.041 g/l on day 9, maintaining high values until the end of the period. The 30% variant reaches 0.994±0.041 g/l on day 10, and 60% reaches 0.876±0.023 g/l on day 11. In contrast, the 90% and 100% concentrations show a lower accumulation and a slower growth, with maximum values of 0.551±0.010 g/l and 0.639±0.108 g/l, respectively, at the end of the monitored period. The control shows a constant evolution, reaching 1.089±0.024 g/l on day 11. The most representative results of the biomass of *Spirulina platensis* algae cultivated on media with the addition of RFS, obtained at the end of the exponential phase, were as follows: 10% - 1.041±0.023 g/l BAU (absolutely dry biomass); 30% - 0.899±0.062 g/l BAU, 60% - 0.876±0.023 g/l BAU, 90% - 0.473±0.085 g/l BAU; 100% - 0.509±0.059 g/l BAU.

Tab. 6. Results of monitoring the biomass of *Chlorella vulgaris* algae cultivated on wastewater from the recirculating system, g/l

Analysis days	Concentrations experienced					
	10%	30%	60%	90%	100%	Control
1	0,712±0,013	0,712±0,013	0,712±0,013	0,712±0,013	0,712±0,013	0,712±0,013
2	0,913±0,038	0,742±0,007	0,683±0,037	0,556±0,012	0,304±0,032	0,861±0,007
3	0,979±0,025	0,957±0,013	0,126±0,014	0,400±0,012	0,564±0,064	0,868±0,012
4	1,053±0,039	0,727±0,015	0,089±0,012	0,505±0,096	0,534±0,046	0,883±0,007
5	1,157±0,092	0,764±0,019	0,115±0,020	0,556±0,070	0,853±0,026	0,965±0,039
6	1,224±0,064	0,920±0,012	0,319±0,019	0,497±0,012	0,363±0,099	1,046±0,058
7	1,246±0,068	1,002±0,021	0,482±0,037	0,675±0,023	0,504±0,082	1,021±0,064
8	1,247±0,071	0,988±0,020	0,527±0,027	0,690±0,012	0,608±0,087	0,994±0,039
9	1,261±0,074	0,942±0,022	0,534±0,068	1,024±0,015	0,735±0,012	1,076±0,083
10	1,343±0,026	1,076±0,024	0,823±0,041	1,373±0,098	0,935±0,089	1,150±0,037
11	1,529±0,015	1,558±0,025	1,130±0,013	1,558±0,025	1,075±0,012	1,224±0,064

Monitoring of the changes in the biomass of *Chlorella vulgaris* took place over 11 days, a period in which the exponential growth phase of the culture was manifested. Starting with the 12th day, as in *Spirulina platensis*, the stationary and decline phases are triggered and therefore further monitoring of the algal biomass is not of interest. On the first day, the initial biomass is the same for all variants, 0.712 g/l, which indicates uniform conditions at inoculation. Between days 2 and 6, the biomass growth differs depending on the concentration of the waste water: for 10% a constant growth is observed up to 1.224 g/l, and for 30% the growth is moderate, reaching 0.920 g/l. At 60%, the growth is very low initially, indicating an inhibitory effect of the high concentration, while at 90% the growth is slow and fluctuating. In the case of 100% wastewater, the biomass increases very slowly, ranging between 0.304 and 0.363 g/l. The control shows a uniform increase up to 1.046 g/l on day 6. On days 7–11, the biomass reaches maximum values for 10% and 30%, reaching 1.529 and 1.558 g/l, which indicates favorable conditions for rapid algae development. The 60% and 90% variants continue to grow more slowly, while 100% remains with lower values, 1.075 g/l. The control shows a stable increase up to 1.224 g/l. When cultivating *Chlorella vulgaris* algae on media with the addition of wastewater, the productivity values for the end of the exponential growth phase were as follows: 10% - 1.529±0.015 g/l; 30% - 1.558±0.025 g/l; 60% - 1.130±0.013 g/l; 90% - 1.558±0.025 g/l; 100% - 1.075±0.012 g/l; control - 1.224±0.064 g/l.

The activities led to the identification of the optimal nutrient medium for the cultivation of *Spirulina platensis* and *Chlorella vulgaris* algae on wastewater, the optimal concentrations being 10% and 60%.

Tab. 7. Monitoring of growth indicators of *Spirulina platensis* algae cultivated on wastewater from the recirculating system

Analyzed period, days	Concentrations experienced											
	10%		30%		60%		90%		100%		Control	
	μ , zile ⁻¹	Cr, g/l BAU	μ , zile ⁻¹	Cr, g/l BAU	μ , zile ⁻¹	Cr, g/l BAU	μ , zile ⁻¹	Cr, g/l BAU	μ , zile ⁻¹	Cr, g/l BAU	μ , zile ⁻¹	Cr, g/l BAU
2	0,183	1,524	0,212	1,631	0,107	1,28	0,033	1,080	0,046	1,111	0,125	1,333
3	0,099	1,256	0,081	1,204	0,124	1,330	0,068	0,856	0,080	0,832	0,123	1,327
4	0,076	1,192	0,073	1,183	0,055	1,136	0,123	1,327	0,124	1,332	0,098	1,254
5	0,058	1,142	0,055	1,136	0,096	1,246	0,101	1,261	0,095	1,245	0,055	1,134
6	0,218	1,652	0,180	1,513	0,159	1,607	0,102	1,264	0,207	1,611	0,146	1,401
7	0,000	1,00	0,000	1,00	0,072	0,848	0,007	1,016	0,104	0,788	0,054	1,134
8	0,021	1,049	0,000	1,00	0,045	1,109	0,046	1,112	0,007	0,984	0,016	1,037
9	0,020	1,046	0,022	1,052	0,013	1,031	0,017	0,962	0,011	1,025	0,017	1,041
10	0,010	0,977	0,021	1,051	0,051	1,125	0,062	1,153	0,160	1,446	0,001	1,003
11	0,000	1,000	0,044	0,904	0,012	1,028	0,066	0,858	0,099	0,796	0,049	1,119

* Note - The calculation was made based on biomass expressed in g/l BAU; μ - algae reproduction rate; Cr - algae reproduction coefficient.

Tab. 8. Monitoring of growth indicators of *Chlorella vulgaris* algae cultivated on wastewater from the recirculating system

Analyzed period, days	Concentrations experienced											
	10%		30%		60%		90%		100%		Control	
	μ , zile ⁻¹	Cr, g/l	μ , zile ⁻¹	Cr, g/l	μ , zile ⁻¹	Cr, g/l	μ , zile ⁻¹	Cr, g/l	μ , zile ⁻¹	Cr, g/l	μ , zile ⁻¹	Cr, g/l
2	0,108	1,282	0,018	1,042	0,018	0,959	0,107	0,781	0,370	0,427	0,083	1,209
3	0,030	1,072	0,111	1,290	0,734	0,184	0,143	0,719	0,268	1,855	0,004	1,008
4	0,032	1,076	0,119	0,760	0,151	0,706	0,101	1,263	0,024	0,947	0,007	1,017
5	0,041	1,099	0,022	1,051	0,111	1,292	0,042	1,101	0,194	1,597	0,039	1,093
6	0,024	1,058	0,081	1,204	0,443	2,774	0,049	0,894	0,362	0,426	0,035	1,084
7	0,008	1,018	0,037	1,089	0,179	1,511	0,133	1,358	0,143	1,388	0,011	0,976
8	0,000	1,001	0,006	0,986	0,039	1,093	0,010	1,022	0,081	1,206	0,012	0,974
9	0,005	1,011	0,021	0,953	0,006	1,013	0,171	1,484	0,082	1,209	0,034	1,082
10	0,027	1,065	0,058	1,142	0,193	1,541	0,127	1,341	0,105	1,272	0,029	1,069
11	0,056	1,138	0,161	1,448	0,132	1,373	0,055	1,135	0,061	1,150	0,027	1,064

* Note - The calculation was made based on biomass expressed in g/l; μ - algae reproduction rate; Cr - algae reproduction coefficient.

In order to select the optimal nutrient media for cultivating *Chlorella vulgaris* and *Spirulina platensis* algae on the investigated wastewater, the algae growth process was analyzed by daily analysis of the reproduction coefficient and reproduction rate. Thus, the values of the reproduction coefficient of *Chlorella vulgaris* algae cultivated on wastewater ranged within the following limits: nutrient medium with 10% wastewater - 1.011-1.282 g/l; nutrient medium with 30% wastewater -0.953-1.448 g/l; nutrient medium with 60% wastewater - 0.184-2.774/l; nutrient medium with 90% wastewater -0.781-1.484 g/l, nutrient medium with 100% wastewater -0.427-1.855 g/l, and in the control variant -0.974-1.209 g/l. The results of the reproduction coefficient certified for the cultivation of *Spirulina platensis* algae on media with the addition of wastewater ranged within the limits: 10% - 0.977- 1.524 g/l BAU; 30% - 0.904-1.631 g/l BAU; 60% - 0.848-1.330 g/l BAU; 90% - 0.856-1.327 g/l BAU; 100% -0.796-1.611 and control - 1.003-1.333 g/l BAU. The results of this indicator reflect the fact that for *Chlorella vulgaris* the most optimal is the nutrient medium with the addition of 10% and 30% wastewater, a compromise between maximum productivity and stability: it offers high biomass values, allows efficient algae reproduction and keeps variability under control, which makes it recommended for reproducible cultivation in experimental or pilot conditions. When cultivating *Spirulina platensis* algae, the highest values of this indicator were recorded in the nutrient medium with the addition of 10% and 30% wastewater, which suggests favorable conditions for the multiplication of algal cells.

The results obtained when determining the growth rate of the algae studied on wastewater from the recirculating fish farming system at the project leader's research station were as follows:

Spirulina platensis: 10% - 0.000- 0.183 days⁻¹; 30% - 0.000 - 0.212 days⁻¹; 60% - 0.012- 0.159 days⁻¹; 90% - 0.007-0.123 days⁻¹; 100% - 0.007-0.207 days⁻¹; control - 0.001-0.146 days⁻¹.

Chlorella vulgaris - 10% - 0.000-0.108 days⁻¹; 30% - 0.006-0.161 days⁻¹; 60% - 0.006-0.734 days⁻¹; 90% - 0.010 - 0.171 days⁻¹; 100% - 0.081 - 0.370 days⁻¹, control - 0.974-1.209 days⁻¹.

The algae growth rate values attest to major results in the sample with the addition of 10% and 30% residual water when cultivating *Spirulina platensis* algae, which confirms favorable conditions for active cell multiplication. In the case of cultivating *Chlorella vulgaris* algae on media with the addition of wastewater, the maximum growth rate was reached in media with 30% and 60% wastewater, where exceptional growth was recorded, suggesting that the algae has an intense capacity for adaptation and multiplication, also attesting to a more uniform growth of algal cells.

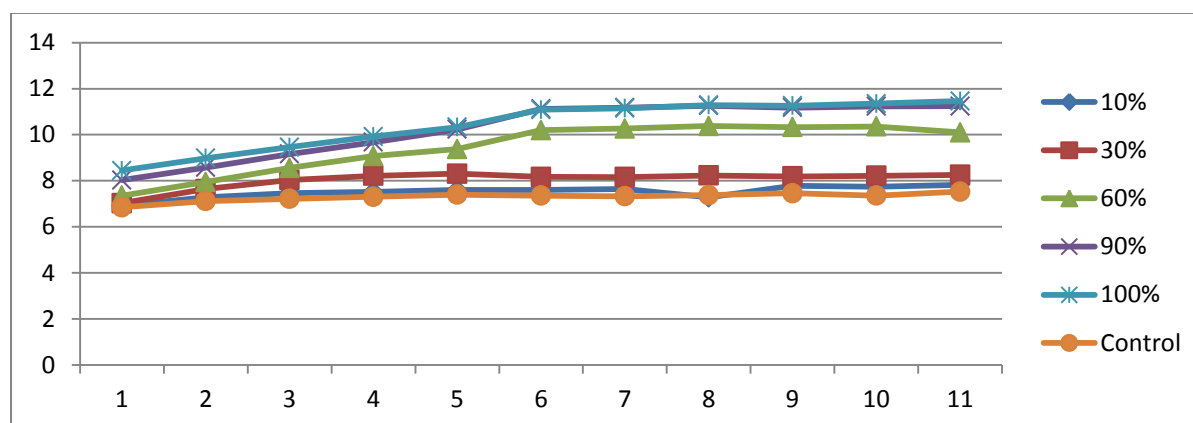


Fig. 1. Changes in pH values of nutrient media with the addition of wastewater used in the cultivation of *Chlorella vulgaris* algae

The analysis of the changes in pH values attested in nutrient media with the addition of wastewater for the cultivation of the *Chlorella vulgaris* algae indicates that at the beginning of the experiment (day 1), the pH values range between 6.85 (control H₂O) and 8.45 (100% wastewater), indicating significant initial differences between the variants. As the days progress, a progressive increase in pH is observed in all wastewater variants, with different intensities depending on the concentration. For the variants with small additions of wastewater (10% and 30%), the increase is moderate and relatively constant, reaching values of 7.83 (10%) and 8.26 (30%) on day 11. These pH ranges remain close to the optimal ones for algae development, suggesting good stability of the environment. At higher concentrations (60%, 90% and 100%), the increase in pH is much more pronounced. Thus, the medium with 60% residual water starts from 7.36 and reaches 10.10 on day 11, and the variants with 90% and 100% reach very high values, 11.24 and 11.46 respectively at the end of the analysis period. This phenomenon indicates the accumulation of metabolites and the possible intensification of photosynthesis, which determines the consumption of dissolved CO₂ and implicitly the increase in the alkalinity of the medium. As for the controls, the variant with distilled water (100% H₂O) shows a slow increase in pH, from 6.85 to 7.53, which reflects a stable environment but with reduced nutrient supply. The pH variant of the culture grown on the standard nutrient medium (control) remains relatively constant, between 7.12 and 7.46, which shows good buffering of the nutrient medium. In conclusion, the data show that small and moderate additions of wastewater (10–30%) ensure a stable pH favorable to cultivation, while high concentrations (60–100%) lead to excessive increases in pH, which can become unfavorable for long-term crop development.

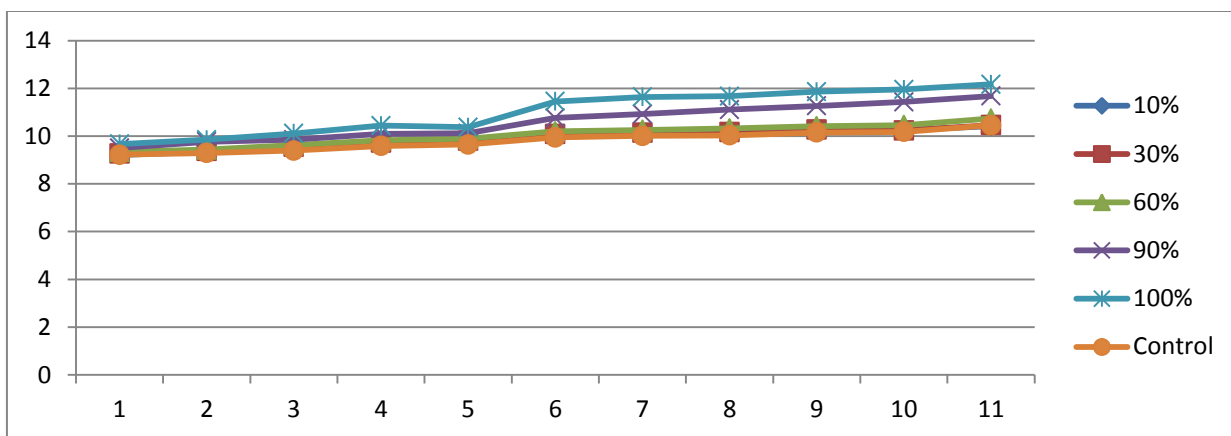


Fig. 2. Changes in pH values of nutrient media with the addition of wastewater used in the cultivation of *Spirulina platensis* algae

The results shown in fig. 2 show that on day 1, the pH values are relatively uniform between the variants, oscillating between 9.21 (10%) and 9.67 (100%), with the control located at 9.22. These high values from the beginning show the specific alkaline environment necessary for the development of *Spirulina platensis*. In the following days, the pH registers a progressive increase in all variants, but the amplitude of the changes differs depending on the concentration of the residual water. At low and moderate concentrations (10%, 30% and 60%), the increase is slow and constant, the pH reaching at the end of the analysis period 10.42 (10%), 10.46 (30%) and 10.73 (60%). These values are maintained in the range favorable for the development of *Spirulina*, without indicating major risks of inhibition. For high concentrations (90% and 100%), the pH values increase significantly, reaching very high levels on day 11: 11.68 (90%) and 12.17 (100%). This pronounced alkalization of the environment is most likely the consequence of the intensification of photosynthesis, with increased consumption of dissolved CO₂ and accumulation of hydroxyl ions (OH⁻). However, such high pH values can become unfavorable for the long-term development of the culture, as they can affect the metabolic stability of the cells. The control variant shows a slow and stable increase in pH, from 9.22 to 10.45 during the 11 days, which confirms that the addition of wastewater accelerates the changes in the nutrient environment. The data obtained show that variants with moderate additions of waste water (10–60%) provide stable and adequate conditions for the cultivation of *Spirulina*, while high concentrations (90–100%) lead to excessive alkalization, which can limit long-term growth and reproduction.

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