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Chlamydomonas acidophila for phosphorus recovery from municipal wastewater treatment plants: Effect of light intensity, temperature, different wastewaters and long-term semi-continuous feeding

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ABSTRACT

Recent years have witnessed a growing scientific and regulatory focus on phosphorus (P) in aquatic environments due to its role in accelerated algae growth, negatively impacting water quality. Agriculture and wastewater treatment plants (WWTPs) stand out as major P sources, prompting tightened discharge standards to meet the European Union Water Framework Directive objectives. Microalgae offer a solution for nutrient recovery, but challenges in mass cultivation and light availability persist. This study explores *Chlamydomonas acidophila*, an extremophilic microalgae, as a viable option for P recovery from WWTPs by understanding the effect of different light intensities and temperatures, as well as the effect of different wastewater characteristics, on *C. acidophila* growth and nutrient uptake.

Batch assays were conducted using growth media, settled wastewater, and final wastewater at various temperatures (10, 16, 20, 25, and 30 °C) and light intensities (15, 40, and 172 μmol photons m^{-2} s^{-1}). Furthermore, two 10 L working volume tanks were fed semi-continuously with a hydraulic retention time (HRT) of three days during 200 days at 70 μmol photons m^{-2} s^{-1}.

The results demonstrated that operational conditions of 20 °C and 40 μmol photons m^{-2} s^{-1} were optimal for *C. acidophila*'s biomass production and nutrient uptake. This characteristic significantly enhances the economic feasibility of *C. acidophila*-based wastewater treatment systems by allowing operation under lower light conditions. *C. acidophila's* adaptability to fluctuating nutrient levels, continuous nutrient consumption at low light, and mitigation of inhibitory effects make it a promising candidate for wastewater treatment. In a long-term semi-continuous treatment, removals of 3.6 mg PO_4^{3-} L^{-1} d^{-1}, 12 mg NH_4^+ L^{-1} d^{-1}, and up to 70 mg NO_3^- L^{-1} d^{-1} were achieved. It can be therefore concluded that *Chlamydomonas acidophila* has high nutrient assimilation capacity at low light intensities and could be a potential candidate for long-term wastewater treatment processes.

1. Introduction

In recent years, there has been an increasing scientific and regulatory interest in the presence of P in the aquatic environment. The overabundance of P results in accelerated growth of algae, leading to undesirable impacts on water quality. Agriculture is one of the major sources of P input in water bodies due to overapplication of non-organically bonded P-fertilizers [1]. Another significant source of P in the environment is WWTP, discharging effluent rich in essential growth nutrients such as nitrogen and phosphorus. Consequently, P discharge standards have been tightened to meet the objectives of the European Union Water Framework Directive, especially in sensitive areas [2]. The European Community's urban wastewater treatment directive aims to reduce eutrophication where discharges bring in high loads of nutrients. In the UK, 62 rivers and canals, 13 lakes and reservoirs and 5 estuaries have been identified to be eutrophic areas [3].

Microalgae have been used worldwide in biological wastewater treatment research for the recovery of nutrients from different waste effluents [4]. Beneficially, algae can assimilate nitrogen and phosphorus, and their biomass can be used in different application fields. However, using microalgae for wastewater treatment remains challenging. It is acknowledged that light availability is one of the greatest challenges for microalgae cultivation and a high photosynthetic efficiency is essential to decrease the costs of microalgal biomass

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production [5–7]. Moreover, the efficiency of wastewater treatment differs from one group to another group of microalgae depending upon the characteristics of wastewater [7]. The extremophilic microalgae, *C. acidophila*, growing at a pH of 2–3, appears to be a promising candidate for nutrients recovery since this microalgae requires a low light intensity [8].

In laboratory culture, many researchers report promising results regarding nutrient removal, however scaling-up from the laboratory to the field is a challenging task due to different factors affecting the scale-up process [9]. Municipal wastewater can be low in nutrients which can be a challenge to enable sufficient cell growth to successfully remove nutrients. Therefore, understanding the effect of different light intensities and temperatures, as well as the effect of different wastewater characteristics, on *C. acidophila* growth and nutrient uptake is crucial for the feasibility of this technology. Microalgae are commonly utilized in the treatment of final effluent, but *C. acidophila* offers an intriguing alternative. As a mixotrophic organism, it can potentially consume organic carbon, broadening its utility in waste management. Furthermore, its extremophile nature enhances its resilience, making it a potentially effective option for treating primary effluent. These unique characteristics position *C. acidophila* as a valuable resource in advancing effluent treatment technologies.

This manuscript's primary objective is to evaluate the feasibility of using *C. acidophila* as an alternative technology for recovering P from WWTPs by studying the combined effect of different light supplies and temperatures on biomass production and nutrient uptake from wastewater. Additionally, the study aims to investigate the long-term growth and nutrient consumption of *C. acidophila* in different wastewaters at different points in the WWTP.

2. Material and methods

2.1. Microorganisms

The strain of *C. acidophila* used in the study was obtained from the Göttingen collection of algae for culture (Sammlug von Algenkulturen Göttingen [SAG], Germany). For the inoculum, cells were cultured in bottles of constant working volume 1 L, maintained at room temperature and light conditions, and aerated with air bubbling.

Periodic transfer of the microalgae was performed to a standard medium developed by Escudero et al., [8] consisting of $(NH_4)_2SO4$ (1000 mg L^{-1}), K_2HPO_4 (200 mg L^{-1}), $MgSO_4$ (20 mg L^{-1}), Na_2EDTA (130 mg L^{-1}), $FeSO_4{\cdot}7H_2O$ (80 mg L^{-1}), $MnCl_2{\cdot}4H_2O$ (0.18 mg L^{-1}), $ZnCl_2$ (0.052 mg L^{-1}), $Na_2MoO_4{\cdot}2H_2O$ (0.063 mg L^{-1}), and $CoCl_2{\cdot}6H_2O$ (0.018 mg L^{-1}). The pH of the standard medium was approximately 3 after preparation. To obtain cells for experiments, the microalgae were harvested by centrifugation at 2500 rpm for 3 min and washed three times with distilled water. The centrifugation step was repeated to remove all culture medium before use in the different assays.

2.2. Wastewater effluents

The wastewater samples used in the study were collected from the Laighpark wastewater treatment plant located in Paisley (Lat/Long: 55.85319901, −4.42134052) immediately prior to each experiment. Depending on the requirements of each experiment, either primary settled effluent, which is raw wastewater that had undergone primary settlement of bulky organic and inorganic solids, or final effluent, which is the wastewater that had undergone biological treatment and was deemed suitable for discharge into the adjacent river, was collected. The average characteristics of the collected samples are shown in Table 1.

Table 1
Average characteristics of the collected wastewaters.

	Settled effluent (after primary treatment)	Final effluent (after secondary treatment)	Discharge standard UK *
PO_4^{3-} (mg L^{-1})	1–5	1–5	1–2[a]
COD (mg L^{-1})	90–215	25–35	125[a]
NH_4^+ (mg L^{-1})	12–33	1–5	5[b]
NO_3^- (mg L^{-1})	0.3–1.3	10–40	10–15[c]
pH	5.8–6.5	5.8–6.5	6–9[b]

[a] [10].
[b] [11].
[c] [12].

2.3. Experimental set-up

2.3.1. Influence of temperature and light intensity on growth and nutrients consumption of C. acidophila

The effects of temperature and light intensity on the biomass and nutrient removal by *C. acidophila* were investigated using a factorial experimental design. The study involved five batch mode trials, where 50 mL of settled, primary effluent was added to 125 mL glass Erlenmeyer flasks. The pH of the samples was adjusted to 2–3 using 5 N sulfuric acid, and algal cells were harvested from the 1 L long-term culture vessels and added to the flasks to achieve an initial cell concentration of approximately 1.0E7 cells mL^{-1}. The trials were carried out at five different temperatures (10, 16, 20, 25, and 30 °C) and three different light intensities (15, 40, and 172 μmol photons m^{-2} s^{-1}). The samples were run in quadruplets at a constant temperature of 22 °C and 100 rpm in the incubator for seven days. The pH, cell concentration, ammonium and phosphate concentrations were monitored every 2–3 days. To control for evaporation loss, foam bungs were used, and any water lost was replaced by adding distilled water to the cultures before sampling.

2.3.2. Influence of wastewater characteristics and HRT on algal growth in semi-continuous mode

In a semi-continuous mode, two identical 12 L tanks with a working volume of 10 L were installed in an insulated chamber at ambient temperature. During weekdays, the feeding schedule alternated every 2 h with the pumps operating for 2 h to feed the tanks (at a flow rate of 139 mL h^{-1}), followed by 2 h of no feeding. This cycle continued throughout the day, resulting in a total of 12 h of feeding and 12 h of non-feeding over a 24- h period, leading to a HRT of three days. The pumps were switched off during weekends.

The supply influent tanks (10 L) were filled with 3.5 L of the corresponding media (standard growth media, primary settled effluent, final effluent) and treated with 5 mL of sulfuric acid (5 N) to decrease the pH to between 2 and 3. Algae were collected from stock cultures, centrifuged at 2500 rpm for 3 min to produce a 50 mL concentrated algae broth, and used to inoculate the tanks with an initial cell concentration of around 9.00E5 cell mL^{-1}. The tanks operated from 14 to 200 days under continuous illumination at a rate of approximately 70 μmol photons m^{-2} s^{-1}, and mixing was ensured by four air stones operating at a rate of 5 L min^{-1}. Orthophosphate and ammonium levels, cell numbers, temperature, and pH were measured daily during the week.

2.4. Chemical analysis

The pH was analysed according to the Standard Methods for the Examination of Water and Wastewater [13]. Cell concentration was determined using the Celeromics Technologies S.L Micro Counter®. PO_4^{3-} analysis was conducted using the standard method 4500 P-E A.P. H.A [13] after centrifuging the samples at 13,000 rpm for 3 min.

Determination of NH_4^+ was carried out according to the colorimetric method based on the Nessler protocol [14].

2.5. Statistical analyses

Tests of significant differences between the parameters evaluated were carried out using Prism 8.4.2 for Mac. Repeated measures ANOVA was applied considering the different treatments as factors and their changes over time as repetitions. Two-way ANOVA was used with a significance level of $p < 0.05$. For further details on significance level, see Appendix A and B.

3. Results

3.1. Influence of light intensity and temperature on the growth and nutrient consumption of *Chlamydomonas acidophila*

The present study aimed to assess the effects of different temperatures and light intensities on the growth and nutrient uptake of *C. acidophila*. Five temperatures (10, 16, 20, 25, and 30 °C) and three light intensities (15, 40, and 172 μmol photons m^{-2} s^{-1}) were tested using batch tests. The range of light intensities was selected based on the average daily light irradiance, with the objective of encompassing low, medium, and high light intensity conditions for algal growth [15]. The results are summarized in Figs. 1 and 2, except for the 10 °C data, where no cell growth or nutrient uptake was observed.

At 16 °C and 30 °C, reduced cell growth was observed across all light intensities tested. The highest cell concentration was obtained at 20 °C and 25 °C, with cell concentrations of 23E6 and 25E6 cells mL^{-1}, respectively. At 16 °C, the highest cell concentration of 8.47E6 cells mL^{-1} was observed for cultures exposed to 40 μmol photons m^{-2} s^{-1}. Although cell growth decreased at 30 °C, increasing light had a positive impact on cell growth compared to samples incubated at 16 °C. For instance, at the highest light intensity, the cell concentration was 3.44E6 cells mL^{-1} at 16 °C, whereas at 30 °C, the cell concentration was 2.42E7 cells mL^{-1}, almost ten times higher than at 16 °C.

While no significant ($p > 0.05$) differences were observed in cell growth for cultures exposed to 40 and 172 μmol photons m^{-2} s^{-1} at 20 °C, higher light energy did not promote greater biomass production. However, for samples exposed to 25 °C, more light resulted in increased cell growth, and significant ($p < 0.0001$) differences were found for cell growth at each light intensity.

After 7 days of incubation, all measured nutrients were consumed in the cultures, except for cultures grown at 10 °C. The removal patterns for ammonium and orthophosphates varied on day 1 and day 4, with the most significant difference in removals reported after 4 days (Table 2).

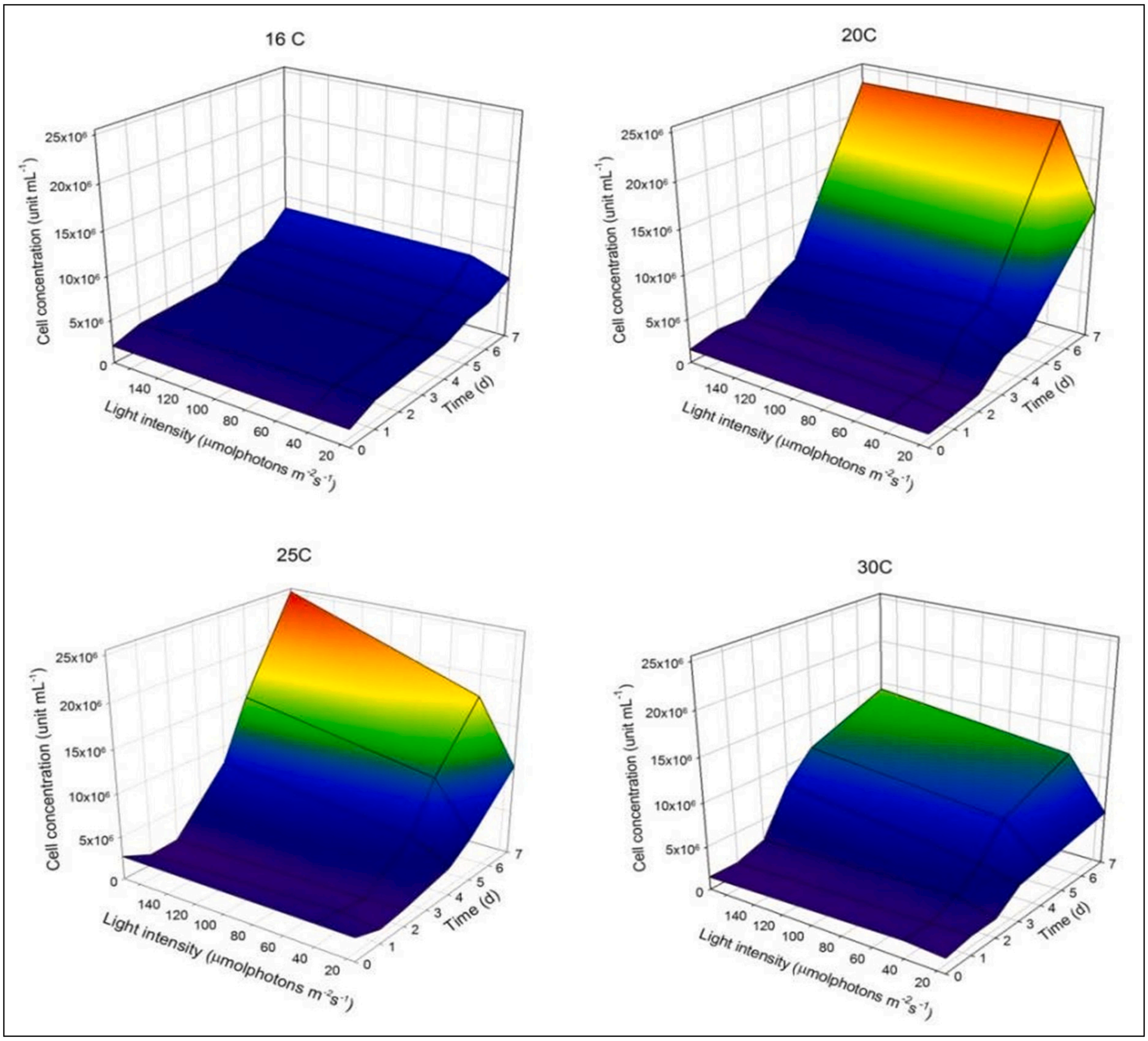

Fig. 1. Changes in the cell concentration in the medium during incubation at different light intensities and different temperatures.

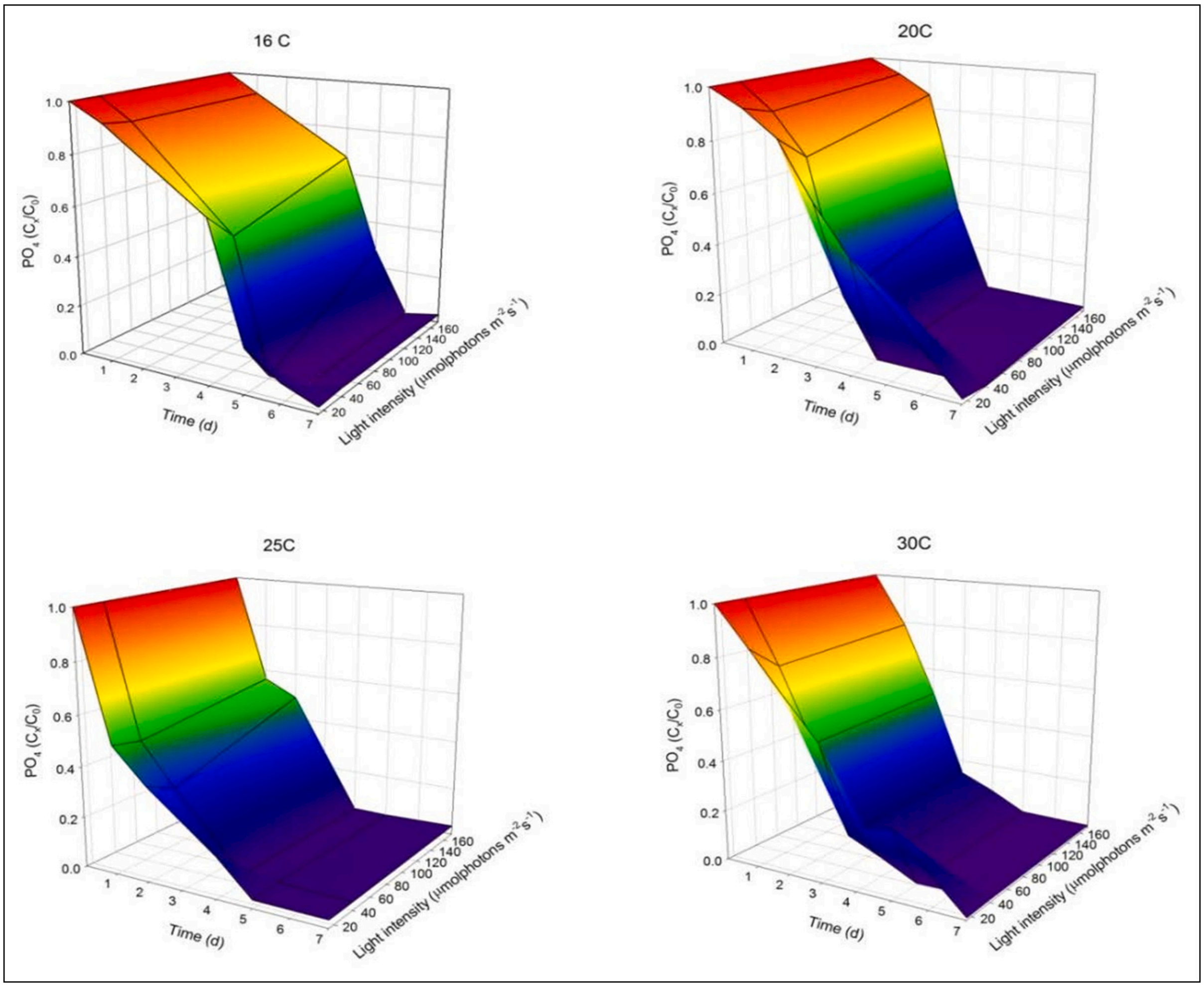

Fig. 2. Removals of PO_4^{3-} in the medium during incubation at different light intensities and different temperatures, expressed as the ratio between PO_4^{3-} concentrations in the media (C_x) and the initial PO_4^{3-} concentration (C_0).

Table 2
Average orthophosphate and ammonium removal efficiencies (percent) on day 1 and day 4 of the incubation period under different light and temperature conditions ($n = 4$):

Temp (°C)	Light intensity (µmol photons m^{-2} s^{-1})	Initial P conc. (mg L^{-1})	P removal in % day 1	P removal in % day 4	Initial N conc. (mg L^{-1})	N removal in % day 1	N removal in % day 4
16	15		7.1 ± 3.0	36.1 ± 2.9		14.0 ± 0.6	28.0 ± 1.0
	40	5.7	7.8 ± 1.4	47.6 ± 1.7	35.6	14.2 ± 0.6	30.6 ± 1.8
	172		8.3 ± 3.0	32.7 ± 10.0		14.3 ± 1.6	24.6 ± 4.3
20	15		6.7 ± 2.9	55.2 ± 3.0		5.4 ± 1.4	29.9 ± 0.5
	40	5.2	10.2 ± 1.8	99.2 ± 0.9	33.6	6.2 ± 1.3	47.5 ± 2.3
	172		6.2 ± 4.5	97.3 ± 1.8		4.0 ± 1.3	42.6 ± 6.3
25	15		49.5 ± 2.6	96.7 ± 2.2		24.4 ± 1.2	34.7 ± 0.9
	40	3.9	51.7 ± 4.1	96.0 ± 2.7	23.9	25.1 ± 1.4	54.2 ± 3.2
	172		43.9 ± 4.3	97.0 ± 2.1		20.6 ± 2.3	68.5 ± 5.4
30	15		15.2 ± 3.9	50.7 ± 3.5		3.6 ± 10.1	41.6 ± 6.7
	40	3.4	24.3 ± 6.1	92.7 ± 9.3	23.7	7.8 ± 5.4	69.1 ± 4.1
	172		21.1 ± 6.0	91.5 ± 6.6		7.8 ± 4.6	88.2 ± 12.8

In the first 4 days of incubation, the lowest phosphate removal was observed at the lowest temperature tested (16 °C), with an average reduction of 39 % of the initial P over the three light intensities evaluated. In contrast, at 20 °C, 25 °C, and 30 °C, almost all present phosphate was assimilated after the fourth day of the experiment. At 25 °C, even at a low light intensity of 15 µmol photons m^{-2} s^{-1}, the elimination rate was almost 97 %. At all other temperatures, an increase in light intensity from 15 to 40 µmol photons m^{-2} s^{-1} caused a significant ($p < 0.05$) (up to 45 %) increase in phosphate consumption, but any additional increase in light had no further positive effect. Ammonium removal showed a similar trend to phosphates, but only at low temperatures. At 25 °C and 30 °C, the rate of ammonium consumption increased with increasing irradiance, such that the highest removal rate was measured at 30 °C and 172 µmol photons m^{-2} s^{-1}.

3.2. Semi-continuous wastewater treatment using *C. acidophila*

3.2.1. Evaluation of semi-continuous treatment of primary settled effluent by *C. acidophila*

In this study, initial tests were performed to evaluate the growth behaviour of *C. acidophila* under semi-continuous feeding conditions with wastewater and standard growth media. The experiment was conducted over a 14-day period with a continuous light intensity of 70 μmol photons m^{-2} s^{-1} and an HRT of 3 days (Fig. 3).

The cell growth patterns of the microalgae were similar in both media during the first five days of the experiment, with the number of cells increasing linearly in both tanks from 1.17E6 and 1.47E6 to 6.13E6 and 7.31E6 cells mL^{-1} for growth media and wastewater, respectively. The growth rate was 0.4 d^{-1} for both media. After day 5, the cell concentration in both tanks plateaued and remained relatively constant, with an average of 8.18E6 cells mL^{-1} for the tank supplied with wastewater and 6.36E6 cells mL^{-1} for the tank fed with growth media. Although the cell growth in the tank fed with wastewater seemed slightly higher, it could not be concluded that the cell growth in this media was significantly higher due to the high variability of the cell counts.

The pH in the wastewater tank increased slightly during the first two days but decreased after a slight adjustment of the influent pH to around 2.0. After that, the pH remained stable at around pH 2.9 for the tank fed with growing media and slightly lower (pH 2.5) for the tank fed with wastewater for the duration of the incubation.

After a start-up period of 5 days, little or no nutrient removal was observed in both tanks. It was only after this period that cell numbers stabilized and a reduction in the concentrations of NH_4^+ and PO_4^{3-} was observed (Fig. 4). In the tank fed with growth media, NH_4^+ removal began at around day 5, while PO_4^{3-} removal was only seen after day 8. In contrast, in the tanks with wastewater, removal of these two nutrients occurred at the same time (day 5).

During the steady-state operation, recovery values of around 10 mg NH_4^+ $L^{-1}d^{-1}$ and 3.5–2.4 mg PO_4^{3-} $L^{-1}d^{-1}$ were achieved for both systems (Fig. 5). Notably, despite different initial nutrient concentrations in the influents, both tanks showed similar removal rates. Thus, it can be concluded that a start-up period is required for nutrient removal in both tanks, and that recovery values of NH_4^+ and PO_4^{3-} can be achieved at comparable rates using growth media or wastewater as the nutrient source.

3.2.2. Semi-continuous treatment of primary and secondary effluents using *C. acidophila*

The growth and nutrient removal of microalgae were investigated in two photobioreactors (PBRs) fed with either primary settled effluent or final effluent. During the initial 50 days of incubation with a HRT of 3 days, the mean cell count was 1.32E7 cells mL^{-1} and 1.42E7 cells mL^{-1} in the tanks supplied with primary settled and final effluent, respectively (Fig. 6A). The pH varied greatly in the first 50 days of the experiment in both tanks, but when the HRT was decreased to 2 days, it stabilized, ensuring optimal conditions for microalgae growth and nutrient assimilation (Fig. 6B).

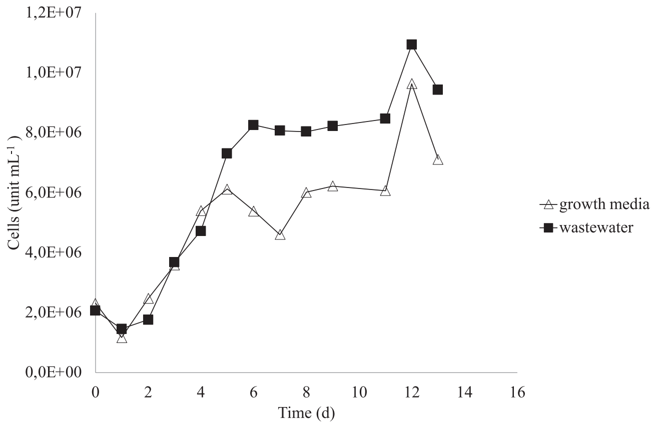

Fig. 3. Changes in the cell concentration in the semi-continuous feeding set-up using *C. acidophila*, cultivated in growth media and primary settled effluent. Each point represents mean value for three replicate determinations with standard deviation.

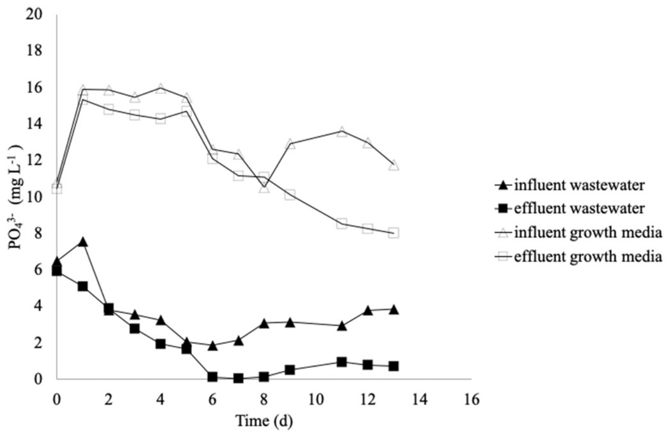

Fig. 4. Evolution of P concentration in the influent and tank using wastewater and growth media during the experiment.

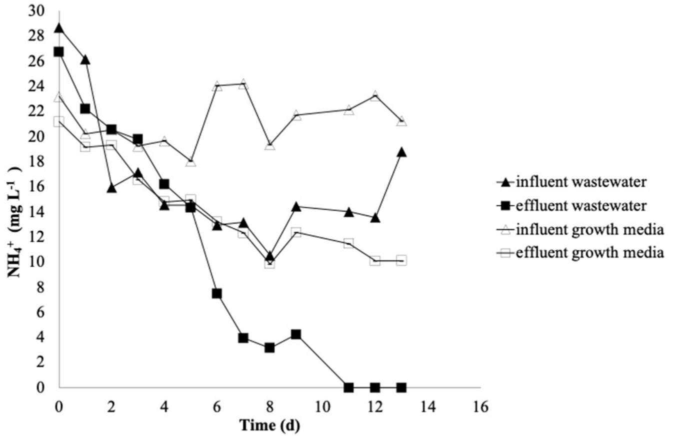

Fig. 5. Evolution of NH_4^+ concentration in the influent and tank using wastewater and growth media during the experiment.

After 100 days of incubation, the cell count in the final effluent tank decreased abruptly to concentrations around 8.0–9.0E7 cells mL^{-1}, similar to the cell concentration in the settled effluent tank, and remained low till the end of the experiment. The pH of the final effluent tank fluctuated after 150 days of operation, which coincided with a fluctuation in the cell's growth and nutrient consumption.

Under an HRT of 3 days, the algae completely consumed the available nutrients after a first acclimatization phase of six days, reaching recovery rates of 2.5 mg PO_4^{3-} L^{-1} d^{-1} (Fig. 7A); 10 mg NH_4^+ L^{-1} d^{-1} (Fig. 7B); 1.5 mg PO_4^{3-} L^{-1} d^{-1} (Fig. 8A); 14 NH_4^+ L^{-1} d^{-1} (Fig. 8B) 20 mg and NO_3 L^{-1} d^{-1} (Fig. 8C) in primary settled effluent and final effluent fed tanks, respectively. In the tank fed with primary settled effluent, after the reduction of HRT from 3 to 2 days, and with an increased influent concentration, the orthophosphate removal rates increased to 5.8 mg PO_4^{3-} L^{-1} d^{-1} (Fig. 7A), and ammonium removal to around 11 mg NH_4^+ L^{-1} d^{-1} (Fig. 7B). Furthermore, in the tank supplied with primary effluent, strong attachment of algal cells to the tank walls and formation of cell flocs was observed therefore it was difficult to obtain a

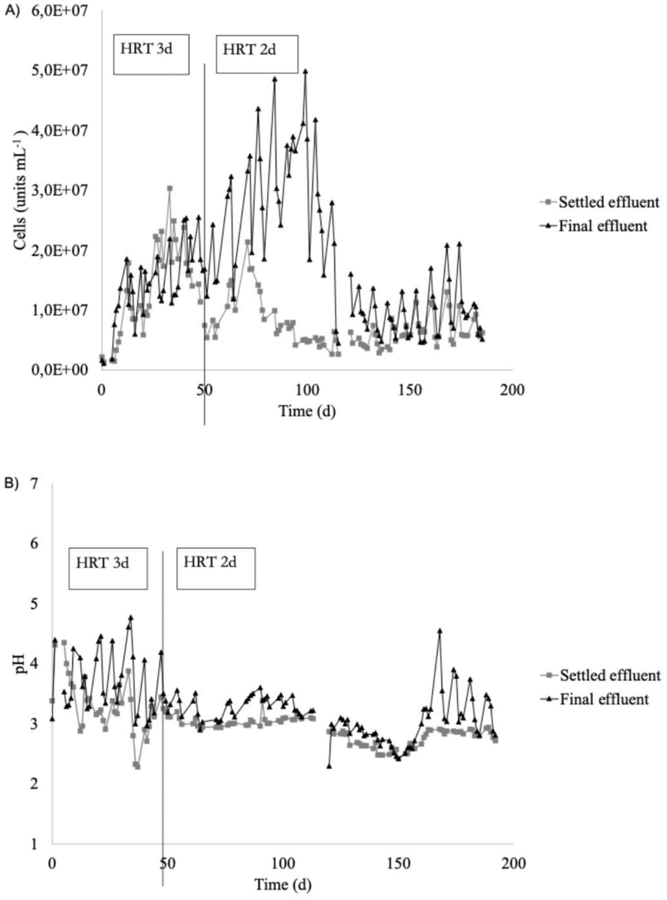

Fig. 6. Changes in the cell concentration (A) and pH (B) for *C. acidophila* operated semi-continuously effluent in primary settled effluent and final effluent.

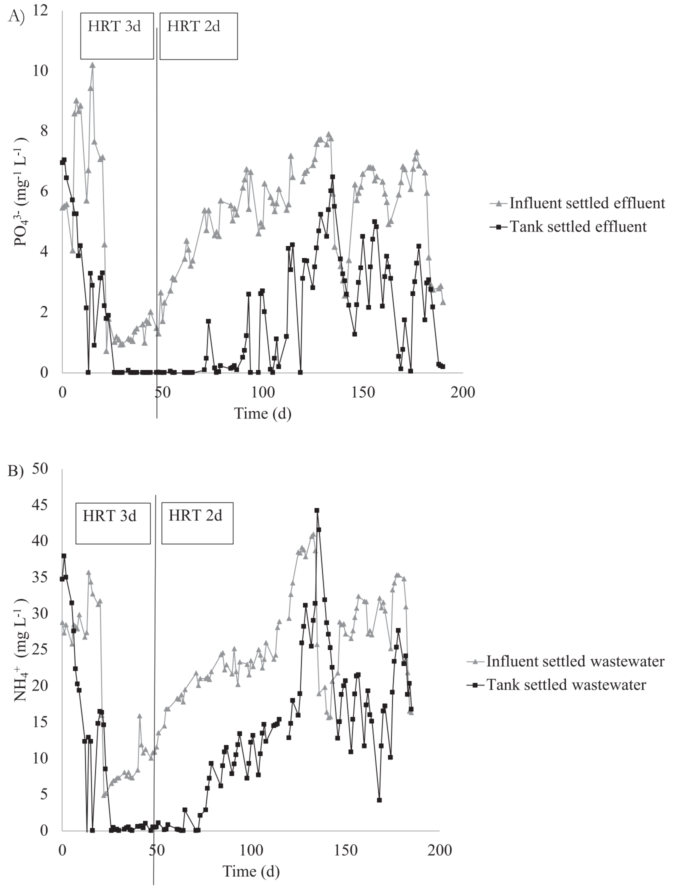

Fig. 7. Changes in PO_4^{3-} (A) and NH_4^+ (B) concentrations in the influent and effluent for *C. acidophila* cultivated in primary settled effluent. Each point represents mean value for three replicate determinations with standard deviation.

representative concentration of cells in this tank.

However, on day 140 of the experiment, the nutrient concentration in the tank exceeded the nutrient concentration of the influent being a sign of insufficient nutrient assimilation by the algae and therefore nutrient accumulation in the tank. The cells might not have sufficient time to assimilate required nutrient, resulting in stress and therefore the accumulation of nutrients in the tank and the strong variation in pH. Another possible explanation is the potential wash out of the tanks. In the tank fed with final effluent, after the reduction of HRT from 3 to 2 days, and with an increased influent concentration, the orthophosphate removal rates increased to 3.6 mg PO_4^{3-} mg^{-1} L^{-1} d^{-1}, ammonium removal to around 12 mg NH_4^+ L^{-1} d^{-1}, and up to 70 mg NO_3^- L^{-1} d^{-1}. Even if the P removals were higher under an HRT of 2 days, complete removal of this nutrient was not achieved. However, a complete removal of NH_4^+ and NO_3^- was observed.

In both tanks, around day 140, a change in the nutrient uptake was observed, but in the tank fed with final effluent, the system recovered after a few days and high nutrient consumptions were achieved at the end of the experiment.

4. Discussion

4.1. Influence of light intensity and temperature on the growth and nutrient consumption of C. acidophila

The present study investigated the effect of temperature and light intensity on algae growth and nutrient uptake. Results showed that growth and nutrient uptake were strongly influenced by temperature. Algae photosynthesis, which is responsible for glucose production, is mainly carried out by enzymes, and at low temperatures, these enzymes do not work efficiently, leading to reduced glucose production and inhibited growth [16]. The present study found that inhibited growth and lower nutrient uptake occurred at 10 and 16 °C, indicating that these temperatures are insufficient for achieving high biomass production and adequate nutrient uptake while treating wastewater. These findings are consistent with previous studies that have reported similar results, indicating that low temperatures negatively impact algal growth and nutrient uptake [17–19].

Using *C. acidophila*, the highest cell growth and nutrient consumption occurred at 20 °C, with a light intensity of 40 μmol photons m^{-2} s^{-1}. Increasing light intensity did not lead to further increases in either parameter. At higher temperatures, such as 25 °C or 30 °C, insufficient light can hinder cell growth, as the energy needed for photosynthesis becomes limited [20]. This demonstrates that when light intensity falls below optimal levels, biomass production slows, highlighting the importance of maintaining proper light conditions for maximizing microalgal growth in applications like wastewater treatment. At 25 °C, increasing light intensity up to 172 μmol photons m^{-2} s^{-1} led to an increase in cell concentration and nutrient uptake. However, low light intensity can also affect cell growth, and temperatures above the optimal range can negatively impact growth [21]. Therefore, it appears that a combination of two stress factors (slightly higher temperature than optimal together with slight low light intensity) is responsible for slower growth at under 40 μmol photon m^{-2} s^{-1} at 25 °C.

At the highest temperature examined of 30 °C, cell growth was generally impaired, although nutrient removal rates were higher compared to the algae cultures grown at lower temperatures. These

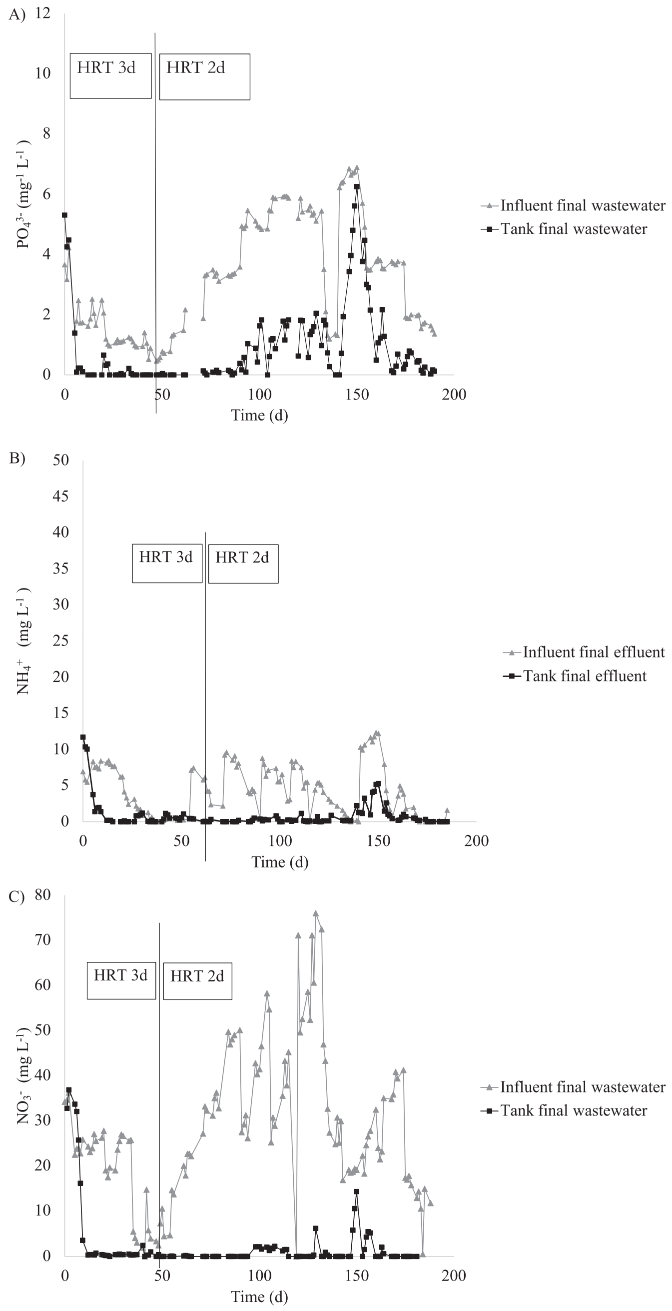

Fig. 8. Changes in PO_4^{3-} (A), NH_4^+ (B) and NO_3^- (C) concentrations in the influent and effluent for *C. acidophila* cultivated in final wastewater. Each point represents mean value from three replicate determinations with standard deviation.

results are consistent with previous studies that have reported that algae growth rate increases with increasing temperature until the optimum point is reached, after which further increases in temperature lead to a rapid decline in cell growth [16]. Under stress, cell division is one of the processes that microalgae suppress to survive, which may be the reason why the cells consumed a greater amount of P at 30 °C, even though cell numbers did not increase in the media.

Overall, the results of this study suggest that the optimum temperature for algae growth and nutrient uptake is 20 °C, with a light intensity of 40 μmol photons m^{-2} s^{-1}. These findings are important for designing photobioreactors that can be used for wastewater treatment. By optimizing the growth conditions, it may be possible to improve the efficiency of algae-based wastewater treatment systems, which have the potential to provide a sustainable and cost-effective solution for wastewater treatment.

The illumination in an algae cultivation tank consumes a substantial amount of energy, making it a primary concern for system operation. Different microalgae species have varying light intensity requirements, and insufficient light can negatively impact their growth and performance. In a recent investigation by Maltsev et al. [22], a wide range of microalgae species were evaluated for their maximum growth at varying light intensities. The results indicated that 83 % of the microalgae species tested required a light intensity exceeding 100 μmol photons m^2s^{-1} for their maximal growth. Therefore, utilizing microalgae species that require lower light intensities could contribute significantly to the economic feasibility of a microalgae-based system for wastewater treatment. Thus, *C. acidophila* seems promising to be implemented for wastewater treatment.

4.2. Semi-continuous wastewater treatment using *C. acidophila*

4.2.1. Evaluation of semi-continuous treatment of primary settled effluent by *C. acidophila*

Results showed that *C. acidophila* grew slightly better in the presence of wastewater than in growing media, despite lower initial nutrient concentrations. The mixotrophic character of *C. acidophila* may explain why the cells grew well in the presence of wastewater despite lower initial nutrient concentrations. Mixotrophic organisms can acquire nutrients from both organic and inorganic sources, which allows them to thrive in environments with variable nutrient availability [23]. In our experiment, the cells grew similarly well in the growth media with optimal nutrient concentrations, as they did in the presence of wastewater with lower initial nutrient concentrations. This finding suggests that *C. acidophila* may be particularly well-suited for wastewater treatment applications, where nutrient availability can fluctuate over time.

The cells consumed up to 70 % NH_4^+ and 70–99 % PO_4^{3-} during the experiment, indicating high nutrient assimilation capacity. These findings are in line with previous studies that have investigated the use of microalgae for wastewater treatment. For instance, Feng et al., [24] evaluated the nutrient removal efficiency of *Chlorella vulgaris* and showed that the algae could effectively remove NH_4^+, NO_3^-, and PO_4^{3-}. Ruiz-Marin et al. [25] studied immobilized mixotrophic algae *Scenedesmus obliquus*, which also showed high nutrient removal rates initially, but decreased after 10 days. This could be due to protein synthesis limitations and comparatively short HRT. However, in contrast to Ruiz-Marin et al. [25], the current study found that the system remained stable during the whole experiment, showing a constant growth and nutrient assimilation. Furthermore, the nutrients' consumptions reported in the present study were higher than those reported by Riaño et al. [26], who evaluated the treatment of fish processing wastewater using *Chlorella sorokiniana* and *Spirulina platensis*. The authors reported nitrogen removal rates of 55–61 % and phosphate removals of 48.4–47.8 %, which were lower than the removal rates observed in the present study. In this study, it is possible to compare nutrient removal efficiencies between batch and semi-continuous systems. Despite operational differences, the two modes share similar principles of nutrient uptake over time. In the semi-continuous system, the feeding pumps were switched off every two hours and left off during weekends, effectively turning the system into a batch mode during those intervals. This allows for a fair comparison, as both systems experienced periods without nutrient replenishment, during which nutrient consumption could still be measured. Consequently, nutrient removal in the semi-continuous system can be directly compared to the batch test results. These results suggest that *C. acidophila* could be a more suitable candidate for long-term semi-continuous wastewater treatment than other species, with an HRT of 3 days.

4.2.2. Semi-continuous treatment of primary and secondary effluents using C. acidophila

In the study, both reactors performed similarly with an HRT of 3 days, but problems arose when the HRT was decreased to 2 days in the tank fed with primary settled effluent, such as unwanted cell growth, blockage of influent tubes due to large particles, formation of algae flocs, and algal settling in the tank. The cause of these problems might be due to a variety of abiotic and biotic factors, such as light penetration issues, temperature variations, and competition with fungi and bacteria, which can affect the overall reactor performance. Bacteria and fungi are the largest biological components in wastewater, feeding on the wastewater nutrients such as ammonium and phosphate, and potentially competing with cultured algae. In this study, the concentration of these biological components, together with higher solid concentration in the primary settled effluent than in the final effluent and poor aeration in the tank, might have been the cause of biofilm and floc formation in the tank. The biofilm formation might have led to poor light penetration and poor microalgae performance, as light intensity decreases almost exponentially with distance away from the irradiated surface. However, in the tank supplied with final effluent, it was not observed biofilm formation on the tank surfaces, and more light reached the algae culture, resulting in higher growth in the tank.

One of the operational downsides of using microalgae in wastewater treatment is the high competition with other microorganisms for nutrients and light, which can lead to the collapse of the culture [27]. Most microorganisms in wastewater operate within a pH range of 6.5 to 8, which is also the range for most algae used in wastewater treatment [28–30]. Therefore, the growth and performance of these algae can be negatively affected by competition for resources with other microorganisms, resulting in reduced biomass production and phosphorus removal efficiency. To overcome this challenge, microalgae with unique growth conditions can be employed. *C. acidophila* is a microalgae that can thrive in acidic conditions with a pH as low as 2.5 and is therefore the dominant species in the wastewater, minimizing competition with other microorganisms [31,32]. Thus, using *C. acidophila* for P removal can provide a competitive advantage by reducing competition for nutrients and light, leading to higher biomass production and P removal efficiency.

It is also important to notice that bacterial activity may contribute to remineralization, that could explain unexpected nutrient fluctuations observed during the experiment. Bacteria can break down organic matter in wastewater, releasing inorganic nutrients back into the system, which could lead to variations in nutrient concentrations that are not solely attributable to microalgal uptake [33]. However, this study did not specifically investigate bacterial activity or its role in remineralization. Moreover, given the harsh medium conditions used in this experiment, it is likely that bacterial activity was minimal or absent. Further studies are required to explore the extent of bacterial involvement and validate its potential contribution to nutrient dynamics in such systems.

5. Conclusion

In conclusion, the study highlights the potential of *C. acidophila* as a viable candidate for long-term wastewater treatment. Several key findings support the efficacy and practicality of employing this extremophilic microalgae in wastewater treatment applications:

1. The identification of an optimum growth temperature of 20 °C and a light intensity of 40 μmol photons m^{-2} s^{-1} for *C. acidophila* is a key finding of our study. This differentiates *C. acidophila* from most microalgae, as 83 % of species require light intensities above 100 μmol photons m^{-2} s^{-1} for optimal growth. This trait significantly enhances the economic feasibility of *C. acidophila*-based wastewater treatment systems by enabling operation under lower light conditions.
2. Adaptability to Fluctuating Nutrient Availability: *C. acidophila* exhibits a notable ability to thrive in environments where nutrient concentrations may fluctuate. This adaptability positions it as an excellent candidate for wastewater treatment applications, where nutrient availability can vary over time.
3. *C. acidophila* demonstrates ability to continuously consume nutrients even at low light intensities, highlighting its efficiency in wastewater treatment. Its mixotrophic nature allows it to assimilate inorganic carbon from wastewater without requiring additional CO_2 bubbling, further simplifying the operational requirements.
4. The naturally low pH of *C. acidophila* helps mitigate the inhibitory effects of bacteria and fungi typically found in municipal wastewater. This characteristic positions *C. acidophila* as a potential alternative for treating primary settled effluent from municipal WWTPs, offering a robust solution for nutrient removal.

C. acidophila is a promising candidate for wastewater treatment, demonstrating growth in low light, resilience to fluctuating nutrients, and consistent nutrient consumption. This study supports its potential in improving nutrient recovery systems.

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CRediT authorship contribution statement

Lena S.D. Procopio: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Ania Escudero:** Writing – review & editing, Formal analysis, Conceptualization. **Colin Hunter:** Data curation.

Declaration of competing interest

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

Glasgow Caledonian University reports financial support was provided by INTERREG IVB NWE. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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