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Selection of microalgae in artificial digestate: Strategies towards an effective phycoremediation

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ABSTRACT

Digestate is a complex by-product of anaerobic digestion and its composition depends on the digestor inputs. It can be exploited as a sustainable source of nutrients for microalgae cultivation but its unbalanced composition and toxic elements make the use challenging. Screening algae in a simplified synthetic digestate which mimics the main nutrient constraints of a real digestate is proposed as a reproducible and effective method to select suitable species for real digestate valorisation and remediation.

Growth performance, nutrient removal and biomass composition of eight microalgae exposed to high amounts of NH_4^+, PO_4^- and organic-C were assessed. Using a score matrix, *A. protothecoides*, *T. obliquus*, *C. reinhardtii*, and *E. gracilis* were identified as the most promising species. Thus, three strategies were applied to improve outcomes: i) establishment of an algal consortium to improve biomass production, ii) K^+ addition to the medium to promote K^+ uptake over NH_4^+ and to reduce potential NH_4^+ toxicity, iii) P starvation as pretreatment for enhanced P removal by luxury uptake.

The consortium was able to implement a short-term response displaying higher biomass production than single species (3.77 and 1.03–1.89 mg mL^{-1} respectively) in synthetic digestate while maintaining similar nutrient remediation, furthermore, its growth rate was 1.6 times higher than in the control condition. However, the strategies aiming to reduce NH_4^+ toxicity and higher P removal were not successful except for single cases. The proposed algal screening and the resulting designed consortium were respectively a reliable method and a powerful tool towards sustainable real digestate remediation.

1. Introduction

Environmental pollution resulting from various anthropogenic activities poses a substantial threat to terrestrial and aquatic ecosystems (Schwarzenbach et al., 2010). The use of conventional methods for pollution control and remediation is usually expensive and sometimes inefficient, thus it is becoming increasingly important to investigate novel and sustainable solutions (Al-Obadi et al., 2022; Awasthi et al., 2021; Brunner and Rechberger, 2015; Elsaid and Aghezzaf, 2015).

Among these solutions, the use of microalgae to mitigate environmental pollutants (Khan et al., 2018; Koul et al., 2022) has emerged as a promising biotechnology for the treatment of organic waste streams. Microalgae efficiently uptake nutrients from various wastewater streams and convert them into valuable bioproducts such as PUFA, essential amino-acids and bio-active metabolites (Sathasivam et al., 2019). Compared to other photosynthetic organisms such as crops, microalgae have much higher productivity in terms of ton ha^{-1} $year^{-1}$ (100–280 compared to 10–20) (Benedetti et al., 2018; Bošnjaković and Sinaga, 2020), therefore they fix more inorganic carbon contributing to the mitigation of climate change. In addition, differently from higher plants, many species of microalgae can implement a mixotrophic metabolism, thus they also use organic matter as a source of energy and C (Candido et al., 2020; Proietti Tocca et al., 2024; Wang et al., 2024). Besides, exploiting wastewater as a source of nutrients (C, N, P) for microalgal cultivation would also drop the costs by about 50%, from 10 € kg^{-1} to 5 € kg^{-1} (Bauer et al., 2021; Rossi et al., 2023). All these features make microalgal biotechnology very attractive and give added value to the process of wastewater remediation (Koutra et al., 2018).

Among wastewaters, digestate is gaining interest as a plentiful source

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Abbreviations

μ_{max}	Maximum growth rate
AD	Anaerobic Digestion
CTR	Control
CW	Artificial digestate
CWk	Artificial digestate added with potassium
CWst	P-starved cells grown in artificial digestate
N	Nitrogen
Nt_e	Maximum density
P	Phosphorous
PolyP	Poly-phosphate

of nutrients (i.e. macronutrients, micronutrients and vitamins) for microalgal cultivation. Digestate is generated by anaerobic digestion (AD) (Bankston and Higgins, 2020; Chong et al., 2021; Guan et al., 2024; Laiq Ur Rehman et al., 2019; Rude et al., 2022; Xia and Murphy, 2016) and its valorisation would make the entire AD process more sustainable and reduce costs of algal biomass production to 4.3 € kg-1 (Rossi et al., 2023). AD converts organic by-products into digestate and renewable energy, i.e., biogas. Even if AD is considered an eco-friendly alternative to traditional waste disposal methods, the digestate resulting from the process must be appropriately managed (Laiq Ur Rehman et al., 2019).

Digestate is a complex mixture of organic and inorganic compounds whose composition is largely variable and depends on the anaerobic digestor feedstock (Carchesio et al., 2014; Laiq Ur Rehman et al., 2019; Risberg et al., 2017; Tambone et al., 2017; Vitti et al., 2021). Regarding algal cultivation main drawbacks of digestate are the excess of nutrients and their unbalanced stoichiometry (Vitti et al., 2021; Risberg et al., 2017; Tambone et al., 2017). Indeed, digestate may limit and even inhibit microalgal growth due to its abundant concentration of reduced N forms (Wang et al., 2021; Xia and Murphy, 2016) whose chemical equilibrium is pH dependent (pK = 9.40 at 20 °C): NH_3 (ammonia) which diffuses across membranes and NH_4^+ (ammonium) which enters the cells through ion transporters (Britto and Kronzucker, 2008; Coskun et al., 2017). Although NH_4^+ is directly assimilated into organic N in plastids, both forms of reduced N are toxic to the photosynthetic apparatus (by binding the Mn-cluster of the OEC complex or by uncoupling the proton gradient across thylakoids) (Källqvist and Svenson, 2003; Britto and Kronzucker, 2002; Esteban et al., 2016).

Even excessive external P can induce growth inhibition and cell damage due to excessive P incorporation into acid-insoluble polyphosphates (PolyP) which may alter enzyme synthesis (Li et al., 2018). Therefore, it is of paramount importance to figure out which algal species are tolerant to these unfavourable conditions and can grow in digestate (Fernandes et al., 2022).

In this study, a synthetic digestate was used instead of a real one to reduce the wastewater complexity while keeping the main constraints (high availability of NH_4^+, PO_4^{4-} and organic-C) for algal growth (Vasseur et al., 2012). Screening algal species based on growth and remediation parameters allowed the selection of the most suitable species. Three strategies were then applied to further increase their growth and remedial capacity.

i) Establishment of a microalgal consortium which is a stable culture of at least two species gaining advantages from the co-cultivation due to mechanisms of symbiosis or commensalism; consortia are known to increase fitness and resilience of microalgal species as compared to monospecific cultures (Subashchandrabose et al., 2011). We hypothesized that a co-culture of the top four species (according to the previous screening) would constitute a functionally robust consortium of organisms capable of facing the many challenges digestate poses.

ii) K^+ enrichment of growth medium, improving K^+ over NH_4^+ uptake. Since NH_4^+ cannot freely cross the cell membrane it requires ammonium transporters (AMT) to enter the cell (Coskun et al., 2017). Nevertheless, low-affinity K^+ transport system (LATS) transports not only K^+ but also NH_4^+ due to their closely similar hydrated radius (Andrews et al., 2013; Britto and Kronzucker, 2008; Coskun et al., 2017; Szczerba et al., 2009). The similarity leads to a well-known interaction between K^+ and NH_4^+ acquisition both in plants and algae causing K^+ limitation when the reduced N is abundant (Andrews et al., 2013; Coskun et al., 2017) as well as NH_3/NH_4^+ toxicity (Britto and Kronzucker, 2008; Szczerba et al., 2009). Hence, we hypothesized that a higher K^+ concentration in the external environment would hinder the influx of NH_4^+ and reduce the potential toxicity of reduced N forms (Britto and Kronzucker, 2008; Szczerba et al., 2009).

iii) P-starvation applied to algae before they grow in synthetic digestate to stimulate the cellular process called luxury uptake (Azad and Borchardt, 1970; Yao et al., 2011). This mechanism allows algae to take up and store quotas of P as polyphosphate (PolyP) more than immediately needed ensuring a supply of P during long-term periods of environmental stress (Li et al., 2018; Solovchenko et al., 2019a). As already reported P-starved cells enhance P uptake (Solovchenko et al., 2019b); therefore, we hypothesized that more P remediation would be achieved by subjecting algae to a period of P starvation before cultivation in digestate.

Data on growth, removal yield and algal biochemical composition provide interesting insights into the physiology of algal cells exposed to a synthetic digestate; results represent the first attempt to design and validate strategies to boost digestate exploitation as a nutrient source by algae towards a rational and physiology-based digestate remediation.

2. Materials and methods

2.1. Microalgal strains

Auxenochlorella protothecoides (CCAP 211/8D[1]), *Tetradesmus obliquus* (CCAP 276/3A), *Euglena gracilis* (CCAP 1224/5Z), *Chlorella vulgaris* (CCAP 211/11B), *Anabaena* sp. (CCAP 1403/4A) *Chlamydomonas reinhardtii* (RCC125[2]), *Synechocystis* sp. (PCC6803[3]), *Arthrospira platensis* (SAG 85.79[4]) were chosen for the experiments according to their known phycoremediation capacities (Table 1). Microalgae species were inoculated and maintained in freshwater media (Table 1) until beginning the experiments in a controlled environmental chamber at a constant temperature of 20 °C and 100 μmol m^{-2} s^{-1}, under a 24 h light regime.

2.2. Experimental design

Species selection was carried out using the artificial digestate as growth medium on cells previously grown in standard media. The artificial digestate was obtained from the Conway medium (Tompkins et al., 1995) modified according to Vasseur et al. (2012) with final concentrations of 250 mg L^{-1} NH_4Cl, 150 mg L^{-1} NaH_2PO_4, 465 mg L^{-1} Na-Acetate (5 times higher than the concentrations used by Vasseur et al. (2012) to better mimic real digestate). The initial pH of the medium was 7.6 thanks to the addition of 10 mM Tris HCl buffer. The medium will be called CW from now on.

An initial cell concentration of 10^5 cells mL^{-1} was used to establish cultures in 250 mL Erlenmeyer flasks filled with 100 mL of CW or

[1] https://www.ccap.ac.uk/.
[2] https://roscoff-culture-collection.org/.
[3] https://utex.org/.
[4] https://uni-goettingen.de/en/184982.html.

Table 1
List of experimental microalgae. Medium refers to the standard medium used to maintain culture an grow algae as control. References of experimental species already used to remediate digestate or wastewater are reported in the last column.

Species	Lineage	ID code	Medium	Reference
Auxenochlorella protothecoides	Chlorophyta	CCAP 211/8D	BG11[a]	Bankston and Higgins (2020); Krzemińska et al. (2019); Wang et al. (2021)
Tetradesmus obliquus	Chlorophyta	CCAP 276/3A	BG11	Chuka-ogwude et al., 2020; Fernandes et al. (2022); Massa et al. (2017)
Chlamydomonas reinhardtii	Chlorophyta	RCC125	BG11	López-Sánchez et al. (2022); Zuliani et al. (2016)
Euglena gracilis	Euglenophyta	CCAP 1224/ 5Z	BG11	Nguyen et al. (2015); Takemura et al. (2020)
Chlorella vulgaris	Chlorophyta	CCAP 211/ 11B	BG11	Drekeke Iyovo et al. (2010); Koutra et al. (2021); López-Sánchez et al. (2022); Zuliani et al. (2016)
Synechocystis sp.	Cyanobacteria	PCC 6803	BG11	Cepoi et al. (2016); Hughes et al. (2018); Trentin et al. (2019)
Anabaena sp.	Cyanobacteria	CCAP 1403/ 4A	3N-BBM[b]	El-Bestawy (2008); Gorain et al. (2019)
Arthrospira platensis	Cyanobacteria	SAG 85.79	ZARROUK[c]	Cicci and Bravi (2014); Kisielewska et al. (2022); Matos et al. (2021); Rao et al. (2024)

[a] M.M. Allen, R.Y. Stanier. Growth and division of some unicellular blue-green algae. Microbiology., 51 (1968), pp. 199–202, 10.1099/00221287-51-2-199.
[b] H.W. Bischoff, H.C. Bold. Phycological Studies IV. Some Soil Algae From Enchanted Rock and Related Algal Species. University of Texas. Publication No. 6318 (1963), p. 95.
[c] C. Zarrouk. Contribution à l'étude d'une cyanophycée: influence de divers facteurs physiques et chimiques sur la croissance et la photosynthèse de Spirulina maxima (Setch et Gardner) Geitler. (Ph.D. Thesis). Université De Paris (1966).

freshwater media (Table 1) as control condition (CTR). Growth was followed in 3 independent biological replicas for a total of 15 days at the experimental condition of 20 °C, 100 μmol photons m^{-2} s^{-1}, under a 24 h light regime.

Equal volumes of *A. protothecoides*, *T. obliquus*, *E. gracilis* and *C. reinhardtii* mono-specific cultures grown in BG11 were co-inoculated in a 250 mL Erlenmeyer flask containing 100 mL fresh BG11 medium to establish a microalgal-consortium. After at least 10 cell duplications, the consortium was found to be stable when, through optical microscope observation and counts, the proportion among the 4 species did not change.

The above mentioned 4 species and their consortium were also grown in CW medium where K^+ concentration was increased to 0.22 mM (almost 5-fold) by the addition of a sterile solution of K_2SO_4 (CWk). Growth was carried out in 3 independent biological replicas at the same environmental conditions as the first experiment.

In addition, they were also grown in CW medium after a period of P starvation as described by A. Solovchenko et al. (2019b) (CWst); cells were harvested through centrifugation (MPW, centrifuge 351e) for 8 min at 3200g, washed with P-free control medium (Table 1) and then incubated at the experimental conditions. Cells were considered P-depleted when no increase in cell number was observed (after about 2–3 generations) and then moved back to the CW medium.

In all cases, growth was followed using either an automatic cell counter (Casy TT, Innovatis AG, Reutlingen, Germany) or a spectrophotometer (UV-1900i, SHIMADZU CORP.) to measure cells mL^{-1} or OD_{750nm}, respectively. Through calibration curves between cell density (as cells mL^{-1} or optical density) and DW (Fig. S1), the cell density of each species was converted and reported as dry weight (DW) per unit of volume and expressed as mg mL^{-1}. Consortium DW was daily measured by directly drying 1 mL sample of the culture.

β-function, a non-linear regression model reported by Yin et al. (2003) (Equation (1)) and also applied to microalgal growth (Mollo et al. (2023), and by Petrucciani et al. (2023)) was used to determine maximum growth rate (μ_{max}). In the β-function N is the algal concentration (mg mL^{-1}), C_m is the maximum growth rate (mg mL^{-1} d^{-1}) which is achieved at time t_m, μ_{max} is the maximum growth rate (d^{-1}) normalized on Nt_m (Equation (2)), t_b is the reference time and t_e is the time at which stationary phase is reached. Nt_e refers to algal density achieved at t_e (maximum density).

Equation (1): β-function

$$\frac{dN}{dt} = C_m\left(\frac{t_e - t}{t_e - t_m}\right)\left(\frac{t - t_b}{t_m - t_b}\right)^{\frac{t_m - t_b}{t_e - t_m}} \quad (1)$$

Equation (2): maximum growth rate normalized on the density achieved ad Tm

$$\mu_{max} = \frac{C_m}{Nt_m} \quad (2)$$

Productivity (P, mg mL^{-1} d^{-1}) was calculated as the biomass (mg mL^{-1}) produced during the total elapsed time (d) from T_b (inoculum) to T_e (time when the maximum density Nt_e is reached) (Equation (3)). The formula is reported below where Nt_b refers to the algal density at time T_b (day 0).

Equation (3): algal productivity

$$P = \frac{Nt_e - Nt_b}{T_e - T_b} \quad (3)$$

2.3. Nitrogen and phosphorous remediation

Total ammonium content was daily calculated using a Spectroquant photometric ammonium test (Supelco, Merck). An aliquot of 1 mL algal culture was centrifuged and the NH_4^+ content of the supernatant was immediately measured by spectrophotometry (UV-1900i, SHIMADZU CORP.). Ammonium content was used to evaluate N remediation after 15 days as % of initial NH_4^+/NH_3 content.

Phosphorous remediation was quantified based on the biomass P amount obtained by TXRF analysis of microalgal composition in elements (see paragraph 2.5). Aliquots of 10 mL algal culture were collected 15 days post-inoculum, washed twice with UP water and centrifuged. The algal pellet was then re-suspended in 2 mL of UP water: 1 mL was used to assess DW per mL culture, and the remaining to analyse P concentration per mL culture. Based on DW and P quota, the total content of P within biomass was calculated and P remediation was then expressed as % of the initial concentration.

2.4. Score matrix

A score matrix based on three growth parameters (μ_{max}, productivity, Nt_e) (see paragraph 2.2) and two remediation parameters (nitrogen and phosphorous remediation) (see paragraph 2.3) was created; values were calculated on data deriving from algae grown in CW medium. Each

parameter had the same weight in the matrix. The score matrix converted algal values within each parameter into a score from 0 to 1 where 0 and 1 were respectively the lowest and the highest data recorded. Scores from each algal species were then averaged and the mean values were ranked to evaluate the most suitable species to remediate a digestate. Four species with the highest scores were further selected.

2.5. Elemental composition

The elemental composition of algal species in each growth condition was determined when algae reached the early stationary phase. C and N biomass quotas were determined by an elemental analyser (ECS 4010, Costech Italy) connected to the ID Micro EA isotope ratio mass spectrometer (Compact Science Systems, Lymedale Business Centre, Newcastle-Under-Lyme, United Kingdom) to obtain C and N stable isotope ($\delta^{13}C$ and $\delta^{15}N$) ratios as reported in Petrucciani et al. (2022). Acquisition and data analysis were performed using EAS- Clarity software (Costech Analytical Technologies Inc., Milano, ItalyOrganic composition).

The concentration of macronutrients and micronutrients (P, S, K, Ca, Fe, Cl) was measured with Total Reflectance X-Ray Fluorescence (TXRF) spectrometer (S2 Picofox, Bruker AXS Microanalysis GmbH, Berlin, Germany). A volume of 10 mL algal cultures was washed twice with UP water and resuspended in 1 mL of UP water before analysis. An HNO3 solution of gallium was used as internal standard for a final concentration of 5 μL L-1 (Petrucciani et al., 2022). Deconvolution and element quantification were performed using SPECTRA 6.1 software (Bruker AXS Microanalysis GmbH, Berlin, Germany).

2.6. Pigment quantification

Algae were collected in the early stationary phase through centrifugation at 1500*g* for 5 min; a volume of 2 ml of 100% (v/v) methanol was added to the pellet and kept overnight in the dark at −20 °C (Ritchie, 2006). The colourless pellet and supernatant were separated by centrifugation at 13,000 g for 5 min. The supernatant was measured spectrophotometrically (UV-1900i, SHIMADZU CORP.) to quantify chlorophyll *a*, chlorophyll *b* and carotenoids.

Chlorophyll content (μg mL^{-1}) was calculated according to Wellburn (1994) (Eq. (4) and (5) for chlorophytes and euglenophytes), and Ritchie (2006) (Eq. (6) for cyanobacteria):

$$\text{Chlorophyll } a = 16.72 \bullet \text{Abs}_{665nm} - 9.16 \bullet \text{Abs}_{652nm} \quad (4)$$

$$\text{Chlorophyll } b = 34.09 \bullet \text{Abs}_{652nm} - 15.28 \bullet \text{Abs}_{665nm} \quad (5)$$

$$\text{Chlorophyll } a = 12.95 \bullet \text{Abs}_{665nm} \quad (6)$$

The total content of carotenoids (μg mL^{-1}) was calculated using the equation presented by Wellburn (1994):

$$\text{Carotenoids} = (1000\text{Abs}_{470nm} - 1.63\text{Chl } a - 104.96\text{Chl } b) / 221 \quad (7)$$

2.7. Macromolecular pools

Proteins were measured according to the Lowry colorimetric method described by Peterson (1977) on algae biomass collected at the early stationary phase by centrifugation (13,000 g for 5 min). A spectrophotometer UV-1900i, SHIMADZU CORP. was used for absorbance measurements. Quantification was carried out by interpolating absorbance data (λ 750 nm) in a standard curve constructed with known concentrations of bovine serum albumin (BSA).

To characterize whole cell macromolecular composition FTIR absorbance spectra were acquired (1800-800 cm^{-1}): aliquots of 50 μl of cell suspension were dried at 80 °C overnight on silicon supports and analysed with a Tensor 27 FTIR spectrometer (Bruker Optics, Ettlingen, Germany). As described by Giordano et al. (2001) relative abundances of lipids, carbohydrates and proteins were calculated via band integrals of deconvolved spectra, with OPUS 6.5 software (Bruker Optics GmbH, Ettlingen, Germany). According to Palmucci et al. (2011), semi quantification of carbohydrates and lipids was then achieved by comparing the total protein content determined as described above with the FTIR absorbance ratios.

2.8. Statistical analysis

All data are reported as mean of three to six ($3 \leq n \leq 6$) independent biological replicates followed by the standard deviation (±SD). The results were analysed using the software Graphpad Prism 9.5.0 (GraphPad Software, San Diego, CA, USA). Two-tailed *t*-test and one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test were used to analyse growth parameters (μmax, N_{te}, T_e, T_m, Productivity) and remediation values (N and P) of tested algae. All statistical analyses were performed with a significance level of $\alpha = 0.05$. Letters or asterisk (*) were used in figures and tables to distinguish significantly different groups ($p < 0.05$).

Principal Component Analysis (PCA) was carried out using PAST 4.13 (Hammer et al., 2001). PCA was performed on cellular composition (content of chlorophyll *a and b*, carotenoids, proteins and semiquantities of lipids and carbohydrates, weight of C, N, P, S, K, Ca, Fe, Cl per DW, $\delta^{13}C$, $\delta^{15}N$ as dependent variables) among different growth conditions within each species (independent variables). Data were normalized using z-values [(n-mean)/SD].

3. Results

3.1. Screening of algal species

3.1.1. Growth in artificial digestate

Data presented in Fig. 1 and Table S1 detail the growth of eight algal species in CW and CTR media. All species were able to grow in CW except *Anabaena* sp. Which rapidly died showing a negative μ_{max} value (-0.40 ± 0.08 d^{-1}) in the treated condition. *C. reinhardtii* showed the highest μ_{max} among CW as well as CTR grown algae (0.55 ± 0.12 d^{-1} in CW); nonetheless, a higher μ_{max} was not related to higher cell density (Nt_e). The cell density of CW cultures was generally lower than the value in the CTR condition.

Adoption of CW as growth medium had also an effect on the growth curve shape (Fig. 1) where the inflection point (T_m) was usually reached in a shorter period compared to what occurred in CTR (Table S1). Similarly, the stationary phase (T_e) was reached earlier in CW cultures than in CTR ones.

3.1.2. N and P remediation

Fig. 2 reports N and P remediation of algae in CW condition. On average, considering Figs. 1 and 2, a higher removal of the elements was achieved with a higher algal growth. The highest N-remediation was achieved by *T. obliquus* with 93.6 ± 0.2% of removed N. Similar values were obtained by *C. vulgaris* (92.2 ± 0.1%), *E. gracilis* (91.3 ± 0.2%), *C. reinhardtii* (89.9 ± 0.1%) and *A. protothecoides* (88.7 ± 0.4%). The lowest removal value was recorded by *Anabaena* sp. (32.3 ± 1.9%).

Removal of P was much lower (on average less than 10% of the initial available P was removed) as compared to N removal; *E. gracilis* achieved the highest removal percentage (22 ± 1%).

3.1.3. Score matrix

The score matrix (Table 2a) made from data reported in Table S2 highlighted that half the species were particularly promising on the base of analysed parameters and were selected for further experiments. In order of score the selected species were *C. reinhardtii* (0.812), *A. protothecoides* (0.769), *E. gracilis* (0.728) and *T. obliquus* (0.600) (Table 2b). Cyanobacteria were largely affected by the experimental growth condition and showed the lowest values among all the tested

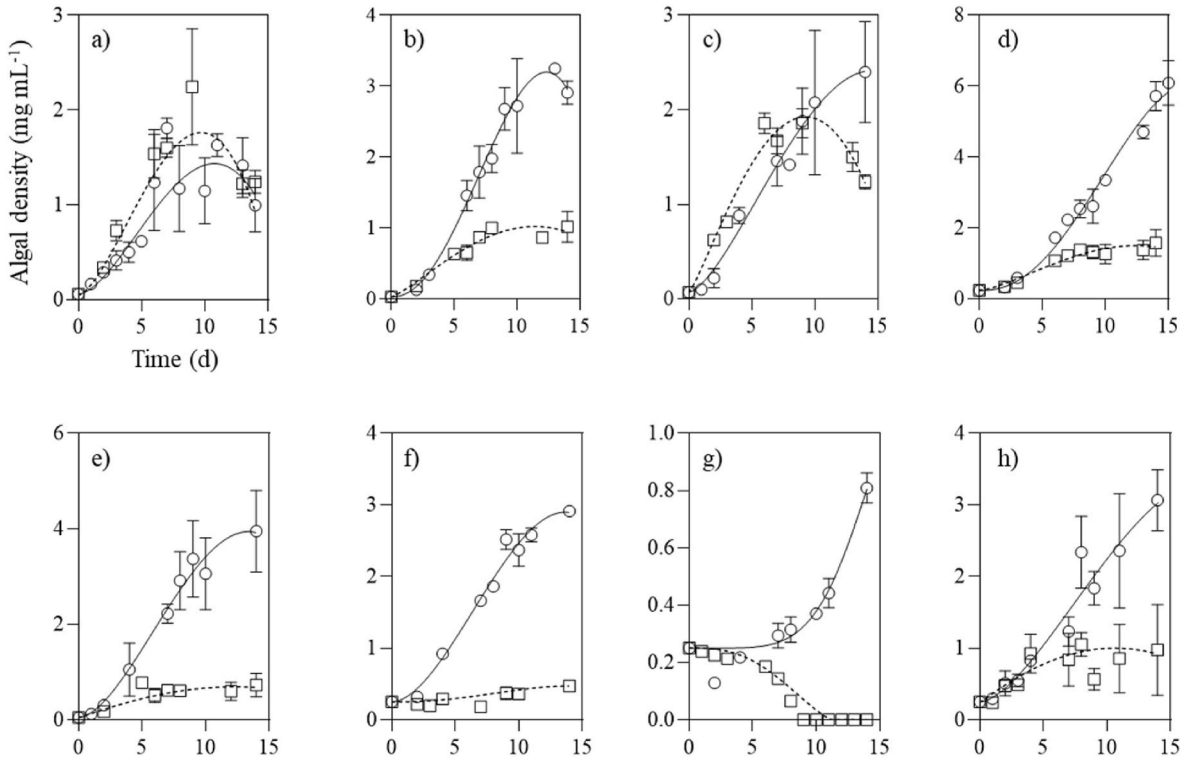


Fig. 1. Growth curve of algae grown in CTR (circle and solid line) and CW conditions (square and dashed line): experimental data (symbols) and the regression curve (solid and dashed lines) are shown. Species are reported in the following order: a) *A. protothecoides*, b) *T. obliquus*, c) *C. reinhardtii*, d) *E. gracilis*, e) *C. vulgaris*, f) *Synechocystis* sp., g) *Anabaena* sp., h) *A. platensis*. Experimental data are reported as mean ± SD. Regression curve of the experimental data was made using the β function non-linear regression.

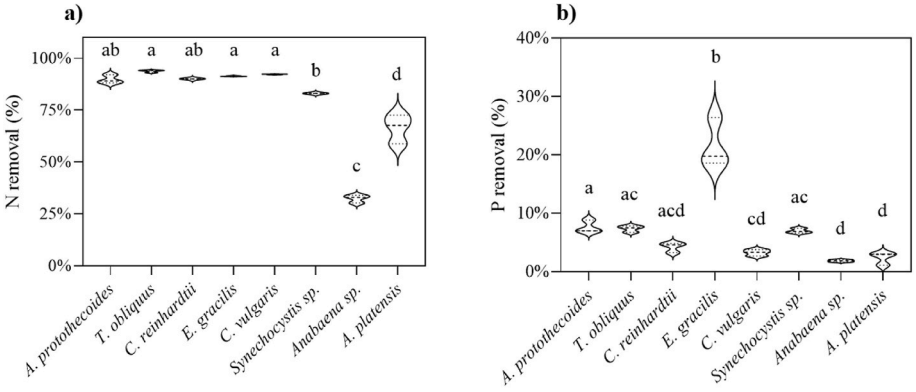


Fig. 2. Nitrogen (a) and phosphorous (b) removal (%) in CW medium at the end of the experiment by tested microalgae. Letters represent significant differences among tested species.

species.

3.2. Improvement of algal growth and nutrient remediation

3.2.1. Consortium growth in a synthetic digestate

The algal consortium composed of *C. reinhardtii*, *A. protothecoides*, *E. gracilis* and *T. obliquus* was established in control condition; its growth rate and Nt_e were second only to the corresponding values in *A. protothecoides* and *E. gracilis*, respectively (Fig. 3b, Table S1). Once exposed to CW medium it showed a 1.6 times higher growth rate than in the CTR condition ($p = 0.0005$) and slightly lower, but statistically significant, Nt_e during the stationary phase than in the CTR condition ($p = 0.0241$) (Fig. 3b–Table S1). Moreover, Nt_e of CW grown consortium was significantly higher than the values of CW grown monospecific cultures ($p < 0.0001$) (Fig. 3b–Table S1).

N remediation (94% ± 0.2%) was consistent with what was observed for the single species while P removal (2.9% ± 1.2%) was on average significantly lower than single species remediation values ($p = 0.0048$, 0.0104, 0.2896, 0.0014 when comparing with *A. protothecoides*, *T. obliquus*, *C. reinhardtii*, *E. gracilis*, respectively) (Figs. 3c and 2).

3.2.2. Addition of K^+

The growth response of algae to the addition of K^+ was species-specific as reported in Table 3 and Fig. S2. *T. obliquus* was the only selected species whose growth was enhanced when K^+ was added as compared to growth in artificial digestate (CW): Nt_e was significantly higher than the CW one ($p < 0.0001$). Moreover, CWk grown cells of *T. obliquus* μ_{max} and Nt_e were equal to those in CTR condition

Table 2
Score matrix reporting score values deriving from original data. A score ranging from 0 to 1 was assigned to each data where 0 was the lowest value recorded for a specific parameter and 1 the highest value: a) assigned scores to each species and parameter; b) average score and score position of each species. A red-green (0–1) colour scale is also provided.

a)

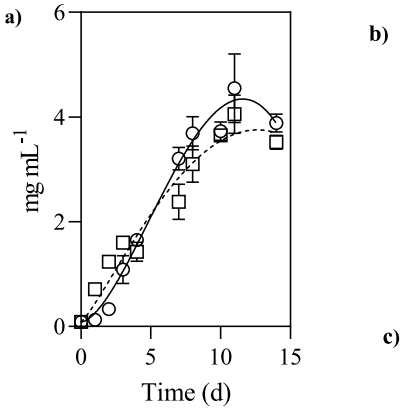
Species	μ_{max}	Productivity	Nt_e	N removal	P removal
A. protothecoides	0.736	0.944	0.954	0.920	0.292
T. obliquus	0.728	0.450	0.544	1.000	0.279
C. reinhardtii	1.000	1.000	1.000	0.939	0.120
E. gracilis	0.341	0.530	0.806	0.963	1.000
C. vulgaris	0.589	0.327	0.355	0.977	0.071
Synechocystis sp.	0.412	0.156	0.338	0.832	0.259
Anabaena sp.	0.000	0.000	0.000	0.000	0.000
A. platensis	0.562	0.458	0.491	0.555	0.026

1
0.5
0

b)

Species	Average Score (Score position)
A. protothecoides	0.769 (2)
T. obliquus	0.600 (4)
C. reinhardtii	0.812 (1)
E. gracilis	0.728 (3)
C. vulgaris	0.464 (5)
Synechocystis sp	0.399 (7)
Anabaena sp	0.000 (8)
A. platensis	0.418 (6)

μ_{max}: maximum growth rate
Productivity: biomass produced during the total elapsed time from T_b (inoculum) to T_e
Nt_e: maximum algal density reached at T_e
N removal: NH_4^+ removal %
P removal: P removal %



Parameter	Growth condition CTR	CW
μmax (d^{-1})	0.27 ± 0.03 [a]	0.44 ± 0.01 [b]
Tm (d)	4.83 ± 0.80 [a]	2.33 ± 0.07 [b]
Te (d)	11.59 ± 0.24 [a]	12.77 ± 0.87 [a]
Nte (mg mL^{-1})	4.36 ± 0.23 [a]	3.77 ± 0.18 [b]
R^2	0.98 ± 0.01	0.90 ± 0.09

	Nitrogen	Phosporous
Remediation (%)	94.0% ± 0.2%	2.9% ± 1.2%

Fig. 3. a) Growth curve of the consortium in CTR and CW media. Experimental data (symbols) and regression curves (solid and dashed lines) are shown. The regression curve of the experimental data was made using the β function non-linear regression. b) Growth parameters based on regression curve of consortium cultured in CTR and CW media. c) Nitrogen and phosphorus remediation percentage. All data are reported as mean ± SD. Letters represent significant differences between the growth conditions.

(respectively $p = 0.6205$ and $p = 0.8109$) (Table 3).

A. protothecoides was not influenced in a significative way by the addition of K^+: N_{te} was comparable to values of cultures in CTR and CW condition while μ_{max} was slightly lower than the CW one ($p = 0.0133$) but equal to μ_{max} of algae grown in CTR condition ($p = 0.0867$) (Table 3).

C. reinhardtii and *E. gracilis* were negatively affected by the treatment. Both species showed lower N_{te} than the ones of CTR and CW cultures (Table 3) and in *E. gracilis* the difference among values was even greater.

Growth of the consortium in K^+ enriched CW medium was an in-between response to those of single species: an increase of Nt_e compared to CW condition was observed ($p = 0.0018$) (Table 3) differently from what observed in *C. reinhardtii* and *E. gracilis* (Table 3). Nonetheless the Nt_e of CWk grown consortium was lower than the CTR one ($p = 0.0001$) (Table 3), thus a total recovery of the maximum density (as for *T. obliquus*) was not achieved.

Change in external N concentration was evaluated to assess if a potential competition between NH_4^+ and K^+ for the transporters would affect cell capacity to remove it (Fig. 4a). *T. obliquus* and *A. protothecoides* grown in CWk medium achieved remediation values higher than 90% and equal to those in CW medium. On the other hand, lower N remediation was recorded in CWk grown cultures of *C. reinhardtii* and *E. gracilis* (concurrently with a lower growth, Table 3, Fig. S2) as compared to their respective values in CW grown cultures (Fig. 4a). Concerning the consortium, a high amount of N was removed from the CWk medium even if statistically lower than the amount removed in CW condition ($p = 0.0025$) (Fig. 4a).

In parallel also the K/N ratio measured in algal biomass changed according to the species (Table S3). In *T. obliquus* and *C. reinhardtii* the K/N ratio was higher when K^+ availability was higher in the medium while in *A. protothecoides* and *E. gracilis* it was statistically similar to the one in the CW condition (Fig. 4b). Uniquely the K/N ratio shown in the algal consortium was not affected by the treatments (CW and CWk) as compared to the CTR condition, therefore by the N and K availability (Fig. 4b).

Table 3
β function parameters (μ_{max}, T_m, T_e, Nt_e) of non-linear regressions made on experimental growth data of selected algae (*A. protothecoides, T. obliquus, C. reinhardtii, E. gracilis*) and their consortium grown in each experimental condition (CTR, CW, CWk, CWst). Data are reported as mean ± SD. Letters represent significant differences between the growth conditions.

Species	Growth condition	μ_{max} (d^{-1})			T_m (d)			T_e (d)			Nt_e (mg mL^{-1})			R^2		
A. protothecoides	CTR	0.34	±	0.05	ab	3.52	±	0.79	a	9.91	±	0.18	a	1.65	±	0.36	ab	0.89	±	0.07
	CW	0.40	±	0.07	a	2.79	±	0.60	ab	9.62	±	0.41	a	1.80	±	0.11	a	0.95	±	0.02
	CWk	0.23	±	0.01	b	6.91	±	1.57	b	12.03	±	0.61	ab	1.50	±	0.27	ab	0.97	±	0.04
	CWst	0.23	±	0.02	b	6.17	±	2.71	ab	14.38	±	1.93	b	1.05	±	0.18	b	0.99	±	0.00
T. obliquus	CTR	0.24	±	0.01	a	7.30	±	0.81	a	12.20	±	0.66	a	3.43	±	0.23	a	0.99	±	0.01
	CW	0.40	±	0.04	b	2.69	±	0.34	b	11.58	±	1.04	a	1.03	±	0.10	b	0.94	±	0.01
	CWk	0.23	±	0.03	a	8.47	±	0.26	a	12.95	±	1.06	a	3.37	±	0.30	a	0.99	±	0.01
	CWst	0.25	±	0.01	a	5.73	±	0.26	c	11.31	±	0.21	a	0.93	±	0.04	b	0.95	±	0.02
C. reinhardtii	CTR	0.28	±	0.02	a	4.00	±	0.42	a	12.42	±	1.03	a	1.96	±	0.24	a	0.95	±	0.04
	CW	0.55	±	0.12	b	1.88	±	0.54	b	9.47	±	0.21	b	1.89	±	0.16	b	0.98	±	0.02
	CWk	0.57	±	0.15	b	1.51	±	0.77	b	7.98	±	0.35	b	0.81	±	0.20	b	0.78	±	0.17
	CWst	0.20	±	0.01	a	8.25	±	0.22	c	12.40	±	0.11	a	0.68	±	0.08	a	0.98	±	0.01
E. gracilis	CTR	0.17	±	0.01	a	10.42	±	0.42	a	16.25	±	0.42	a	5.57	±	0.42	a	0.97	±	0.01
	CW	0.19	±	0.04	a	4.02	±	0.70	b	12.46	±	1.35	ab	1.52	±	0.28	b	0.94	±	0.04
	CWk	0.16	±	0.02	a	2.02	±	1.33	bd	13.49	±	2.95	a	0.68	±	0.02	c	0.91	±	0.05
	CWst	0.28	±	0.03	b	1.45	±	0.83	d	8.53	±	0.38	b	0.82	±	0.10	c	0.74	±	0.02
Consortium	CTR	0.27	±	0.03	a	4.83	±	0.80	a	11.59	±	0.24	a	4.36	±	0.23	a	0.98	±	0.01
	CW	0.44	±	0.01	b	2.33	±	0.07	b	12.77	±	0.87	a	3.77	±	0.18	b	0.90	±	0.09
	CWk	0.31	±	0.02	a	3.70	±	0.41	ab	11.59	±	0.06	a	2.90	±	0.03	c	0.98	±	0.01
	CWst	0.21	±	0.01	c	7.50	±	1.28	c	12.26	±	0.79	a	0.99	±	0.20	d	0.98	±	0.02

μ_{max}: maximum growth rate.
T_m: inflection point. Time at which μ_{max} is achieved.
T_e: time at the end of the growth where Nte is achieved.
Nt_e: maximum algal density.
R^2: coefficient of determination.

3.2.3. Effect of P starvation

The pretreatment involving P starvation highly affected Nt_e and μ_{max} (Fig. S2). Nt_e was significantly lower than the value achieved without pretreatment even in those species whose growth in the CW was comparable to the growth in CTR condition (*A. protothecoides*, *C. reinhardtii*) (Table 3). Starvation also affected the consortium growth: in particular, μ_{max} and Nt_e values were the lowest recorded among all growth conditions (Table 3).

In mono-cultures P remediation was not enhanced by the pretreatment: removal percentages of P were always lower than those in CW condition (Fig. 5a). On the contrary, the consortium P removal was comparable to the one achieved without P starvation ($p = 0.0623$).

Nevertheless, the P percentage of algal biomass (g P per g DW) was higher in pretreated cells of *A. protothecoides* and *C. reinhardtii* than in CW grown cells (Fig. 5b). P percentage in *E. gracilis* grown in CWst and in CW conditions was remarkably higher if compared to the P amount in CTR condition (8% rather than 1%) (Fig. 5b) but no significative difference between the two treated conditions was observed. Similarly, in CWst and CW grown consortia, P concentration was 2–4 times higher than in the CTR grown consortium.

3.3. PCA analysis

PCA analysis pictured microalgal physiological parameters in different growth conditions. The sum of PC1 and PC2 accounted for 75.5%, 67.0%, 68.3%, 70.1%, 85.4%, 86.4%, 98.7%, 68.9% of the total variance respectively for *A. protothecoides*, *T. obliquus*, *C. reinhardtii*, *E. gracilis*, *Synechosystis* sp., *C. vulgaris*, *Anabaena* sp., *A. platensis* (Fig. S4). PCA was also carried out on the consortium and a total variance of 72.6% was explained (Fig. 6).

In all the species analysed, CTR and CW conditions were clearly separated according to PC1. Loading values revealed that for the majority of green algal species (*A. protothecoides, T. obliquus*, *C. reinhardtii*, *E. gracilis, C. vulgaris* and the consortium) pigment content weighted more than other parameters on PC1 thus explaining the variance between the two conditions. On the contrary, cyanobacteria (*Synechocystis* sp., *Anabaena* sp. and *A. platensis*) elemental composition (except for C and N) was more relevant for the separation between control and treated cells. Lastly, in *C. vulgaris* the organic composition in terms of macromolecular pools was responsible for the separation.

Considering the four species selected for the further improvement of growth conditions, in *A. protothecoides* CW and CWk were closely related while CWst was separated according to PC2, due to significant changes in P, S, K, Ca, Fe, Cl amounts. Contrarily, *T. obliquus* showed a great separation between CW and CWk, according to both PC1 and PC2 with elemental composition (except for C and N) weighting the most on PC2. In *C. reinhardtii* CW was separated from both the treatment CWst and CWk differently: CW and CWst were divided according to PC1 and thus to pigment content while CW and CWk were divided according to PC2 and thus to macromolecular and P, S, K, Ca, Fe, Cl amounts. *E. gracilis* showed low separation among the treatment CW, CWk and CWst, while the three were weightily separated from the CTR according to PC1. This was observed also for the consortium, while PC2 clearly separated CW from both CWk and CWst due to changes in P, S, K, Ca, Fe, Cl amounts.

4. Discussion

4.1. Growth in the synthetic digestate and species selection

The synthetic digestate as modified by the present study was suitable for challenging and screening algae. Indeed, particularly the Nt_e value of most tested species exposed to CW condition was lower than in CTR condition, in particular, cyanobacteria were more negatively affected than other groups (Fig. 1, Table 2, Table S1). Indeed, as observed by Collos and Harrison (2014), cyanobacteria are less tolerant to NH_4^+ rich media than other groups; nonetheless, due to the highly variable

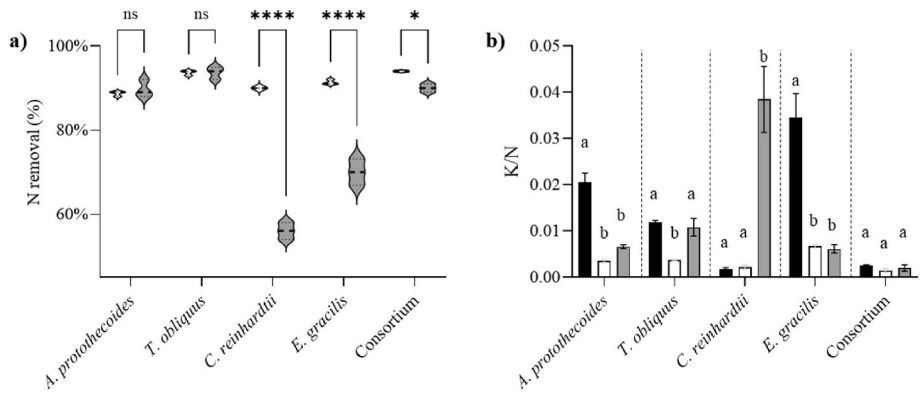


Fig. 4. a) Removal percentage of nitrogen of selected species and consortium in CW (white) and CWk (grey) conditions. b) K to N ratio in algal biomass of selected species and consortium in CTR (black), CW (white) and CWk (grey) conditions. Asterisks (*) represent the degree of significative difference between the growth conditions for each species (ns $p > 0.05$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$). Letters represent significant differences among the growth conditions for each species.

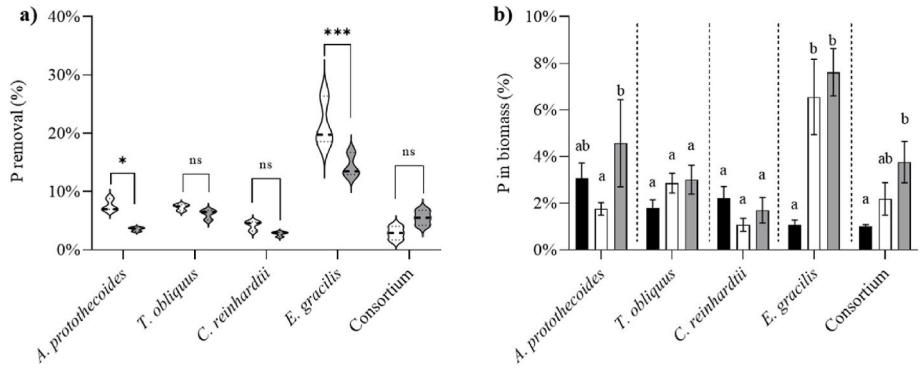


Fig. 5. a) Removal percentage of phosphorous of selected species and consortium in CW (white) and CWst conditions (grey). b) Percentage of phosphorus in algal biomass (g P per g DW) of selected species and consortium in CTR (black), CW (white) and CWst (grey) conditions. Asterisks (*) represent the degree of significative difference between the growth conditions for each species (ns $p > 0.05$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$). Letters represent significant differences among the growth conditions for each species.

composition of a real digestate, cyanobacteria could still be used in some cases (e.g., *A. platensis*, Rao et al. (2024)). The lower Nt_e could be related to at least three factors: 1) un-balanced N:P ratio, 2) high P concentration *per se*, 3) high N concentration provided as NH_3/NH_4^+ *per se*.

1) In synthetic digestate (see paragraph 2.2), the molar ratio among the three major supplied elements was 4.5 C: 3.7 N: 1 P. Since average elemental stoichiometry in microalgae is 106 C: 16 N: 1 P (Redfield ratio, Geider and La Roche, 2002; Shilton et al., 2012), C and N availability in CW medium may have limited P assimilation and formation of new cells. As reported by Qian et al. (2024), for wastewater remediation, and by Psachoulia et al. (2024), for digestate remediation, an N/P ratio of 10 would efficiently support algal species growth. The nutrient unbalance of the growth medium is acknowledged to be one of the main factors affecting biomass production (Chong et al., 2021) and CW composition could explain both the lower number of cells achieved as compared to Nt_e in CTR condition, but also the scant P remediation observed (always lower than 22 %, Table 3). Despite a low removal percentage, it should be noted that P content (in terms of % DW) of algae grown in synthetic digestate was higher than reported in literature (e.g., *C. reinhardtii* with a content of around 2% compared to *C. reinhardtii* grown in limited resource condition with a maximum content of 1.2% as reported by Isanta-Navarro et al. (2024)).
2) Providing excessive P has already been observed to negatively impact cell growth (Cembella et al., 1984; Li et al., 2018). While the experimental P concentration (150 mg L^{-1}) supplied to algal cultures was lower than the average toxic concentration level in chlorophytes (250 mg L^{-1}, Li et al., 2018; Lobakova et al., 2023), P concentration in CW was ≈5.5 times higher than in BG11. Therefore, exposing algae to such a high amount without any prior acclimation could *per se* constitute a growth-hampering factor.
3) Based on the literature, several mechanisms of NH_3/NH_4^+ toxicity have been suggested. i) loss of available nutrients due to cation exchange in the attempt to regulate electrochemical potential (e.g. Mg^{2+}, Ca^{2+}) (Lu et al., 2005). ii) reduced uptake of K^+ due to the competition for K^+ LATS (Coskun et al., 2017). iii) energy loss due to active efflux of the extra ammonium (Collos and Harrison, 2014), iv) detrimental effects on photosynthetic apparatus (Drosou and Pantazis, 2023). The last mechanism is particularly suggested by our data. Indeed, based on PCA analysis (Fig. 6, Fig. S4) photosynthetic pigments weighted more than other factors on the separation between CW and CTR grown cells; in particular, the chlorophyll content of chlorophytes was lower as compared to amounts in CTR algae (Fig. S3). As highlighted by other authors (Collos and Harrison, 2014), reduced N source (NH_3/NH_4^+) affects the photosynthetic apparatus possibly leading to a chlorophyll content reduction; nevertheless, a lower chlorophyll content was not observed in reduced N grown chlorophytes by Giordano et al. (2003); Lau et al. (1997). Once inside the cell, NH_3/NH_4^+ pool favours both the uncoupling of photophosphorylation and the photodamage of PSII due to direct destruction of the manganese cluster in the PSII oxygen-evolving complex (Britto and Kronzucker, 2002; Drath et al., 2008; Markou et al., 2016; Markou and Muylaert, 2016; Zhu et al.,

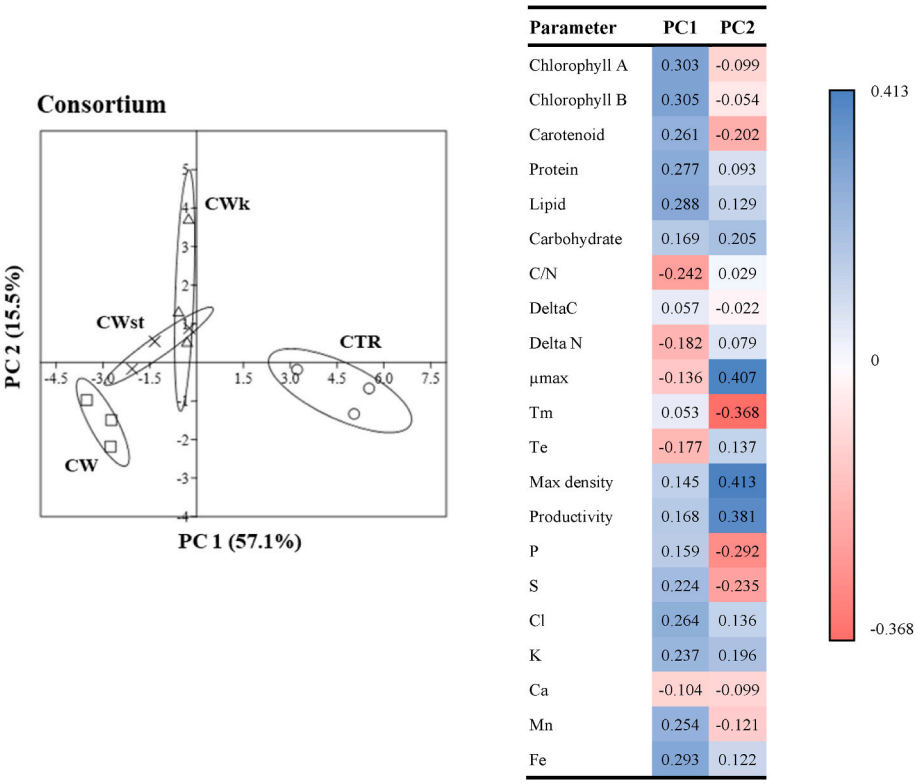


Fig. 6. Principal component analysis of consortium based on cell composition. It is reported the scatter plot, with the percentage of variance explained by each PC, and a heatmap with the loading values of each parameter. Different symbols indicate different experimental conditions: ○ CTR, □ CW, Δ CWk, × CWst.

2000). Toxicity of NH_3/NH_4^+ on the photosynthetic apparatus may have led to a reduction in activity (as observed by Shen et al. (2020)) and thus to a lower maximum number of cells achieved during growth.

Nonetheless, growth rates were not strongly affected by the algal exposure to the synthetic digestate (Tables S1 and 3). The observation could be explained by the lower energy budget (in terms of reducing power) required to assimilate NH_4^+ into amino acids instead of NO_3^- (Norici et al., 2022; Chai et al., 2021; Collos and Harrison, 2014; Andrews et al., 2013). The spared energy could then be allocated to growth (Andrews et al., 2013) and/or cell maintenance (involving repair mechanisms to cope with stresses) (Zhu et al., 2023).

Since algal consortia are cultures resembling more natural conditions in which different species of microorganisms live together as a community, exchange metabolites and contribute to the complete degradation of organic matter and pollutants (Subashchandrabose et al., 2011), they are supposed to be more resilient cultures. Co-cultures of different species can even perform entire metabolic pathways usually absent in single strains (Brenner et al., 2008) thanks to the combination of species-specific reactions. Therefore, based on the results (Table 2, Table S1), an algal consortium was developed consisting of the three best-performing chlorophytes and the euglenophyte. The higher resilience of co-cultured species (consortium) as compared to single species was confirmed (Brenner et al., 2008; Safonova et al., 2004). Although the literature is furnished with natural consortia enriched with bacteria (Gururani et al., 2022), to the best of our knowledge we presented here the first artificial algal consortium developed by selecting suitable algae; this laboratory-based approach may suggest a step forward towards a sustainable digestate remediation.

4.2. Addition of K^+

Since K^+ and NH_4^+ ions are known to compete for K^+ LATS (Coskun et al., 2017)algae may experience K^+ limitation in NH_4^+ rich media. Indeed, the K^+ amount in dry biomass of all species except for *C. reinhardtii* drastically dropped in cells exposed to CW medium (Table S3). The strategy to increase K^+ concentration in CW medium was then tested to favour algal growth. The K^+ enrichment caused a higher K/N ratio in the biomass except for *E. gracilis* where it was constant (Fig. 4b). The finding was consistent with K quotas in the corresponding biomass; N quotas of CWk grown cells not necessarily decreased in dry weight, it also increased in *A. protothecoides* as compared to CW grown cells (Table S3). The effect of a higher K^+ availability on growth was, therefore, species-specific; indeed, *T. obliquus* was the only species showing an improvement in maximum cell density (Table 3). N removal was not affected by the enrichment unless a lower maximum density was achieved (in *C. reinhardtii* and *E. gracilis*) (Figs. 3 and 4a).

These results suggested that i) K^+ enrichment may not be a good strategy to enhance algal growth and to alleviate NH_4^+ toxicity in NH_4^+ rich digestate, and ii) species-specific transporters with different substrate affinity exist. The consortium response did not particularly benefit from the K^+ enrichment (Table 3). Its K/N ratio was not affected by a change in N or K availability (Fig. 4b) confirming a robust behaviour towards environmental changes. Nevertheless, as also observed in *T. obliquus*, a higher chlorophyll concentration in CW K^+ enriched consortium was observed if compared to the content in CW grown consortium (Fig. S3), suggesting that the strategy could ameliorate the toxic effect of reduced N on the photosynthetic apparatus even without a direct impact on biomass productivity.

4.3. P starvation

The strategy to pretreat algal cells with a P starvation period to enhance P luxury uptake and, therefore, increasing the removal of digestate P was not successful (Fig. 5a). Despite a type of luxury uptake called overcompensation, or overshoot, does exist as a fast response to P

starvation and it is already demonstrated in algae (Brown and Shilton, 2014; Cembella et al., 1984), reduced P intracellular concentration, lower growth (both Nt_e and μ_{max}) and even less P remediation in CWst grown algae were always observed as compared to the values in CW and CTR grown algae. Hampered growth was also observed in other research studies carried out on algae (*Micractinium simplicissimum* and *Chlorella regularis*) or yeast (*Saccharomyces cerevisiae*) (Lobakova et al., 2023; Gerasimaite et al., 2014; Li et al., 2018) following luxury uptake: in these micro-organisms a production of cytosolic acid-insoluble PolyP and/or short-chain PolyP was observed; PolyP interfered with protein folding and biomolecules synthesis acting as chaperone (Brown et al., 2004; Vasilieva et al., 2022; Xie and Jakob, 2019) and leading to cell death or suppression of cell duplication (Lobakova et al., 2023). PolyP synthesis is carried out in the cytosol but the molecules are rapidly transported to the vacuole through specific transporters (Gerasimaite et al., 2014) where they also chelate metals (Xie and Jakob, 2019). On the contrary, an overproduction of PolyP due to luxury uptake may uncouple cytosolic production and transportation to vacuoles. Since acquired P cannot be allocated into safe P storage (Lobakova et al., 2023), morphological alteration of the cell structure finally leads to a reduced P intracellular concentration and external P remediation. Indeed, in our data measured in early stationary phase biomass P content in CWst cells was like the one in CW cells (Fig. 5b) and final P removal of CWst cultures was always lower or comparable to one in CW condition (Fig. 5a).

5. Conclusion

Digestate is a tough kind of wastewater to deal with and the high concentrations of ammonium, phosphates and various organic molecules make its remediation quite difficult. Nonetheless, its chemical load is an interesting source of algal nutrients once suitable algal species are selected to tolerate and efficiently use such medium. Adopting a synthetic digestate to grow algae allowed us to simplify experimental constraints and propose a method i) to identify suitable species by screening algae on the basis of growth and remediation performance, ii) to delve into the physiological short-term response of exposed cells and iii) to improve their remediation ability. Indeed, *T. obliquus, C. reinhardtii, A. protothecoides, E. gracilis* were selected. To boost valorisation and remediation of the synthetic digestate addition of K^+ to the medium and pretreatment with P-starvation were applied without success. In particular addition of extra K^+ elicited species-specific modulation of growth suggesting algal K^+ transporters have different affinity and sensibility to NH_4^+ with only *T. obliquus* being favoured. On the other hand, pre-treating algae with P-starvation always led to negative results suggesting that a P overshoot may have led to the synthesis of toxic cytoplasmatic PolyP which damaged the entire cell. Finally, the development and use of an algal consortium made from the score matrix selected species and grown in synthetic digestate led to higher biomass production as compared to the value of single species (up to three times higher), high nutrient removal (94% removal of N) and 1.6 times higher growth rate than in replete standard medium. Compared to monocultures a constructed consortium may represent a robust tool in a remediation process due to short-term modulation of species relative abundance and metabolic activity. Our research on synthetic digestate represents an initial and reproducible approach towards the effective remediation of a real digestate.

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CRediT authorship contribution statement

Mollo Lorenzo: Writing – review & editing, Writing – original draft, Investigation, Data curation, Conceptualization. **Petrucciani Alessandra:** Writing – review & editing, Supervision. **Norici Alessandra:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any financial or personal relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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