Biomass and Bioenergy 211 (2026) 109155

Contents lists available at ScienceDirect

Biomass and Bioenergy

journal homepage: www.elsevier.com/locate/biombioe

Exploring a microalgal consortium-based approach for the remediation of an agrifood-derived digestate

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A B S T R A C T

This study investigates the potential of employing a microalgal consortium to remediate agrifood-derived digestate while simultaneously producing valuable algal biomass. Digestate, a nutrient-rich by-product of anaerobic digestion, poses environmental risks if improperly managed, yet it can serve as an economical nutrient source for microalgal cultivation. The research explored the functioning of a consortium composed of three chlorophytes, *Tetradesmus obliquus*, *Chlamydomonas reinhardtii*, and *Auxenochlorella protothecoides*, through a three-step approach. Initially, a screening step identified a 7% digestate dilution as the lowest concentration that did not inhibit algal growth, enabling physiological adjustments that allowed the cells to acclimate to moderate stress. In the subsequent *optimisation step*, acclimated cells were tested as free-living cultures or immobilised in alginate beads, with or without the addition of the plant growth-promoting bacterium *Azospirillum brasilense*. Immobilisation was found to accelerate the onset of exponential growth, although its benefits varied with the media, while the bacterium's effect was more pronounced on maximum cell density than on growth rate. The final *remediation step* evaluated the consortium's performance in reducing key pollutants under batch cultivation conditions simulating parameters relevant for scale-up. Results demonstrated efficient removal of nitrogen and phosphorus, and a complete removal of heavy metals such as cadmium (Cd), chromium (Cr), and lead (Pb) when algae were immobilised. This study demonstrates that, despite the considerable potential for further optimisation, digestate can be effectively valorised as a nutrient source for selected microalgal consortia. When properly applied, this consortium based remediating systems not only support sustainable digestate management but also enable the production of biomass with promising applications.

1. Introduction

Anaerobic digestion (AD) is an efficient process that uses anaerobic microorganisms to convert solid organic wastes into biogas, which can then be upgraded into biomethane [1–3]. This process also produces a nutrient-rich liquid waste called digestate, which has a chemical composition determined by the characteristics of the digester feedstocks [4,5]. While most of the organic matter in the feedstock is used by bacteria and converted into biogas, a majority of N (nitrogen) and P (phosphorus) are preserved during the anaerobic process and are found in the digestate, reaching values up to 100 g L^{-1} and 10 g L^{-1}, respectively [5]. Therefore, digestate is a valuable source of nutrients that needs to be properly utilised both economically and environmentally. Digestate is often pre-treated by physical (e.g., solid–liquid separation or filtration), chemical (e.g., precipitation, oxidation) or biological (nitrification–denitrification) methods to mitigate toxicity [6,7]. Biological treatments are effective and sustainable options for digestate remediation, particularly for nitrogen removal [8]. However, their performance is often limited by the variability of digestate composition and the presence of inhibitory compounds like ammonia, nitrite, or heavy metals [9,10]. In addition, they may also require external carbon sources, long retention times, and high energy input [11]. Moreover, they can produce undesired by-products and are generally less effective at removing recalcitrant compounds and heavy metals [9,12]. Nonetheless, there is increasing attention to the potential applications of digestate, particularly in cultivating microalgae [3,4,13].

Microalgae are unicellular, photosynthetic organisms that have gained attention for their rapid growth, ability to capture carbon dioxide, and production of valuable biomass [14–17]. However, the cultivation of microalgae is often constrained by high initial investments and by the costs of water and nutrients needed for growth, resulting in an average cost of 10 € kg^{-1} of biomass produced [4,18]. To reduce these costs, it is possible to use nutrient-rich wastewaters as growth media for the algae, potentially lowering the production costs to as little as 5 € kg^{-1} [4,13]. This approach not only reduces the economic burden but also lessens the environmental harm by reducing dependence on synthetic nutrients and preventing adverse effects such as eutrophication and the emission of greenhouse gases [19,20].

This article is part of a special issue entitled: Circular bioeconomy published in Biomass and Bioenergy.

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https://doi.org/10.1016/j.biombioe.2026.109155
Received 25 March 2025; Received in revised form 5 November 2025; Accepted 19 February 2026
Available online 3 March 2026

Digestate could be a great fit for this purpose due to its C, N and P content and the improved bioavailability of these elements resulting from the previous anaerobic digestion process, which transforms nutrients into simple organic molecules (such as acetate), phosphates, and ammonia [21]. However, the tendency of ammonia to evaporate and its known toxicity for microalgae, especially at high NH_3/NH_4^+ concentrations, pose significant challenges for its use [22–26]. To reduce ammonia evaporation, careful control of the pH is needed to keep it below the pK_a (≈9.4) [27], while toxicity can be reduced by diluting the medium [4,5,28] or adding extra carbon [29,30]. The ratio between these two main nutrients must also be carefully balanced to achieve proper algal growth and effective remediation of the medium [31,32].

The selection of the right algal species plays a crucial role in achieving efficient phycoremediation [28]. Additionally, recent findings suggest that using a consortium of microalgae can outperform monocultures in key metrics of productivity, stability, and remediation efficiency [28,33–35]. For instance, Qin et al. [36] reported that a mixed *Chlorella–Scenedesmus* consortium achieved approximately 10 % higher COD and total phosphorus removal from wastewater than *Chlorella* monocultures under identical conditions. More recently, Mollo et al. [37] observed up to an 8.4-fold increase in protein content in a three-species chlorophyte consortium compared to the corresponding monocultures, alongside enhanced biochemical stability under fluctuating conditions. These improvements arise from complementary resource use and from enhanced resilience, whereby dynamic shifts in species composition buffer the community against environmental fluctuations.

The species composition within the consortium plays an important role in determining its performance and efficiency. For example, different species can functionally complement each other by completing metabolic pathways necessary for the degradation of molecules and pollutants within the digestate [38–40]. In addition to microalgal consortia, algae-bacteria consortia are gaining popularity. Bacteria, especially PGPB (Plant Growth Promoting Bacteria), can deliver phytohormones (e.g. IAA) to microalgae, metabolise organic carbon using the oxygen released by algae, and release CO_2 for use in photosynthesis [41–43]. An innovative method for co-cultivating algae and bacteria is through alginate immobilisation [44–48], which keeps both organisms close to each other and encourages communication and metabolite exchange. Despite numerous studies investigating microalgal growth on digestate, research on the immobilised co-cultivation of microalgae with PGPB in digestate-based media is extremely limited. The use of alginate bead immobilisation for simultaneous cultivation of algae and PGPB has shown promise in other wastewater contexts [49,50], yet its potential in agrifood-derived digestate remains largely underexplored.

This article explores the potential of using agrifood-derived digestate as a growth medium for microalgae cultivation and aims to develop a protocol to optimise algal growth while efficiently remediating the digestate. A consortium of three chlorophyta (*Tetradesmus obliquus*, *Chlamydomonas reinhardtii*, and *Auxenochlorella protothecoides*), previously established [35], was used as a biological remediating system. A three-step protocol was adopted to: 1) select the appropriate digestate dilution (*screening step*), 2) optimize algal growth by assessing the growth promotion by *Azospirillum brasilense* as a PGPB bacterium and by the immobilisation on alginate beads (*optimisation step*), 3) evaluate the remediation yield in batch flask cultures with aeration and a 16:8 h light: dark cycle to approximate key environmental variables relevant for scale-up (*remediation step*). Finally, the composition of the algal biomass was analysed to determine the effect of the digestate on the algal consortium and the potential applications of the microalgae.

2. Materials and methods

2.1. Experimental design

A microalgal consortium previously established by co-inoculating an equal cell number of *Auxenochlorella protothecoides* (CCAP 211/8D[1]), *Tetradesmus obliquus* (CCAP 276/3A[1]), and *Chlamydomonas reinhardtii* (RCC125[2]) was used. This specific microalgal consortium was selected based on previous studies [35] in which the three species, initially chosen from the literature for their well-documented tolerance to ammonium and high efficiency in digestate remediation, were identified as the most promising after cultivation in a synthetic digestate rich in ammonium, organic carbon, and phosphates. The combination of these strains showed high stability and nutrient removal efficiency under such conditions.

The consortium was maintained and propagated in the synthetic freshwater BG11 medium, at 20 °C and illuminated with continuous (24 h d^{-1}) white fluorescent lamp (100 μmol photons s^{-1} m^{-2} at the tubes' surface) until the beginning of the experiment. The initial species composition (number of cells) of the consortium was: 62% *A. protothecoides*, 7% *T. obliquus* and 32% *C. reinhardtii*.

Digestate samples were obtained from an anaerobic digester located in Marche region (Italy). The digester was fed with chicken manure, silages and olive mill wastewaters. Before use, digestate was centrifuged at 28,000 g and the supernatant was further filtered at 0.22 μm to remove most of the suspended and dissolved solids, and to remove any microorganisms. Removal of microorganisms was necessary to clearly distinguish the contribution of algae and *A. brasilense* (see 2.1.2) in the culture and avoid potential influential from indigenous microorganisms. Filtration through a 0.22 μm membrane was preferred over autoclaving to sterilize the medium, in order to avoid nutrient precipitation and potential leakage. The characterisation of the digestate is reported in Table 1.

2.1.1. Screening step

The first phase of the experimental design (*screening step*) was carried out on not-acclimated cells using a digestate content of 2, 4, 7, 10, 20, 30, 40 and 50%. Digestate dilutions were carried out in BG11 medium.

Table 1
Characterisation of digestate used to prepare cultivation media. The digestate was obtained from an anaerobic digester fed with silages, chicken manure and olive mill waste waters. Data are expressed as mean ± standard deviation.

Parameter	Concentration
pH	8.8 ± 0.1
Conductivity (mS cm^{-1})	16.6 ± 0.43
Alkalinity (mg$CaCO_3$ L^{-1})	9174 ± 667
sCOD (mgCOD L^{-1})	7352 ± 194
TKN (mg L^{-1})	898 ± 293
N-NH_3 (mg L^{-1})	652 ± 192
N-NO_2 (mg L^{-1})	0.10 ± 0.10
N-NO_3 (mg L^{-1})	1.20 ± 0.20
P_{tot} (mg L^{-1})	163 ± 113
P-PO_4 (mg L^{-1})	100 ± 39
S-SO_4 (mg L^{-1})	1164 ± 21
K (mg L^{-1})	5933 ± 398
Ca (mg L^{-1})	1179 ± 63
Cr (mg L^{-1})	0.60 ± 0.08
Pb (mg L^{-1})	0.03 ± 0.00
Cd (mg L^{-1})	6.84 ± 9.00
Br (mg L^{-1})	9.09 ± 0.50

[1] https://www.ccap.ac.uk/.
[2] https://roscoff-culture-collection.org/.

The *screening step* was set in glass tubes containing 30 mL of different digestate dilutions. The dilution gradient was selected to cover a broad range of digestate concentrations and assess both nutrient availability and potential toxicity. Previous studies have shown that digestate concentrations above 10% can inhibit algal growth due to factors such as high ammonia content, turbidity, and the presence of organic or metallic toxicants [28,51–57]. The upper limit (50%) was included to determine the inhibition threshold, while the lower concentrations (2–10%) allowed us to identify the optimal range supporting growth.

Exponentially growing algal cells from the maintenance cultures were used as inoculum to reach a fixed initial algal concentration of 5×10^5 cells mL^{-1}. The cultures were grown at 20 °C, mixed manually twice per day and were continuously illuminated (24 h d^{-1}) with white fluorescent lamp at an intensity of 100 μmol photons m^{-1} s^{-1} measured at the surface of the tubes. Growth was daily monitored for 10 days where a stationary phase was reached by each condition. Three independent biological replicates were established. A BG11 growing consortium was used as control for this phase.

2.1.2. Optimisation step

The second phase (*optimisation step*) was carried out on cells acclimated for at least 10 generations to the selected digestate dilution. This phase aimed to assess: 1) the effects of algae immobilisation onto alginate beads, 2) the effects of co-inoculating *Azospirillum brasilense* (a N-fixing and IAA-producing bacterium, DSM1690[3]), 3) the differences between digestate dilutions made with tap water (TW) or BG11. Cultures were set up in 250 mL Erlenmeyer flasks filled with 100 mL of BG11 for the control condition, or with the digestate diluted at the concentration selected during the *screening step* (dilutions were carried out using either BG11 or tap water). Exponentially growing algal cells from the acclimated cultures were used as inoculum to reach a fixed initial algal concentration of 5×10^5 cells mL^{-1}. A bacterial cell concentration 10^3 times higher than that of the algae was selected [45], with the bacteria previously cultured and maintained in LB medium [58]. The ratio is required to rapidly colonize the bead matrix, maintain sufficient IAA production throughout the incubation period, and ensure effective nutrient exchange between bacteria and algae. The cultures were grown at 20 °C, mixed manually twice per day and were continuously illuminated (24 h d^{-1}) with white fluorescent lamp at an intensity of 100 μmol photons m^{-1} s^{-1} measured at the surface of the tubes. Growth was daily monitored for 10 days. Three independent biological replicates were established.

Algae immobilisation was carried out as reported by de-Bashan et al. [45]: samples of the consortium and the bacterium were centrifuged at 2000 g for 5 minutes. The cellular pellets were then washed twice with a sterile solution of 0.7% KCl and finally both resuspended in 4 mL of the same solution. The resuspended cells were then mixed together and added with 32 mL of sterile 2% alginate. The solution gently mixed on a shaker for 15 minutes and thus dripped from a sterilised syringe into a 2% $CaCl_2$ solution. The formed beads (with a diameter of about 3 mm and a volume of 15 μL) were left 1 h in the solution and finally were washed in the KCl solution before transferring in the experimental growth medium. An average of 6.7 beads per mL of culture was established. The number of beads per unit of culture volume was determined based on the maximum carrying capacity of each bead, which was assessed at 1.4×10^6 cells per bead. This capacity was calculated by growing immobilised algae in BG11 medium and monitoring when algal cells began to be released into the growth medium. Once the microalgae cells started to be released, the concentration of cells per bead remained constant (thus called maximum carrying capacity). Considering that the average maximum cell density of the consortium in a BG11 medium is around 0.9 to 1×10^7 cells mL^{-1}, the number of beads avoided the reaching of the maximum carrying capacity and the dispersing of cells within the medium.

2.1.3. Remediation step

The third experiment (*remediation step*) was carried out on algae acclimated in the 0.22 μm filtered digestate diluted with tap water and on algae grown in BG11 (control condition). Cultures were set in 500 mL Erlenmeyer flasks filled with 200 mL of growth medium. Immobilised and free cultures were inoculated in the *optimisation step likewise*. Growth was followed daily for a total of 11 days. The cultures were aerated with air at a flow rate of 0.2 L min^{-1}. Growth conditions were 20 °C, 16h:8h light:dark photoperiod, 100 μmol photons s^{-1} m^{-2} (white fluorescent lamp). A 16:8 h light:dark photoperiod was intended to reintroduce a diel cycle and allow the algal consortium to synchronise its physiological processes. Circadian regulation plays a key role in coordinating cell division, nutrient uptake, and biomass composition, thus providing additional insights into the metabolic dynamics of the cultures under non-constant illumination.

2.2. Growth analysis and consortium analysis

Cell density and size were assessed using an automatic cell counter (Casy TT, Innovatis AG, Reutlingen, Germany). Together with cell concentration (cell mL^{-1}), average cell diameter and volume (μm and fL) were also recorded. Cell concentration of immobilised cultures was assessed by collecting a predetermined number of beads with a sterile spoon and then transferring them in a 15 mL plastic tube containing a known volume of 2% $NaHCO_3$ solution. The tubes were transferred to a horizontal shaker and gently agitated for 30 min or until the complete dissolution of the beads. The solubilised samples were analysed with the automatic cell counter to attain cell density inside the beads. The β-function [59], implemented for algal growth studies [35,60–62] was used as non-linear regression model to determine maximum growth rate (μ_{max}), achieved at the inflection point of the curve (T_m), and maximum density (Nt_e), achieved at the end of growth (T_e).

Morphological characteristics and proportion among species were investigated through imaging flow cytometry (IFC) (FlowSight®, Amnis Corp., Seattle, WA) and analysed by INSPIRE software package (Amnis Corp.) [35,62].

2.3. Biochemical characterisation of algal biomass

Samples of algae were collected to characterise the biomass in terms of pigments, proteins, carbohydrates, lipids and elements. Chlorophylls quantification was carried out by extracting them with methanol from the algal pellet. The pigments were then quantified spectrophotometrically [63]) and reported as fg per unit of biovolume (fL). The colorimetric Lowry method was used to quantify proteins in algal samples as reported by Peterson [64]. The combination of protein quantification and FTIR (Fourier Transform Infrared Spectroscopy) analysis was employed to carry out a low-cost and effective semi-quantification of carbohydrates and lipids according to Palmucci et al. [65]. FTIR spectroscopy provides biochemical fingerprints based on the vibrational transitions of chemical bonds and is thus employed to estimate the relative content of macromolecules. FTIR spectra allow for the estimation of the relative proportions of different macromolecular pools within the cell. By quantifying the protein fraction using Lowry analytical method, the relative abundances of the remaining two pools, lipids and carbohydrates, can be inferred. Relative bands of FTIR spectra (acquired through a Tensor 27 FTIR spectrometer, Bruker Optics, Ettlingen, Germany) were deconvolved with the OPUS 6.5 software (Bruker Optics, Ettlingen, Germany) to obtain the band integrals associated with biomass pools according to Giordano et al. [66]. Elemental composition was made according to [67]. 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) was used to obtain C and N

[3] https://www.dsmz.de/.

quotas together with the profile of stable isotopes ($\delta^{13}C$ and $\delta^{15}N$). A Total Reflectance X-Ray Fluorescence (TXRF) spectrometer (S2 Picofox, Bruker AXS Microanalysis GmbH, Berlin, Germany) was used to obtain Cr, Br, Ca, Cd, Cl, Co, Cu, Fe, K, Mn, Ni, P, Pb, S and Zn. Elemental composition was reported as % of the cell dry weight.

2.4. Photosynthetic analysis

The F_v/F_m ratio was used as an indicative parameter to measure the photosynthetic efficiency of the algal consortium. The F_v/F_m was measured using a Dual Pulse Amplitude Modulation (PAM) 100 fluorimeter (Heinz Walz GmbH, Effeltrich, Germany) and the Dual PAM v1.8 software (Walz GmbH, Effeltrich, Germany) was used to obtain data. Samples were collected from algal cultures in exponential and stationary phase and concentrated at 3×10^6 cells mL^{-1} by centrifugation. Analysis was carried out as reported by Baker [68].

2.5. Remediation analysis

The bioremediation capacity of acclimated consortium cultures was evaluated by calculating the removal efficiency during the cultivation period, i.e., the difference between the concentration of the pollutants contained in the diluted digestate at the beginning of the *remediation step* and at the end of algal growth. Concentration of pollutants was also calculated when algae were found in the exponential phase. The pollutants evaluated were COD, macronutrients (N and P), and main ions. The COD was measured following Standard Methods (APHA, 012), whereas the main nutrient species (NH_4, NO_2, NO_3, and PO_4) and other ions (Na, K, Ca, Mg, Cl, and SO_4) were analysed using ion chromatography (Dionex ICS-1000 and 1100).

2.6. Statistical analysis

All experiments were performed in at least 3 independent biological replicates. At least 3 instrumental replicas were carried out for digestate characterisation before and after phycoremediation. Analysis of macromolecules (proteins, pigments and FTIR analysis) was performed in at least three instrumental replicas per biological sample. Data are reported as mean ± standard deviation. Graphpad prism 9.5.0 (GraphPad Software, San Diego, CA, USA) was used to perform statistical analysis. All statistical analysis were performed with a significance level of $\alpha = 0.05$. Asterisks (*) were used in figures to distinguish significantly different groups ($p < 0.05$). 2-way and 3-way ANOVA analysis were carried out depending on the number of factors considered (i.e. growth condition, growth medium, presence of *A. brasilense*, growth phase). Tukey's *post-hoc* test was used to carried out multiple comparison among all experimental conditions.

Principal Component Analysis (PCA) was carried out using Quasar 1.9.1 [69] on: 1) the 1800-800 cm^{-1} range of the FTIR spectra (dependent variable) of algal biomass between different growth condition (independent variables); 2) the consortium IFC morphological data (dependent variable) between different growth condition (independent variable).

3. Results

3.1. Screening step

The growth rates (μ_{max}) of algae exposed to different concentrations of digestate showed significant differences compared to the control condition as well as among the various digestate dilutions ($p < 0.0001$, Fig. 1a). The growth rates ranged from 0.46 ± 0.09 to 0.19 ± 0.07 d^{-1} (Fig. 1a) for algae growing in digestate, while the control (BG11) had a growth rate of 0.45 ± 0.04 d^{-1} (Fig. 1a). The growth rates of the control culture and the cultures grown in digestate were comparable up to digestate concentrations of 7%., but a decline in growth rates was observed with increasing digestate concentrations (Fig. 1a). Similarly, the Nt_e showed the highest cell concentration at 2% digestate concentration (Fig. 1b), while Nt_e decreased up to $6.5 \times 10^5 \pm 1.7 \times 10^4$ cells mL^{-1} with increasing concentration of digestate. The 7% dilution was the highest digestate concentration where a Nt_e significantly higher than the initial inoculum (2.6×10^6 1.2×10^5 cells mL^{-1}) was registered. The different medium composition also affected the inflection point (T_m) of the curve ($p < 0.0001$), thus the time at which μ_{max} was achieved: CTR cultures reported a T_m of 3.02 ± 0.29 d^{-1} while it increased to 5.37 ± 0.21, 6.05 ± 0.61, 6.57 ± 0.27 d^{-1} respectively for the 2%, 4% and 7% digestate conditions (the ones where algal growth was significant). The impact of these parameters on the growth curve is illustrated in Fig. S1.

3.2. Optimisation step

A 7% diluted digestate was selected for the optimisation and remediation steps as it represented the highest concentration that still supported consistent algal growth, with a specific growth rate comparable to the control. At the same time, it marked the lowest digestate concentration showing measurable inhibitory effects, thus allowing the physiological responses of the consortium to be meaningfully assessed under moderate stress. This made the 7% dilution an ideal condition to both test remediation strategies and gain insights into the adaptive mechanisms of the algae. The acclimation of the consortium improved the growth performance as compared to the *screening step* (Fig. 2); indeed, the μ_{max} of the consortium significantly increased from 0.44 ±

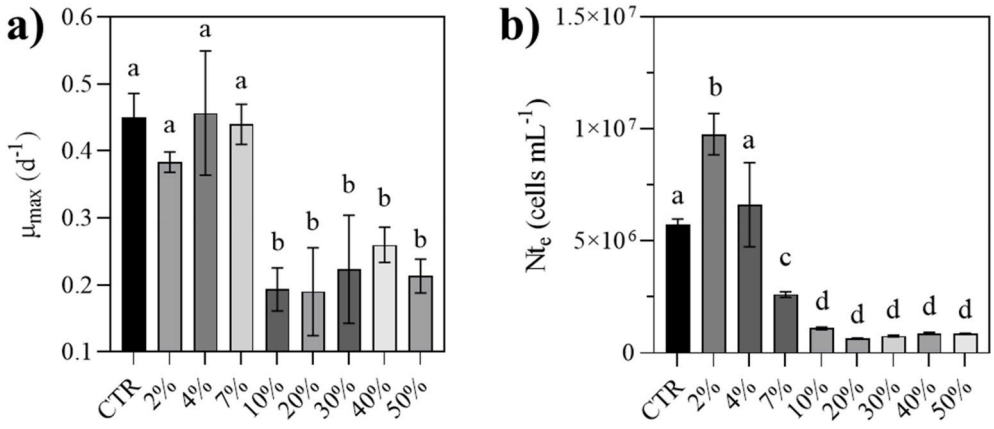

Fig. 1. Growth analysis of algal consortium grown in different percentages of digestate. Growth rates (a) and maximum densities (b) were obtained through non-linear regression of experimental growth curves. Data are expressed as mean ± standard deviation. Letters represent significant differences among the conditions ($p < 0.05$).

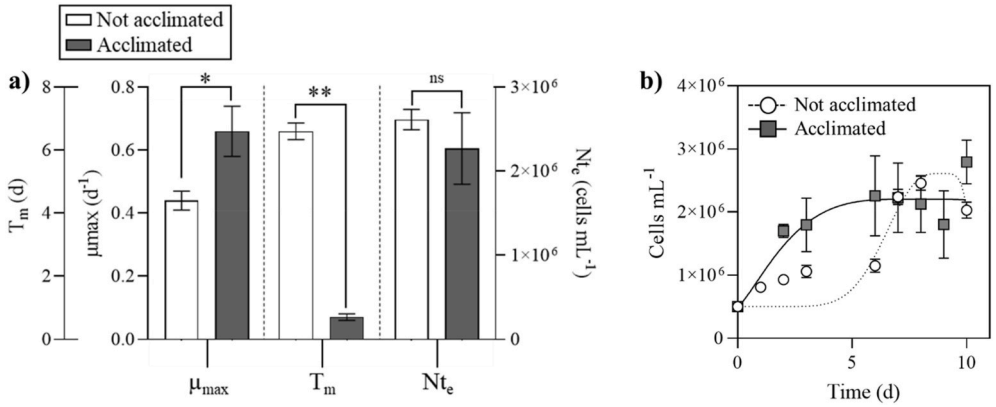

Fig. 2. Comparison of a) growth parameters and b) growth curves of consortium cultures grown in 7% digestate without or with an acclimation phase of at least 10 generations (*screening* and *optimisation step*). Data are expressed as mean ± standard deviation. Asterisks indicate significant differences between acclimated or not acclimated cells (ns $p > 0.05$, *$p < 0.05$, **$p<0.01$, ***$p < 0.001$, ****$p < 0.0001$). For the growth curves experimental data (symbols) and the regression curve (solid and dashed lines) are shown.

0.03 to 0.66 ± 0.08 d^{-1} ($p = 0.0181$) while the T_m decreased from 6.57 ± 0.27 to 0.72 ± 0.11 d^{-1} ($p = 0.0011$). That resulted in a very short lag phase in the acclimated cultures of the *optimisation step*. On the other hand, the Nt_e was not affected by the acclimation ($p = 0.2827$).

Among the different growth conditions tested in the *optimisation step* (free living algae, immobilised algae, immobilised algae with bacteria and digestate diluted with tap water or BG11), the immobilisation of the algae in alginate beads significantly promoted growth as demonstrated by a higher μ_{max} of the consortium (Fig. 3a, $p = 0.0003$) and as observable in the growth curves reported in Fig. 3c, d, 3e. Interaction between the culture medium and the immobilisation factor was significant ($p = 0.0100$) since cultures in tap water diluted digestate (7% TW, Fig. 3a and e) did not benefit from the immobilisation process, at least in terms of growth rate. In addition to the immobilisation, the presence of the PGPB *A. brasilense* (Beads + A.b., Fig. 3a) did not contribute in a significant way to the variation of the growth rate ($p = 0.4555$).

The presence of the bacterium was more significant when Nt_e was assessed ($p = 0.0071$), especially in the 7% TW condition where cellular density dropped to 1.7 × 10^6 cells mL^{-1}, around 70% less than the density of free and immobilised cells grown in the same medium (Fig. 3e). In CTR and 7% medium, the consortium density was not affected by *A. brasilense* (Fig. 3b, c, 3d) proving that the interaction between the bacterium and the type of growth medium could significantly modify the maximum density of algal cultures ($p < 0.0001$).

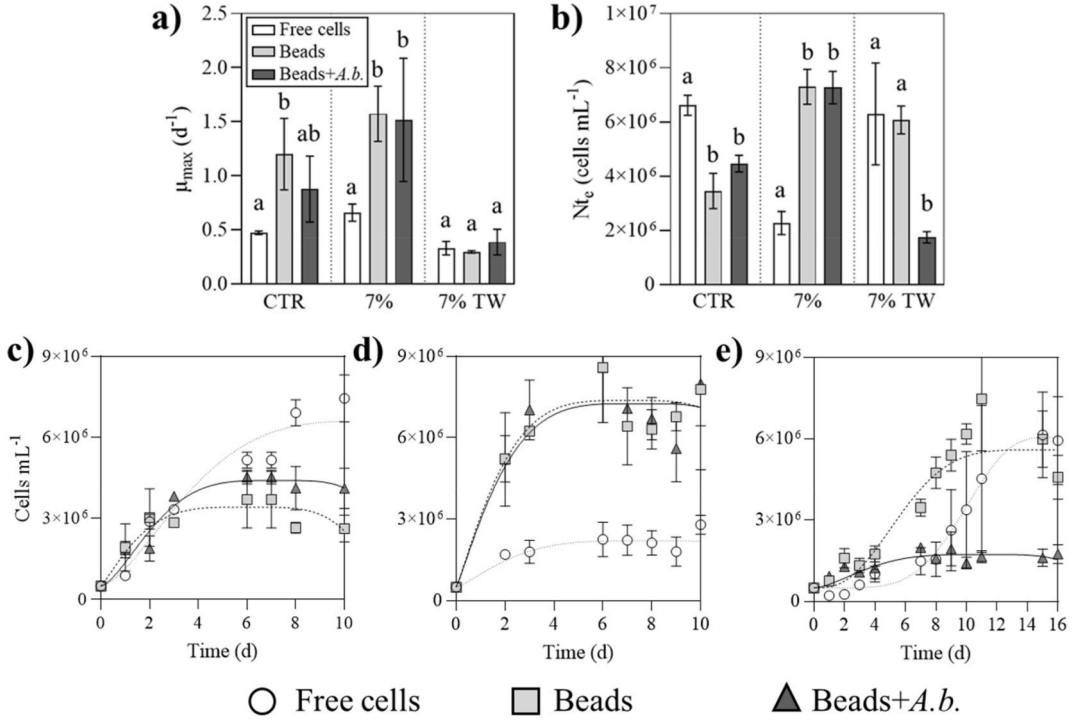

Fig. 3. Growth analysis of algal consortium grown in the BG11 control medium (CTR), 7% digestate (7%) and in 7% tap water diluted digestate (7% TW). Cultures acclimated to their respective growth media were grown as a suspension (free cells), immobilised on alginate beads (beads) or immobilised together with the PGPB *Azospirillum brasilense* on alginate beads (beads + *A.b.*). Growth rates (a) and maximum densities (b) were obtained through non-linear regression of experimental growth curves. Growth curves are reported in the following order: a) CTR, b) 7%, c) 7% TW. Data are expressed as mean ± standard deviation. Letters represent significant differences among the growth conditions within the same growth medium ($p < 0.05$). For the growth curves experimental data (symbols) and the regression curve (solid and dashed lines) are shown.

In terms of species composition, the immobilisation process was the main source of variation among all tested conditions while the addition of *A. brasilense* did not modify the proportion between the three algae in a significant way (Fig. 4). *A. protothecoides* was the most representative species of the consortium in BG11 (CTR) and 7% BG11 diluted digestate (7%), always representing at least half of the cells within the cultures. While the abundance of *C. reinhardtii* increased at the expense of *A. protothecoides* when immobilised in BG11 medium, the proportion of all species remained unchanged in the 7% condition. On the contrary, in the digestate diluted with tap water (7% TW) the immobilisation process favoured *T. obliquus* which reached more than 60% of all the algal cells. Again, *A. protothecoides* was the one who suffered more from the immobilisation.

3.3. Remediation step

3.3.1. Analysis of the microalgae

Consortium growth in the 7% tap water diluted digestate and in BG11 was monitored for a period of 11 days, till the stationary phase was achieved. As shown in Fig. 5a and c, free cells showed the same rate but a higher maximum cell density than the immobilised cells grown in the same medium. Overall, algae as free cells in BG11 reached the highest cell density within all experimental conditions, followed by those as free cells grown in digestate and then by both the immobilised consortia). Notably, in the BG11 beads condition, a certain release of cells into the medium was observed, and, starting from day 6, released cells started to duplicate reaching a cell density of about 2.4×10^6 cells mL^{-1}, similarly to the cell concentration in the beads (Fig. S2a). The release of cells into the medium was not due to the beads reaching their maximum carrying capacity. Assuming that the cells observed in the medium originated directly from the beads, the total cell concentration (i.e., the sum of the cells remaining inside the beads and those released into the medium) did not exceed 5×10^5 cells/bead on day 6, the first day when a significant concentration of cells in the medium was detected (Fig. S2). This value remains well below the maximum carrying capacity of 1.4×10^6 cells/bead. Based on this assumption, approximately $22 \pm 4\%$ of the cells had been released from the beads on day 6.

Species-specific differences in adhesion to the alginate matrix were observed. *A. protothecoides* accounted for more than 74% of the cells released into the medium (Fig. S3b), while representing only about 30% of the cells that remained attached to the beads (Fig. 5d). The other taxa showed higher retention within the alginate matrix. In digestate cultures, *A. protothecoides* constituted a smaller fraction of the total population compared with BG11 cultures, and its abundance decreased following bead degradation and cell release into the medium.

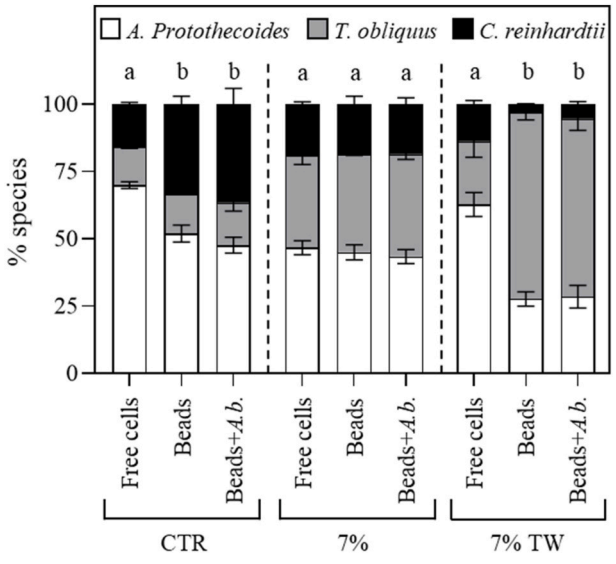

Fig. 4. Relative abundance (%) of the species (*A. protothecoides*, *T. obliquus* and *C. reinhardtii*) in the consortia based on IFC analysis. Data are divided based on the growth medium. Data are expressed as mean ± standard deviation. Letters represent significant differences among the growth conditions within the same growth medium ($p < 0.05$).

For what concern the biomass production (mg mL^{-1}), the highest value was achieved by the consortium grown as free cells in BG11, followed by the immobilised consortium grown in the same medium and the free cells digestate grown consortium. The lowest value was achieved by the immobilised consortium grown in digestate who recorded a maximum density from 60 to 70% lower than the other conditions (Fig. 5b).

The consortium grown in the digestate was predominantly made up of *T. obliquus*, which consistently comprised more than 70% of the population, regardless of its growth phase (Fig. 5d). In contrast, when BG11 was employed as the growth medium, the dominant species shifted to *C. reinhardtii*, followed by *A. protothecoides*, and then *T. obliquus* (Fig. 5d). An exception was observed in the composition of cells released from the BG11 beads, where *A. protothecoides* was the most abundant species, accounting for over 74% of the total cell population (Fig. S2b).

As obtained from the 3-way ANOVA analysis, most of the parameters reported in Table 2 were significantly affected by the growth phase (exponential or stationary), growth medium (BG11 or digestate) and growth condition (free cells or immobilised) and by their interaction (Tab. S1). The immobilisation significantly reduced the photosynthetic efficiency (F_v/F_m) by at least 20% in digestate and 40% in BG11. On the contrary, the growth medium did not affect in a significant way the photosynthetic efficiency when algae were grown as free cells, at least during the exponential phase ($p > 0.9999$).

Similarly, a lower content of chlorophylls in immobilised cells compared to that in free cells was observed (Table 2). Notably, the content of chlorophylls in the immobilised cells was quite low already during the exponential phase, while it did not change significantly during the stationary phase. On the other hand, a significant reduction due to the growth phase was observed for both free cells conditions (Table 2).

The macromolecular composition was highly dependent on the growth phase of the culture when algae were cultivated as free cells (Table 2, Tab. S1): proteins were the largest macromolecular pool in exponential phase; lipids and carbohydrates were more abundant in stationary phase. On the other hand, immobilised cells composition was unaffected by the growth phase. Although the growth medium had a significant impact on algal biomass composition, this effect was far more pronounced in free-cell cultures (Table 2), whereas immobilised cultures displayed largely uniform macromolecular profiles across both media: protein content in digestate-grown culture was on average twice that of BG11-grown culture in both the exponential and stationary phases (Table 2).

Similar to the content of macromolecules, the elemental composition analysis revealed substantial differences between groups (Table 2, Tab. S2). The PCA carried on the elemental composition of the algal biomass described 72.2% of the observed variance (PC1 and PC2 accounted for 40.3 and 31.9% respectively) (Fig. 6). A positive correlation was found between Ca and Cl which grouped closely and whose content was higher when algae were immobilised in beads (independently on the growth medium). With respect to the other elements, the heavy metals Cd, Cr, Pb and Mn were positively correlated with one another (Fig. 7). Their concentrations, reported in Tab. S2, were much higher in immobilised cells than in free cells, reaching values up to 76 times those observed in free cells (as in the case of cadmium).

On the other hand, essential nutrients such as P, K, S and Fe were grouped and their content per unit of biomass was higher during the exponential phase of free cells where algae were in an active metabolic phase (Fig. 6). In addition, the content of these essential nutrients was higher in the free cells grown in BG11 than in the ones grown in

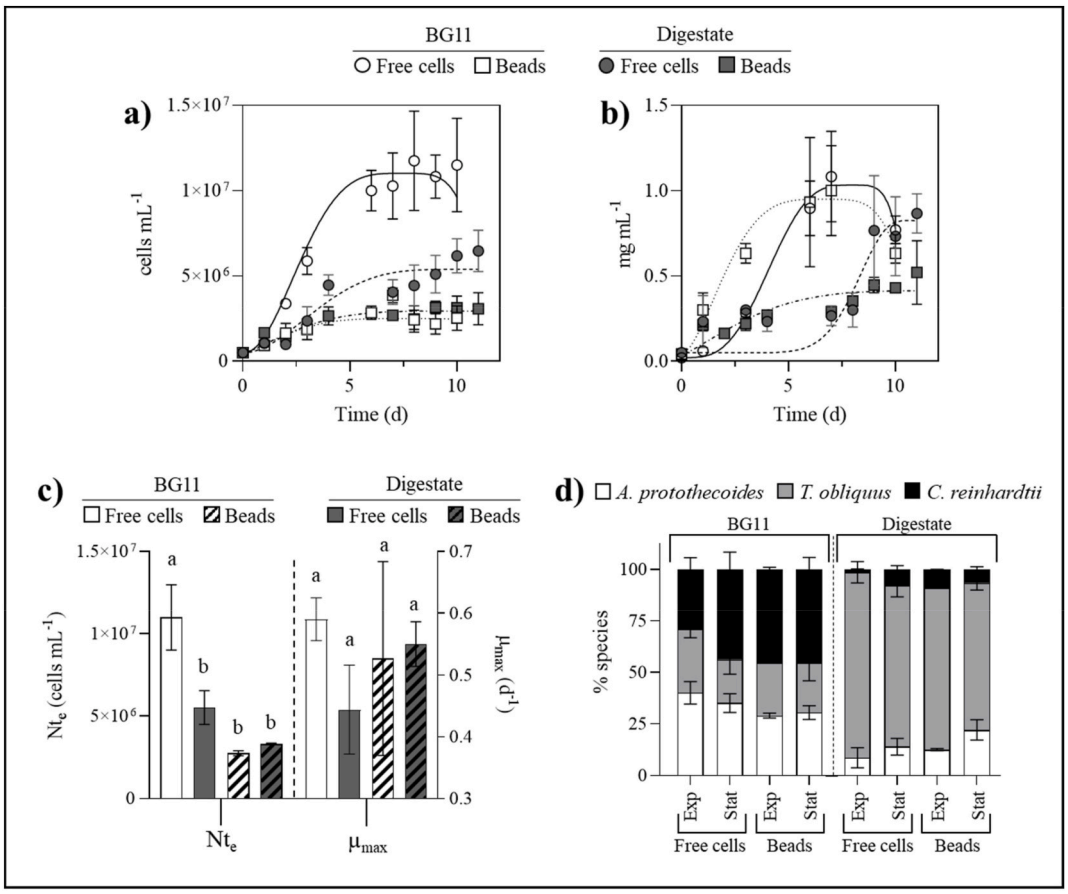

Fig. 5. Growth comparison of consortium cultures grown in 7% tap water diluted digestate (Digestate) and in BG11 as suspended cells (Free cells) or as immobilised cells (Beads). Growth curves expressed as cell number per volume (a) and as dry weight per volume (b) are reported with the experimental data (symbols) and the regression curve (solid and dashed lines). Growth parameters (c) obtained through non-linear regression of experimental cell density growth curves and the consortium species composition during exponential and stationary phases are also reported (d). Data are expressed as mean ± standard deviation. Letters indicate significant differences ($p < 0.05$) between the different experimental conditions.

digestate (Tab. S2). Regarding C and N, their content was mostly unaffected by the type of growth medium and the growth condition (free or immobilised cells), but an important shift in the isotopic composition of N ($\delta_{15}N$) was observed for the digestate grown cultures compared to the algae grown in BG11 (Table 2); indeed, the average value for the BG11 cultures was −13 while the one for the digestate cultures was +34. On the contrary, the C isotopic fractionation was similar between the different growth conditions (Table 2).

3.3.2. *Digestate remediation*

The pH of the medium differed depending on the growth condition of the algal consortium (Fig. 7). When the algae were grown as free cells, the pH decreased to 8.7 on day 7 compared to the initial value (pH = 9.37), then remained practically stable until the end of the experiment, never reaching the initial pH value or the pK_a of ammonia (≈9.4). The pH value of the medium where algae were grown in beads was lower than the other growth condition reaching values of 7.69 ± 0.61.

As it is reported in Table 3, the efficiency of phycoremediation strongly depended on microalgae growth conditions. Noteworthy are the cases of conductivity and Ca within the medium: they both decreased in the free cell condition, while they increased significantly in the bead condition (Table 3).

COD decreased similarly in both growth conditions with removal rates of 40 and 47% when algae were grown as free cells and in beads, respectively. Nitrogen was efficiently removed by free cells: 87% of TKN, 97% of NH_3 and 100% of NO_3 and NO_2 removals were achieved. Phosphorous, also highly present in the digestate, was removed with a yield of 71%. Immobilised algae did not reach the same values; however, the lower remediation of P and N did not prevent the algae from completely remediating heavy metals (Cr, Cd, Pb, Br). This result was not achieved by the free cells, except in the case of lead (Pb), where full remediation was attained in both conditions.

4. Discussion

4.1. *Digestate factors affecting algal growth*

The composition of digestate is highly dependent on the digester feedstock, and it significantly influences algal growth and remediation [4,70]. For instance, while the growth of the present algal consortium was limited and close to zero in the provided digestate at concentrations higher than 7%, the same consortium was able to grow in urban wastewater-derived digestate at concentrations ranging from 10 to 50% [61]. Additionally, while the agrifood digestate in the present work was diluted in BG11 or tap water for the optimisation and remediation steps, the urban wastewater-derived digestate was mixed with primary or secondary effluents from the wastewater treatment plant [61]. However, these findings are consistent with previous studies where digestate concentrations between 1 and 10% were employed [28,51–57]. Among the three algal species used in the present consortium, *A. protothecoides* and *T. obliquus* have been reported in literature for their ability to tolerate and grow in digestate-based media. Krzemińska et al. [71] and Wang et al. [72] demonstrated that *A. protothecoides* could grow in digestate concentrations comparable to those tested in the present study,

Table 2
Photosynthetic efficiency and biomass characterisation of consortia grown in the 7% tap water diluted digestate and in BG11 as free or immobilised cells (Free cells, Beads, respectively) during the *remediation step*. Quantified compounds are expressed as percentage of weight per dry weight. Data are expressed as mean ± standard deviation (n > 3). Different letters indicate a statistically significant difference ($p < 0.05$) among the different sources of variation (growth medium, growth condition, growth phase).

Parameter	Growth phase	BG11				Digestate			
		Free cells		Beads		Free cells		Beads	
F_v/F_m	Exponential	0.59 ± 0.02	b	0.21 ± 0.01	d	0.60 ± 0.01	ab	0.48 ± 0.01	c
	Stationary	0.48 ± 0.05	c	0.22 ± 0.03	d	0.67 ± 0.03	a	0.48 ± 0.02	c
Chlorophyll *a* (%)	Exponential	2.1 ± 0.2	a	0.6 ± 0.0	cde	1.5 ± 0.4	b	0.2 ± 0.0	ce
	Stationary	0.6 ± 0.1	ce	0.6 ± 0.1	de	0.9 ± 0.2	de	0.3 ± 0.1	e
Chlorophyll *b* (%)	Exponential	0.7 ± 0.1	b	0.2 ± 0.0	bc	1.8 ± 0.6	a	0.3 ± 0.1	bc
	Stationary	0.1 ± 0.0	bc	0.2 ± 0.1	bc	0.3 ± 0.1	bc	0.1 ± 0.0	c
Protein (%)	Exponential	34.4 ± 2.7	b	13.2 ± 2.4	c	68.0 ± 13.0	a	15.0 ± 7.4	c
	Stationary	16.1 ± 2.0	c	12.4 ± 0.9	c	41.7 ± 3.3	b	14.8 ± 3.9	c
Lipids/proteins	Exponential	0.2 ± 0.1	bc	0.1 ± 0.0	c	0.1 ± 0.0	c	0.3 ± 0.2	bc
	Stationary	0.8 ± 0.1	a	0.1 ± 0.0	c	0.4 ± 0.1	b	0.3 ± 0.0	b
Carbohydrates/Proteins	Exponential	0.9 ± 0.1	d	2.1 ± 0.3	c	0.7 ± 0.0	d	1.4 ± 0.1	d
	Stationary	6.6 ± 0.6	a	2.4 ± 0.1	c	3.3 ± 0.3	b	2.3 ± 0.2	c
Carbon (%)	Exponential	54.9 ± 2.9	a	50.7 ± 0.9	ab	47.9 ± 3.9	ab	50.9 ± 0.6	ab
	Stationary	55.1 ± 1.1	a	44.7 ± 4.7	bc	49.4 ± 1.3	ab	44.2 ± 2.2	b
Nitrogen (%)	Exponential	11.3 ± 0.0	a	5.9 ± 1.0	c	8.3 ± 0.5	b	5.8 ± 0.5	c
	Stationary	4.0 ± 0.3	d	4.1 ± 0.3	d	4.1 ± 0.4	d	3.9 ± 0.8	d
C/N	Exponential	4.9 ± 0.3	c	8.8 ± 1.2	b	5.8 ± 0.3	c	8.8 ± 0.9	b
	Stationary	13.9 ± 0.6	a	11.1 ± 2.1	ab	12 ± 0.8	ab	11.5 ± 2.2	ab
$\delta^{13}C$	Exponential	−25.4 ± 1.4	cd	−27.5 ± 3.3	d	−24.0 ± 1.3	bcd	−22.4 ± 0.7	bc
	Stationary	−17.0 ± 0.9	a	−22.6 ± 1.0	bc	−24.0 ± 1.0	bc	−20.7 ± 1.7	ab
$\delta^{15}N$	Exponential	−9.5 ± 1.9	c	−14.0 ± 2.5	c	37.6 ± 3.8	a	37.9 ± 2.1	a
	Stationary	−14.5 ± 2.5	c	−14.6 ± 2.3	c	24.6 ± 2.2	b	36.7 ± 2.9	a

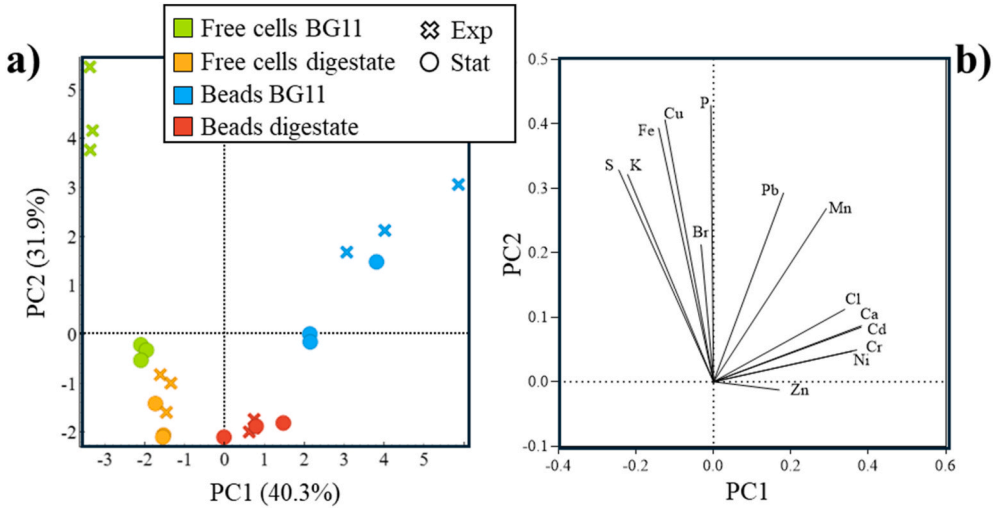

Fig. 6. PCA score plot (a) and loading plot (b) of algal biomass elemental composition. Free cells conditions are reported in green (BG11) and orange (digestate) while beads conditions are reported in blue (BG11) andred (digestate). A cross shape was used to identify algae analysed during the exponential phase, while a circle shape was used for algae in stationary phase. PCA analysis was carried out on 3 samples per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

achieving cell densities at least four times higher than the initial inoculum. Similarly, *T. obliquus*, also included in the present consortium, was shown by Sánchez-Quintero et al. [73] to grow effectively in digestates with dilution factors between 15 and 25 (corresponding to 4–7% v/v digestate), with the best performance obtained at intermediate dilutions. These observations are in good agreement with our results and further confirm the robustness of both *A. protothecoides* and *T. obliquus* in ammonium-rich environments.

Further studies supporting the dilution levels applied in the present work include the cultivation of *Chlorella sorokiniana* in digestate at a dilution factor of 13.5 (≈7.4% v/v digestate), which achieved efficient nutrient removal and stable growth [74]. Higher digestate concentrations can also be tolerated, as reported by Guo et al. [75], where *Chlorella vulgaris* and *Scenedesmus quadricauda* were gradually acclimated to digestate levels ranging from 20% to 60%. However, the ability to use more concentrated digestate strongly depends on the feedstock composition and the applied cultivation conditions, including the adaptation strategy and nutrient balance. In particular, the complete absence of dilution has only been demonstrated under specific experimental configurations, such as when a 0.45 μm microfiltration membrane separated the culture from the digestate, allowing nutrient diffusion while preventing direct exposure to potentially inhibitory compounds [76].

Several factors may have contributed to the inhibition of algal

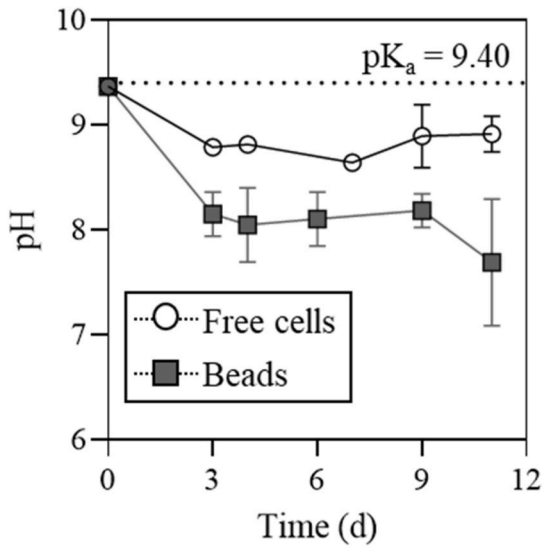

Fig. 7. Change of pH during growth in free cell and bead condition. Values are reported as mean ± standard deviation (n = 3). The pKa of ammonium (9.40 at 20 °C) is reported with a dashed line.

growth by the digestate when concentrations over 7% were used, including light attenuation, NH_3/NH_4^+ concentration, toxic molecules, and recalcitrant organic compounds [72,77]. Notably, even at low digestate concentrations, the medium acquired a very dark colour and increased turbidity (Fig. S4), potentially hindering light penetration. Reduced irradiance could have decreased the photosynthetic activity of algal cells, leading to a lower growth rate and cell density [4,78], although mixotrophic cultures (as in this case due to the presence of organic C) can partially compensate growth reduction caused by the photolimitation [70].

The presence of phenolic compounds (deriving from olive mill wastewaters, one of the feedstocks of the anaerobic digester) and other resistant organic compounds were likely another source of inhibition [79–82] even though some algal species as *T. obliquus* has shown high tolerance towards phenolic compounds [80]. Nonetheless, activity of molecules different from phenols cannot be excluded such as antibiotics and heavy metals released in the chicken manure, and thus in the digestate. Algae have been tested for heavy metals remediation with promising results [86], but excessive concentrations can lead to oxidative stress and accumulation of reactive oxygen species (ROS) intermediates [87]. For example, the digestate Cu and Zn concentrations were much higher than those in BG11 standard medium [88] (14 and 209 μM respectively for Cu and Zn compared to 0.3 and 0.77 μM, Table 1). Despite heavy metals' cellular and molecular interactions being known [83], toxicity thresholds vary with the species, primarily because adsorption mechanisms may establish on the algal cell wall [84,86].

The algal growth was probably impacted by all the cited factors. Moreover, content of carbon (C), nitrogen (N), and phosphorus (P) could also have played an important role. Using COD, TKN, and P_{tot} values (Table 1) as references respectively for C, N and P [52], the digestate showed a high phosphorus content compared with the other elements. The resulting elemental ratio (45C: 5.5N: 1P) deviated markedly from the Redfield ratio (106C: 16N: 1P [31]), which represents the average elemental composition of phytoplankton cells and reflects the stoichiometric balance typically required for optimal algal metabolism and growth.

Table 3
Amount of elements and remediation capacity of the microalgae cultures cultivated in the 7% tap diluted digestate (*remediation step*). Values are expressed as mean ± standard deviation. Letters indicate significant differences among the two growth conditions at the same growth phase ($p < 0.05$).

Parameter	Growth phase	Concentration				Removal efficiency	
		Free cells		Beads		Free cells	Beads
pH	Exponential	8.82 ± 0.03	a	8.05 ± 0.36	a	−0.55 ± 0.03	−1.32 ± 0.36
	Stationary	8.92 ± 0.17	a	7.69 ± 0.61	b	−0.45 ± 0.17	−1.68 ± 0.61
Conductivity (mS cm^{-1})	Exponential	1.56 ± 0.04	a	7.09 ± 0.20	b	4% ± 3%	−335% ± 12%
	Stationary	1.49 ± 0.04	a	6.60 ± 0.08	b	9% ± 3%	−305% ± 5%
Alkalinity (mgCaCO$_3$ L^{-1})	Exponential	495 ± 36	a	205 ± 77	b	44% ± 4%	77% ± 9%
	Stationary	467 ± 99	a	180 ± 16	b	47% ± 11%	80% ± 2%
sCOD (mgCOD L^{-1})	Exponential	367.6 ± 9.7	a	351.4 ± 14.3	a	40% ± 2%	42% ± 2%
	Stationary	368.5 ± 15.6	a	321.8 ± 22.9	b	40% ± 3%	47% ± 4%
TKN (mg L^{-1})	Exponential	35.9 ± 11.7	a	67.0 ± 24.0	a	75% ± 8%	53% ± 17%
	Stationary	19.1 ± 2.6	a	30.9 ± 17.5	a	87% ± 2%	78% ± 12%
N-NH$_3$ (mg L^{-1})	Exponential	25.5 ± 7.5	a	54.1 ± 17.3	b	79% ± 6%	55% ± 14%
	Stationary	3.2 ± 1.3	a	13.0 ± 1.6	a	97% ± 1%	89% ± 1%
N-NO$_2$ (mg L^{-1})	Exponential	0.0 ± 0.0	a	0.0 ± 0.0	a	100% ± 0%	100% ± 0%
	Stationary	0.0 ± 0.0	a	0.0 ± 0.0	a	100% ± 0%	100% ± 0%
N-NO$_3$ (mg L^{-1})	Exponential	0.0 ± 0.0	a	0.0 ± 0.0	a	100% ± 0%	100% ± 0%
	Stationary	0.0 ± 0.0	a	0.0 ± 0.0	a	100% ± 0%	100% ± 0%
Ptot (mg L^{-1})	Exponential	7.2 ± 5.0	a	15.3 ± 1.0	b	58% ± 29%	10% ± 6%
	Stationary	5.0 ± 1.8	a	10.9 ± 3.2	a	71% ± 10%	36% ± 19%
P-PO$_4$ (mg L^{-1})	Exponential	4.4 ± 1.7	a	9.9 ± 0.6	b	59% ± 16%	7% ± 6%
	Stationary	3.0 ± 1.2	a	6.5 ± 1.9	b	72% ± 11%	39% ± 18%
S-SO$_4$ (mg L^{-1})	Exponential	77.6 ± 1.4	a	79.6 ± 0.3	a	5% ± 2%	2% ± 0%
	Stationary	74.1 ± 2.0	a	78.8 ± 0.1	b	9% ± 2%	3% ± 0%
K (mg L^{-1})	Exponential	329.6 ± 22.1	a	283.9 ± 23.6	a	26% ± 5%	36% ± 5%
	Stationary	278.0 ± 5.6	a	292.5 ± 25.5	a	37% ± 1%	34% ± 6%
Ca (mg L^{-1})	Exponential	76.4 ± 4.1	a	547.5 ± 80.3	b	8% ± 5%	−562% ± 97%
	Stationary	60.3 ± 9.4	a	611.6 ± 140.1	b	27% ± 11%	−639% ± 169%
Cr (mg L^{-1})	Exponential	0.03 ± 0.00	a	0.02 ± 0.01	a	41% ± 8%	59% ± 11%
	Stationary	0.02 ± 0.01	a	0.00 ± 0.00	b	57% ± 14%	100% ± 0%
Pb (μg L^{-1})	Exponential	1.50 ± 0.2	a	0.90 ± 0.40	b	40% ± 8%	65% ± 16%
	Stationary	0.00 ± 0.0	a	0.00 ± 0.00	a	100% ± 0%	100% ± 0%
Cd (mg L^{-1})	Exponential	3.8 ± 0.5	a	0.0 ± 0.0	b	26% ± 9%	100% ± 0%
	Stationary	3.2 ± 0.2	a	0.0 ± 0.0	b	38% ± 5%	100% ± 0%
Br (mg L^{-1})	Exponential	0.6 ± 0.0	a	0.1 ± 0.0	b	6% ± 4%	81% ± 7%
	Stationary	0.4 ± 0.0	a	0.0 ± 0.0	b	33% ± 3%	95% ± 6%

Digestate analysis revealed that nitrogen was predominantly in reduced form (NH_3/NH_4^+) (Table 1). Due to the digestate's alkaline pH (8.8), around 20% of the reduced N (346 mg L^{-1}) was present as toxic ammonia (NH_3), while 80% (1387 mg L^{-1}) remained as ammonium (NH_4^+). While NH_4^+ is less harmful, it can still disrupt cellular processes [85,89,90]. However, considering dilution and the pH-buffering capacity of BG11 medium (pH 7.6), the vast majority (98.4%) of N was likely present as NH_4^+.

According to Collos and Harrison [23], chlorophytes thrive at 8 mM NH_4^+, with toxicity emerging above 39 mM. In our screening, NH_4^+ concentrations ranged from 0.9 mM (1% digestate) to 45 mM (50% digestate), therefore digestate concentrations up to 10% (corresponding to 9 mM NH_4^+) fall within the non toxic range. At low concentrations, high cell densities (Fig. 1b) were likely promoted by mixotrophic metabolism thanks to the presence of acetate and reduced nitrogen [70,91,92], which requires less energy than in autotrophic regime supplied with nitrate [22,93,94]. However, growth declined at ≥7% digestate, with reduced μ_{max} from 10% onward (Fig. 1). As reported in the literature [61], The isotopic analysis (Table 2) confirmed the mixotrophic hypothesis and the uptake and assimilation of reduced N; indeed, an $\delta^{15}N$ enrichment was observed, probably associated with the chicken-manure origin of the digestate [95].

Since the same consortium already showed resilience to NH_4^+ [37,61], multifactorial toxicity likely played a role.

4.2. Insights from immobilisation of the algal consortium

Immobilising algae on alginate beads is known to make harvesting easier and more cost-effective [46]. In addition, previous literature has reported an improved algal growth and a greater nutrient remediation thanks to immobilisation [47,96–101]. However, several factors play a crucial role in determining the effectiveness of immobilisation.

First of all, an excessive number of beads may limit light penetration, thus affecting the photosynthetic performance of the cells [102]. However, in the present work, growth reduction was not observed in immobilised cells grown in 7% digestate (Fig. 3b and d), maybe due to the immobilisation within the alginate matrix which promoted a mixotrophic metabolism through enhance nutrient uptake, as also suggested by Xia and Murphy [70].

Unexpectedly, the addition of the bacterium *A. brasilense* did not increase algal growth rate or cell density (Fig. 3a and b). *A. brasilense* is a well-documented producer of indole-3-acetic acid [45,103] and results are in contrast to previous reports [47,48,104–106] showing stimulation of *T. obliquus* [104] and *C. reinhardtii* by this bacterium [105]. In our opinion, a plausible explanation is that suboptimal conditions compromised bacterial viability or IAA biosynthesis: residual disinfectants in tap water (e.g., chlorine), the absence of buffering salts and trace elements supplied by BG11, and the elevated ammonia and heavy-metal concentrations in the digestate may each have inhibited *A. brasilense*.

Algal growth and nutrient removal depend on the support used to immobilise algae (e.g. alginate, carrageenan, chitosan, polyethylene, etc.) and the immobilised species [107–109]. The IFC analysis (Fig. 4) revealed that two species, *T. obliquus* in 7% TW cultures and *C. reinhardtii* in CTR cultures, significantly benefited in terms of abundance from the immobilisation process compared to their respective free cell growth condition. It's interesting to note that there was no change in composition in 7% consortium cultures (digestate diluted with BG11, Fig. 4). A possible reason for the higher *T. obliquus* abundance could be its higher surface to volume ratio as compared to the other species, allowing a higher nutrient uptake, especially in nutrient-poor conditions [60]. Similar findings regarding species composition were obtained from the analysis of the microalgal consortium during the *remediation step*, where *C. reinhardtii* was found to be the most abundant species in BG11 cultures, while *T. obliquus* dominated in digestate cultures (Fig. 5d).

Moreover, maximum cell density achieved by immobilised cells during the *remediation step* was lower than that found during the *optimisation step* (Fig. 3b). One possible explanation for this difference is the integrity of the beads. During the experiment, the beads were subjected to airflow, which was absent in the *optimisation step*. According to literature, intense aeration can lead to losses in the beads' polymeric structure [110], which was [111] confirmed by direct observation of the bead structure (data not shown) and spectroscopic analysis of algal biomass and media (Table 2, Fig. S3, Fig. 6, Table 3). After bead dissolution, high levels of calcium (Ca) and chloride (Cl) were found in the algal biomass, despite washing. FTIR confirmed Ca as calcium carbonate ($CaCO_3$) with a peak at ~1420 cm^{-1} [112]. These elements were absent in free cells (Fig. S3). Their presence is linked to the immobilisation process, where $CaCl_2$ enables monomer crosslinking. [113]. Even the medium was enriched in Ca, further proving the deterioration of the beads (Table 3).

The deterioration of beads is a known problem [114,115], and it could have left debris on the cells. The presence of bead debris on and inside the cells has also been observed by Vasilieva et al. [96] in their study on chitosan-immobilised algae. It can be inferred that a similar phenomenon occurred. However, in their case, the algae were not adversely affected by the presence of these particles, unlike in our situation. Another indication of bead deterioration emerges from the biomass composition data (Table 3): bead cultures displayed elevated carbohydrate-to-protein ratios relative to free-cell cultures even during exponential growth, when protein accumulation is normally favoured. While residual alginate fragments may have adhered to the cells and partially inflated the carbohydrate measurements, we cannot rule out a genuine metabolic shift toward carbon storage in the absence of targeted analyses.

Supporting the hypothesis of an airflow-causing deterioration, algal biomass was enriched in heavy metals (specifically Cr, Cd, and Br) as compared to free cell biomass, probably derived from alginate fragments attached to the algal cells. Indeed, as reported in the literature, the carboxylic, hydroxyl, and other functional groups of the alginate can interact with metals [116,117]. Finally, an inefficient dissolution of the beads was excluded since FTIR biomass analysis of not-bubbled immobilised algae did not report the $CaCO_3$ peak at 1420 cm^{-1} (Fig. S3). As reported in the literature, bead integrity can be improved either by increasing the alginate concentration from 4% to 10% [108,115] or by reducing the airflow rate, as suggested by Lau et al. [118] and Pane et al., [119]. Evidence from studies using orbital shakers supports this approach, showing that gentle mixing does not cause bead degradation [114,120].

Bead degradation and heavy metal accumulation in digestate-grown algae may increase oxidative stress and toxicity, limiting growth, cell density [83,87], and photosynthetic efficiency (F_v/F_m). Immobilised cells showed significantly lower F_v/F_m than free cells in both BG11 and digestate media (Table 2). Since free cells had similar F_v/F_m across media, the medium itself likely wasn't the cause. This is unexpected, as immobilised cells often show equal or better efficiency [97,98]. Alginate fragments from matrix breakdown may have blocked light and nutrients, reducing F_v/F_m [46].

4.3. Insights from the remediation step

4.3.1. Digestate remediation performance

Under the free-cell condition, the results were consistent with those reported in several other phycoremediation studies, reaching an algal biomass density of 1.13 ± 0.08 g L^{-1} at the stationary phase (Fig. 5b) and achieving removal efficiencies of 87% for TKN and 71% for total phosphorus [121]. These values confirm the high potential of microalgae for nutrient removal from digestate-based media, even in the absence of immobilisation supports.

Further evidence supporting the effectiveness of the dilution strategy adopted in the present work is provided by Palikrousis et al. [74], who reported nitrogen and phosphorus removals comparable to those

obtained here, together with slightly higher biomass yields (≈1.5 mg mL^{-1}). Interestingly, their results showed that CO_2 supplementation not only enhanced algal productivity but also improved nutrient removal performance, likely by mitigating the inhibitory effect of ammonium and promoting a more balanced carbon–nitrogen metabolism. Comparable outcomes were also observed by Sánchez-Quintero et al. [73], who achieved similar nutrient removal efficiencies starting from nitrogen concentrations of the same order of magnitude as those tested in this study. However, their findings indicated that COD removal did not exceed 50%, in line with our observations, confirming that organic carbon degradation tends to be less efficient than nitrogen and phosphorus removal under comparable conditions.

In our experiments, COD removal was indeed markedly lower than that of the main nutrients, and the final concentration still exceeded the legal discharge limit of 125 mg L^{-1}, as established by the EU Urban Wastewater Treatment Directive [122,123]. Remaining within biological approaches, the integration of bacteria or fungi into algal consortia could potentially enhance the overall remediation process by improving organic carbon degradation [111,124,125].

The limited COD removal observed in this study may also be related to the chemical nature of the organic matter present in the digestate. The release of extracellular polymeric substances (EPS) by microalgae [126,127] and the presence of recalcitrant organic compounds, such as phenolic derivatives typically found in olive mill wastewater (OMWW) [128,129], could have contributed to the persistence of organic carbon in the medium. This interpretation is supported by the isotopic composition of the biomass. The measured $\delta^{13}C$ values deviated slightly from the control (Table 2), suggesting limited incorporation of carbon from the digestate. In contrast, Chieti et al. [61] reported a stronger shift toward heavier $\delta^{13}C$ values when the same consortium was grown in urban wastewaters, where the isotopic signature of the biomass closely reflected that of the carbon-rich medium. Since wastewater carbon is generally enriched in the heavier isotope due to its animal-derived origin, the weaker enrichment observed here indicates that only a fraction of the digestate carbon pool was metabolically assimilated by the microalgae.

Immobilised cells, on the other hand, proved particularly effective in removing heavy metals from the medium. These results are consistent with previous observations (see Section 4.2) and further confirm the well-known ability of alginate to adsorb and retain substantial quantities of pollutants from aqueous matrices. However, the partial degradation of alginate beads and the adhesion of residues to the microalgal cell walls, as previously reported, may explain the higher heavy-metal content detected by TXRF (Tab. S2).

The deterioration of alginate beads and the potential release of adsorbed metals and other ions warrant careful consideration. Although partial bead breakdown was observed, analysis of the culture medium at the end of the experiment revealed no increase in dissolved heavy-metal concentrations: Cd, Cr, and Pb remained fully removed. Conversely, increases in conductivity and Ca^{2+} levels confirmed bead degradation. These results suggest that, under the tested conditions, metal–alginate complexes remained bound to the biomass rather than being released back into the liquid phase.

Despite most nitrogen being present in soluble form (the pH values of bead cultures were below the pKa; Fig. 7, [27,130]), airflow dynamics and reduced nitrogen uptake by algae, particularly for immobilised cells, may have led to nitrogen losses to the atmosphere. Depending on the metabolic activity and aeration rate, both ammonia volatilisation and assimilation can be stimulated [131,132]. However, reduced metabolic activity alone cannot explain the nitrogen loss observed in free-cell cultures, as most biochemical parameters (Table 2) were comparable to those of BG11 controls. In addition to the presence of heavy metals and other pollutants, a lower nitrogen availability could also account for the reduced μ_{max} and Nt_e values observed in free-cell digestate cultures compared with BG11 (Fig. 5).

Therefore, nitrogen loss was most likely attributable. to airflow-induced ammonia volatilisation. The results suggest that carefully controlling airflow, pH, and the NH_4^+/NH_3 equilibrium could effectively reduce nitrogen losses. Moreover, CO_2-enriched aeration could provide an additional carbon source for free cells, thereby enhancing nitrogen assimilation and minimising volatilisation losses [133].

4.3.2. Algal biomass composition and physiological responses

Regarding algal biomass, the consortium showed distinct physiological and structural responses depending on the cultivation conditions. The reduction in maximum cell density (Nt_e) compared with the optimisation step can be attributed to the different operational parameters applied during the remediation experiments.

Species-specific behaviour was also evident, both in adhesion to the alginate, since some species adhere more strongly to calcium, alginate matrices than others [134,135], and in the relative abundance of taxa across growth phases. Slight shifts in species composition occurred as the consortium moved from the exponential to the stationary phase, a relevant aspect for biomass valorisation where a consistent product is desirable [15,136], though such variability is expected under real wastewater treatment conditions [137,138].

Under the experimental growth conditions (pH $\leq$ pK_a of ammonia, 9.25, Fig. 7), nitrogen was mostly present as ammonium (NH_4^+), which is readily assimilated by microalgae and accounted for the higher protein content in free-cell cultures grown in digestate, up to 68% DW during exponential growth, compared with BG11 controls (Table 3).

However, pH values approaching 9.4 in free-cell systems may have partially shifted the NH_4^+/NH_3 equilibrium toward un-ionised ammonia, increasing its volatility and potential toxicity [130]. While continuous aeration likely supplied CO_2 that helped buffer pH and mitigate ammonia stress, it may also have favoured nitrogen losses through volatilisation.

In immobilised cultures, lower pH values and slower growth likely reduced ammonia volatilisation but also limited nitrogen assimilation. The lower protein content observed in these samples suggests a metabolic reallocation toward carbohydrates and lipids rather than true nitrogen limitation, as nitrogen remained available during the exponential phase (Table 2). Residual alginate fragments adhering to cells after bead dissolution may also have contributed to the apparent increase in carbohydrates.

Ammonia dynamics also affected pigment synthesis and photosynthetic efficiency. Immobilised cultures showed a marked decrease in chlorophyll content (Table 2) and lower F_v/F_m ratios, indicating stress on the photosynthetic apparatus. In contrast, free-cell cultures maintained stable. F_v/F_m values, even exceeding those reported by Mollo et al. [60], suggesting effective tolerance to moderate ammonium levels, possibly supported by CO_2 from aeration. The decline in chlorophyll in immobilised samples, contrary to the expected self-shading effect [119], may be linked to nutrient and gas diffusion limitations within the beads rather than to mixotrophic metabolism [61]. The overall lower pigment content and photosynthetic efficiency in BG11 immobilised samples (Table 3) confirm that the immobilisation process and digestate composition jointly constrained pigment biosynthesis and photosynthetic activity.

5. Conclusion

This study demonstrates that digestate is a potential source of nutrients and can be valorised by selected microalgal consortia. At a 7% dilution of digestate in water, the consortium achieved robust growth and high removal efficiencies for nitrogen and phosphorus, and immobilised algae facilitated near-complete removal of heavy metals (Cr, Cd, Pb) from the medium.

Alginate bead immobilisation proved beneficial for accelerating culture establishment (shortening the lag phase) and enhancing heavy metal sequestration when maintaining bead integrity during operation. The consortium's species composition and biochemical profile shifted

adaptively across different media and growth conditions, underscoring the resilience conferred by multi-species cultivation. Overall, our findings highlight the potential of microalgal consortia as a waste-to-resource platform for digestate treatment, capable of mitigating environmental risks while generating biomass with potential value.

CRediT authorship contribution statement

Lorenzo Mollo: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Alessandra Petrucciani:** Writing – review & editing, Supervision, Methodology. **Alessandra Norici:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors thank Enereco SpA for the financial support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biombioe.2026.109155.

Data availability

The materials and data that support the findings of this study are available from the corresponding author upon reasonable request.

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