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Vitamin B_{12} bioaccumulation in *Chlorella vulgaris* grown on food waste-derived anaerobic digestate

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ABSTRACT

Anaerobic digestion plays a pivotal role in the modern circular economy, as it offers a sustainable solution for converting organic waste into biogas (methane). It also results in a nutrient-rich liquid stream, referred to as digestate. This digestate is extensively applied to agricultural land as fertilizer due to its high macronutrient (N, P) content, but the bioactive micronutrients it contains and their significance for downstream applications remain largely unknown. Here, we investigate whether digestate generated from a vitamin B_{12}-deficient substrate (fruit and vegetable waste) can be enriched in this vitamin through anaerobic digestion, and explore the capability of the microalga *Chlorella vulgaris* to grow in this medium and bioaccumulate B_{12}. Our findings uncover, for the first time, that substantial amounts of B_{12} are synthesized during anaerobic digestion, and that *C. vulgaris* can effectively be enriched with this vitamin when grown in the digestate (10.6 μg $B_{12} \cdot g^{-1}$ dry weight). Additionally, we identified that pH-induced ammonia toxicity was the main inhibitor when growing microalgae in the digestate, which allowed us to significantly enhance productivity at lab- and pilot-scale through pH control. The case of B_{12} synthesis in digestate and accumulation in microalgae highlights the potential for enhancing the value of these waste streams through the identification and utilization of bioactive compounds.

1. Introduction

Anaerobic digestion is an important organic waste treatment technology and is considered a key enabler for the implementation of a truly circular economy [1]. This technology can process a variety of inputs, such as food and agricultural waste, municipal wastewater treatment sludge, and animal slurry [2–5], while producing biogas (methane) and resulting in a liquid digestate. The latter is a nutrient-rich output, consisting of slow-degrading organic substances, ammonium, phosphorus salts and other minerals [6].

Although biogas as an energy carrier is the main product of anaerobic digestion [7], generation of value from the digestate stream could provide additional benefits. If recovered, the significant nutrient content of the digestate is an important resource for the circular economy. Along with conventional practices such as agricultural application [8], using digestate as a nutrient source for microalgal cultivation can offer significant advantages in terms of both wastewater treatment and the production of high-value biomass [9–11]. Photosynthetic microalgae

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synthesize and/or accumulate several valuable metabolites that can be used in a variety of applications in food, feed, nutraceuticals and as biostimulants [12]. Digestate-grown microalgal biomass has been extensively studied as a potential feedstock for biofuel production and, more recently, as animal feed [13,14]. However, growing algae in digestate can be challenging. For example, toxicity from free ammonia, which necessitates dilution of the digestate, can result in low abundance of phosphate and other nutrients that ultimately limit productivity [15]. In addition, replicating laboratory results at scales that are relevant to industry remains a significant bottleneck [16].

Identifying strategies to enhance the nutritional value of algal biomass is equally important to increase derived value. Digestate is usually approached by researchers solely as a macronutrient source; however, hundreds of bioactive metabolites have been shown to be detectable in digestates (e.g., vitamins, hormones, humic acids, amino acids and more) [17,18]. These metabolites are produced by the highly complex microbial consortia that populate anaerobic digesters and degrade organic waste [19]. In agriculture, it has been proposed that the antimicrobial and antifungal properties of such compounds can promote resistance against soil pathogens [18]. In algal cultivation, the use of digestate represents an opportunity for the enrichment of microalgae with vitamins and minerals, increasing the nutritional value of the biomass compared to growth on conventional media [20].

However, knowledge of bioactive micronutrient transfer from digestate to microalgae is still limited. Of particular interest is the fact that high levels of vitamin B_{12} (cobalamin, herein B_{12}) are often present in fermentation products due to *de novo* synthesis by anaerobic bacteria involved in the acidogenesis step of anaerobic digestion [21–23]. Humans and other animals require this molecule as a cofactor for methionine synthase, the key enzyme of central C1 metabolism, and for methylmalonyl CoA reductase involved in odd-chain fatty acid synthesis. Plants do not contain B_{12} since they neither produce it nor require it metabolically, so strict vegetarians are at risk of deficiency, as are the elderly where uptake mechanisms can be impaired [24]. B_{12} deficiency is linked to megaloblastic anaemia and peripheral neuropathy disorders in humans, as well as cardiovascular disease and impaired cognitive function [25]. Although microalgae cannot synthesize B_{12}, many are able to take it up from their environment, either through supplementation to the growth medium or by mutualistic interaction with B_{12}-producing bacteria [26,27]. Therefore, growing microalgae in anaerobic digestate offers the potential for recovering a high-value component that would otherwise be supplemented exogenously at a high cost. Demonstrating that algae grown in digestate can achieve enhanced nutritional content is an important step in the valorisation of this stream as a microalgae medium. This is particularly timely when considering the increasing prevalence of plant-based products in food and feed applications [28].

In this work, we investigate whether fruit and vegetable waste, which like all plants lack B_{12}, can be enriched with this vitamin through anaerobic digestion. If so, we assess the capacity of the microalga *C. vulgaris* grown on the digestate to uptake and become enriched with this vitamin. In addition to analysing the biochemical properties of *C. vulgaris* grown in digestate, we explore ways to optimize algal productivity at pilot-scale, under natural conditions in an outdoor facility.

2. Materials and methods

2.1. Anaerobic digester operation

Fruit and vegetable waste (consisting of 10 kg mango, 15 kg banana, 10 kg melon and 5 kg leafy vegetables which reflect composition of waste from markets in subtropical regions) were homogenized, blended, analysed for chemical oxygen demand (COD) and total solids (TS) using standard methods, and used to feed anaerobic digestion reactors (composition in Table 1) [29]. Two continuous stirred 5 L stainless steel anaerobic digesters equipped with heating/insulating jackets (Lobster Max, Anaero Technology, UK) were inoculated with a starter culture sourced from a food waste anaerobic digestion plant in the southwest of the UK and fed with the blended fruit and vegetable waste. The anaerobic digestion reactors were operated continuously for a total of 11 weeks at 25 °C and 35 °C with a hydraulic retention time (HRT) of 35 days and an organic loading rate (OLR) of 3 g volatile solids (VS) $\cdot L^{-1} \cdot d^{-1}$. The biogas production rate was measured on an hourly basis throughout the digestion process (Gas flowmeter, Anaero Technology, UK) and biogas was monitored in terms of CH_4, CO_2, O_2, H_2, H_2S and N_2 using a gas analyser (GA5000, Geotech, the Netherlands) (Table S1).

Table 1
Physicochemical composition of the fruit and vegetable waste feed and the digestates. The fruit and vegetable waste feed was analysed for total solids (TS) and chemical oxygen demand (COD), and digestates collected at two different time periods (0–4 and 4–7 weeks post-inoculation; AD_1: 0–4 weeks, AD_2: 4–7 weeks) were analysed for TSS, COD, ammonium nitrogen (NH_4^+) and phosphorus as orthophosphate (PO_4^{-3}).

Sample identifier	Weeks after inoculation	Components				
		Solids ($g \cdot L^{-1}$)	COD ($g \cdot L^{-1}$)	NH_4^+ (g $N \cdot L^{-1}$)	PO_4^{-3} (mg $P \cdot L^{-1}$)	pH
Feed	–	149.8[a]	182.1	ND[c]	ND[c]	5.4
AD_1	0–4	7.9[b]	22.1	3.6	88.2	7.8
AD_2	4–7	4.4[b]	11.6	1.9	41.2	7.7

[a] Solids were quantified as Total Solids (TS).
[b] Solids were quantified as Total Suspended Solids (TSS).
[c] ND: not determined, nitrogen and phosphorus in fruit and vegetable waste were largely embedded into non-hydrolysed solids.

The liquid anaerobic digestate effluent produced was collected weekly, stored at 2 °C, and analysed at the end of the anaerobic digestion operation for pH, ammonium nitrogen (NH_4^+), and nitrate nitrogen (NO_3^-) using an electrochemical probe (HQ4100, Hach, USA), and chemical oxygen demand (COD), total suspended solids (TSS) and phosphorus as orthophosphate (PO_4^{-3}) using standard methods [29]. The digestate was collected throughout the process and pooled in two different batches, labelled AD_1 and AD_2 (no significant differences were found between digestates produced at 25 °C or 35 °C). AD_1 consists of all digestate harvested between 0 and 4 weeks post-inoculation, and AD_2 contains digestate from 4 to 7 weeks. Before being used for the cultivation of microalgae, the digestate underwent HTST pasteurization (72 °C for 30 s) for removal of potential pathogens [30] and centrifuged (5000 $\times g$ for 5 min) for removal of excess particulates that cause turbidity, which could otherwise hinder the photosynthetic growth. In addition to samples from the anaerobic digesters of this study (AD_1, AD_2), samples (AD_3, AD_4, AD_5) from independent UK-based commercial anaerobic digesters treating food-waste with category 3 animal by-products were also analysed (for confidentiality reasons related to commercial sensitivity, the names of the industrial partners are not disclosed in this study).

2.2. Microalgae cultivation

2.2.1. Preliminary lab-scale experiments

Non-axenic *C. vulgaris* CCAP 211/52 cultures (Culture Collection of Algae and Protozoa, Oban, Scotland) were grown in TAP (Tris Acetate Phosphate [31]). Cells from a 3-day culture (10^7 cells$\cdot mL^{-1}$) were used to inoculate 1 mL of medium made with different amounts of anaerobic digestate (AD_1) in 24-well plates. The well-plates were placed in an incubator (Infors HT Multitron incubator, Basel, Switzerland), rotated at 120 rpm and illuminated under a photoperiod of 16 h light/8 h darkness with LED lamps and a light intensity of ~80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (SKL 215 PAR 'Quantum' sensor Skye Instruments, UK), at a constant temperature of 25 °C. Cell growth was measured daily as optical density OD_{730} with a ClarioStar plate reader (BMG Labtech, Ortenberg, Germany).

2.2.2. Pilot-scale cultivation in horizontal photobioreactors

C. vulgaris starter cultures of 10 mL were gradually scaled up through consecutive subculturing cycles (5–10× increase in volume in each cycle) in the laboratory to provide inoculum for cultures of 10 L. After acclimation for 7 days in the outdoor Algal Innovation Centre glasshouse (Cambridge, UK), these were used to establish 6 × 10 L cultures in plastic bags with aeration for 7 days. Finally, the bag cultures were used as inoculum (20 % *v*/v, resulting in an initial cell density of 2–3·10^6 cells·mL^{-1}) for 2 × 150 L horizontal tubular photobioreactors (Greenskill Environmental Technology, UK). In addition to natural light, supplemental LED lighting (Philips Master EM LED T8 Tube G13 4 ft., 36 W) was used to provide a minimum photoperiod of 12 h light and ensure consistency throughout the growth cycles. Cultures were aerated by stirring rotating paddles and no additional CO_2 was supplemented. The photobioreactors were operated in 13-day semi-batch cycles, harvesting 80–90 % of the culture and adding an equivalent volume of fresh media after each cycle to return to an initial cell density of 3·10^6 cells·mL^{-1}. Two different sets of experiments were carried out: 3 cultivation cycles using TAP media between 31 August–7 October 2021, and 1 cycle using 2.75 % (*v*/v) digestate between 7 and 20 October 2021. Both media had an initial NH_4^+ concentration of ~100 mg N·L^{-1}. Microscopic observation of the culture (Rebel hybrid microscope, ECHO, USA) and measurement of cell density (Z1 particle counter, Beckman Coulter, USA) were carried out daily to assess cell growth. Abiotic parameters were also measured daily. pH, dissolved oxygen (DO) and NH_4^+ were measured with a portable probe (HQ4100, Hach, USA), and PO_4^{3-} was measured as previously described (Section 2.1). Ambient light and temperature in the glasshouse were also monitored during the experiments (WS1 sensor, Ubibot, UK).

The harvesting process consisted of a dewatering step using membrane filtration (MH1 single membrane, Membranology, UK), which produced a biomass concentrate, followed by a centrifugation step (Avanti J-26, Beckman Coulter, USA) at 2500 × *g* for 5 min to further reduce the concentrate to pellets of 80–90 % water content. The pellets were freeze-fried, reduced to a fine powder with mortar and pestle, and stored at −80 °C until further analysis.

2.2.3. Optimization of algal productivity in digestate

C. vulgaris cultures were grown in conical 500 mL flasks with TAP media placed in an incubator in the laboratory, as described in Section 2.2.1 (25 °C, ~80 μmol·m^{-2}·s^{-1}, 16 h light/8 h darkness). Once the cultures had reached the mid-exponential growth phase, aliquots were placed into 50 mL screw-cap plastic tubes and centrifuged at a speed of 2500 ×*g* for 5 min at 25 °C (Avanti J-26 XP, Beckman Coulter, USA). The supernatant was discarded, and the pellets were then re-suspended in 50 mL of modified TAP media with different digestate dilutions (2, 5, 8, and 15 % *v*/v), corresponding to different ammonium-derived nitrogen (NH_4^+) concentrations (36, 90, 145, and 270 mg·L^{-1}). Also, two additional conditions were assessed for the digestate medium at 90 mg·L^{-1}: (i) addition of 2.4 g·L^{-1} Tris to assess the influence of pH on ammonia toxicity and (ii) addition of 1 g·L^{-1} acetate to assess the influence of organic carbon availability. The resuspended cultures (cell concentrations of 2·10^6 cells·mL^{-1}) were transferred to 100 mL flasks and grown in the same incubator setup as described above for 3 days. Cell density (Z1 particle counter, Beckman Coulter, USA), Photosystem II maximum efficiency (F_v/F_m, measured after 15 min of dark adaptation) (AquaPen-C, PSI, Czechia), and pH (HQ4100, Hach, USA) were measured daily.

C. vulgaris grown as 500 mL cultures in conical flasks (described in the previous paragraph), were used to inoculate 4 × 10 L hanging bags, which were grown under natural lighting conditions and ambient temperature (Algal Innovation Centre glasshouse, Cambridge) until they reached a cell concentration of approximately 2·10^6 cells·mL^{-1}. Then, these cultures were used to inoculate 12 × 5 L hanging bags, corresponding to triplicates of 3 experimental conditions: TAP, digestate, and digestate buffered with Tris (2.4 g·L^{-1}). NH_4^+ concentration was adjusted to 90 mg·L^{-1} in all experimental conditions, resulting in a volumetric use of digestate of 5 % (*v*/v).

2.3. Biomass analysis

2.3.1. Bulk composition analysis

Fourier-transform infrared (FTIR)-attenuated total reflectance (Spectrum Two, PerkinElmer, Germany) was used to determine the composition of the biomass grown on different media in terms of proteins, carbohydrates, and lipids. The instrument had a diamond crystal iATR reflectance cell with a DTGS detector scanning over the wavenumber range of 4000–450 cm^{-1} at a resolution of 4 cm^{-1}. Approximately 3–5 mg of finely powdered freeze-dried biomass was applied to the surface of the crystal and then pressed onto the crystal head. Scans were recorded and baseline-corrected using the spectroscopic software Spectrum (version 10, PerkinElmer, Germany). Standard materials (BSA-KBr, Glucose-KBr, and C16:0-KBr) were used as controls for protein, carbohydrate, and lipid peak identification, respectively. The amide II peak (1545 cm^{-1}) was used for the determination of proteins, the methyl/methylene peak (2800–3000 cm^{-1}) was used for lipid content, and the pronounced peak at 1050 cm^{-1} was selected for carbohydrate quantification as it exhibited a good linear relationship to the glucose standard used [32,33]. FTIR spectra were used for determining the ratio of the different biochemical components in the analysed samples. Protein, carbohydrate, and lipid content were then expressed as percentages of dry biomass weight after determining the protein content by a colourimetric method.

For protein determination, approximately 5 mg of algal biomass was weighed and homogenized by bead milling (TissueLyser II, QIAGEN, Germany) with 0.5 mm stainless beads at a 30 s^{-1} frequency and a concentration of 0.8 g beads·mL^{-1} of biomass suspension. The powdered biomass was suspended in 5 % (*v*/v) SDS extraction medium, incubated at 100 °C for 5 min and centrifuged (5000 ×*g* for 5 min) [34]. The supernatant extracts were diluted (1 in 5) to avoid any interference with the reagent and quantified using the Pierce™ Modified Lowry Protein Assay Kit, a microplate reader (CLARIOstar Plus, BMG, Germany) to measure the absorbance at 750 nm and a standard BSA curve fitted into a quadratic model as recommended by the manufacturer.

2.3.2. Lipid analysis

A total lipid extraction was performed as outlined by Davey et al. [35]. Briefly, samples of ~2 mg of freeze-dried biomass were suspended in 10 mL of (2:1) chloroform and methanol mix, spiked with 200 mL of pentadecanoic acid (C15) (1 mg·mL^{-1}) and sonicated for 30 min on ice. Then, after the addition of 5 mL of water, the tubes were centrifuged at 1000 ×*g* for 3 min at 4 °C to achieve phase separation. The aqueous phase was discarded, the solvent was evaporated from the lower phase with N_2 gas (45 °C; GeneVac EZ-2; SP Scientific, Ipswich, United Kingdom), and the extract was then resuspended with 200 mL of n-heptane.

Three 100 mL aliquots of the extract were transferred to a glass tube and trans-esterified with 3 mL of a 2.5 % *v*/v H_2SO_4/methanol mix, vortexed and placed in a 60 °C water bath for 4 h and then allowed to cool. Following this step, 3 mL of water and 3 mL of hexane were added, and samples centrifuged at 2000 ×*g* for 3 min to achieve phase separation. The upper hexane phase was then separated, re-extracted and evaporated under the conditions described in the previous paragraph. The fatty acid methyl ester (FAME) extracts were resuspended with 120 mL of hexane, separated, and identified using GC (Thermo Scientific Trace GC Ultra) with a Zebron ZB-Wax Capillary GC column (30 m × 0.25 mm, 0.25 μm film thickness, Phenomenex, UK). The injection volume was 1 μL with a 35:1 split ratio with an injector temperature set to 230 °C, using helium as a carrier gas at a constant flow of 1.2 mL·min^{-1}. For the run, the following gradient was used: initial oven temperature 60 °C, 2 min; 150 °C at 15 °C min^{-1}; 230 °C at 3.4 °C min^{-1}. The detector temperature was 250 °C. FAMEs are identified by co-elution with a FAME standard mix (Supelco, 37 Component FAME

Mix). The peaks were identified based on the standard mix peak RT and through library search (NIST). The areas were compared as a percentage of the total identified FAMEs.

2.3.3. Pigment analysis

After lysing the cells (as detailed for protein extraction), the extracts were analysed with High-Performance Liquid Chromatography (HPLC). Two solvent mixes were used, solvent A (ACN: H_2O: MeOH: Hexane: Tris; 80:7:3:1:1) and solvent B (MeOH: Hexane; 5:1), analytical HPLC grade solvents were used throughout this work and sourced from Fisher Scientific and Sigma Aldrich. The solvent gradient was: 1) From 0 to 7 min, 100 % solvent A. 2) From 13 to 14 min, 100 % solvent B. 3) From 15 to 20 min, 100 % solvent A. The flow rate was 0.5 mL·min^{-1} in an Inertsil ODS2 column (C18, 250 mm × 2 mm, 5 mm) and the retention time was compared with commercial lutein (Puritan's Pride Lutein 40 mg) and chlorophyll *a* standards (Sigma-Aldrich).

2.3.4. B_{12} bioassay

Aliquots of 1 mL of microalgae cultures grown on either TAP or digestate were centrifuged at 5000 ×*g* for 5 min; the supernatant was used for extracellular vitamin B_{12} content determination, while the pelleted cells were resuspended in 1 mL of distilled water for determination of intracellular B_{12}. The resuspended cells were boiled in a water bath for 20 min to extract intracellular B_{12} into the liquid phase. The samples were then centrifuged to remove particulates. The same procedure was followed for samples of the food waste used for the anaerobic digestion process and the digestate used for algae cultivation.

The content of B_{12} was determined by a microbiological bioassay method using a recently evolved B_{12}-dependent strain of *Chlamydomonas reinhardtii* (metE7) [36,37]. This strain was incubated for 4 days in a TAP-based medium at ~90 μE·m^{-2}·s^{-1} continuous light, 120 rpm, plus the B_{12}-containing samples, appropriately diluted, in 24-well plates at a total volume of 2 mL per well. Growth was quantified by measuring optical density at 730 nm. B_{12} concentration in the samples was estimated by comparing the optical densities obtained to a standard curve of known B_{12} concentrations (between 8 and 200 pM) fitted with a 4-parameter logistic model [38,39].

2.4. Elemental analysis

To determine the content of macro- and micronutrients and potentially toxic elements of the microalgae grown on TAP and digestate, 0.1 g freeze-dried samples were pre-digested with 10 mL HNO_3 for 30 min, followed by 30 min in an ultrasonic water bath. The samples were then subjected to microwave digestion (UltraWAVE, Milestone, Italy) and the total elemental concentrations in the digested samples were analysed by inductively coupled plasma-optical emission spectrometry (ICP-OES; Varian Vista MPX, USA).

2.5. Microbiome analysis

2.5.1. Metabarcoding of anaerobic digestate samples

We used amplification of 16S rRNA followed by Illumina short-read sequencing to characterize the diversity of microbial communities within two digestate samples. Briefly, DNA was extracted from 750 μL of anaerobic digestate samples (AD_1 and AD_2) using the ZymoBIOMICs DNA/RNA miniprep kit (Zymo Research) according to the manufacturer's parallel purification protocol. 16S rRNA genes were amplified using specific primers (16S V3-V4) and barcoded. PCR reactions were carried out with 15 μL of Phusion® High – Fidelity PCR Master Mix (New England Biolabs); 2 μM of forward (CCTAYGGGRBGCASCAG) and reverse (GGACTACNNGGGTATCTAAT) primers, and ~10 ng template DNA. Thermal cycling consisted of initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 30 s and 72 °C for 5 min. PCR products were mixed and purified with Universal DNA Purification Kit (TianGen, China, Catalog #: DP214). Sequencing libraries were generated using NEB Next® UltraTM II FS DNA PCR-free Library Prep Kit (New England Biolabs, USA, Catalog #: E7430L) following the manufacturer's recommendations and indexes were added. The library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. Quantified libraries were pooled and sequenced on Illumina platforms, according to effective library concentration and data amount required. This process was performed by Novogene (Cambridge, UK). Sequencing datasets generated and analysed during this study are available from the European Nucleotide Archive (ENA), under project accession PRJEB65882.

2.5.2. Pre-processing of sequencing data

250 bp paired-end reads were assigned to samples based on their unique barcode and truncated by clipping the barcode and primer sequence. Paired-end reads were merged using FLASH (v1.2.11) [40] the splicing sequences were called raw tags. Quality filtering on the raw tags was performed using the fastp (v0.23.1) software [41] to obtain high-quality clean tags [42]. The tags were compared with the reference database (Silva database (16S), https://www.arb-silva.de/)using UCHIME Algorithm to detect chimera sequences, and then the chimera sequences were removed [43]. Then the effective tags were finally obtained. Sequences analyses were performed by Uparse software (v7.0.1001, [44]). Sequences with ≥97 % similarity were assigned to the same OTUs. The representative sequence for each OTU was screened for further annotation. For each representative sequence, the Silva Database [45] was used based on Mothur algorithm to annotate taxonomic information. OTUs abundance information was normalized using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analyses of alpha diversity and beta diversity were performed based on this output normalized data. Alpha and beta diversity indices were calculated with QIIME (Version 1.9.1). This process was performed by Novogene (Cambridge, UK)

2.5.3. Bioinformatic downstream analysis

Taxonomic profiles were analysed on the phylum and genus level. The top 10 phyla as well as the top 100 genera were selected using custom R and Perl scripts and dominant taxa were identified for the different samples. Multiple sequence alignments were conducted using the aligned representative sequences to calculate the phylogenetic relationship of OTUs employing MUSCLE (v3.8.31, -maxiters 2 [46]). The reconstructed phylogenetic tree was visualized using custom R and Perl scripts and iTOL [47] alongside relative abundance profiles for genus level. The database of bacterial species that biosynthesize cobamides including cobalamin (vitamin B_{12}) was compiled from the comparative genomics study for cobamide production across 11,000 bacterial species [48]. For cobamide biosynthesis capacity, we divided the dataset into 3 classes: complete biosynthesis, partial biosynthesis, and no biosynthesis and calculated the representation of these classes for each genus using custom R scripts (only the number of species within the dataset were included in the calculation). For the samples sequenced, we also estimated the percentage of the top 100 most abundant genera in each cobamide biosynthesis class. After subtracting the genera that lacked any sequenced species, we calculated the fraction of each cobamide biosynthesis class within each genus, summed up the fractions across all genera for each class, and then divided the totals by the number of genera. For the analysis the Perl SVG extension and the R packages tydiverse [49], ggplot2 [50], openxlsx [51], RColorBrewer [52], ggpubr [53,54], optparse [54] were employed.

2.6. Statistical analysis

Experimental data were expressed as the mean of three replicates (unless stated otherwise) ± standard deviation. Datasets were compared with one-way analysis of variance (ANOVA), using Microsoft Excel 2021. The criterion for statistical significance was set at $p < 0.05$.

3. Results

3.1. Pilot-scale cultivation of algae on digestate

The anaerobic digestion process has been well established [1–4]. Anaerobic digestate from the digestion of fruit and vegetable waste was collected throughout the process and pooled in two different batches, labelled AD_1 and AD_2. AD_1 consisted of all digestate collected between 0 and 4 weeks post-inoculation, and AD_2 consisted of digestate from 4 to 7 weeks. To use digestate for the cultivation of microalgae, parameters required to support effective algal growth were determined (Table 1). An abundance of total suspended solids (TSS) was notable, which if not removed would impede light transmission to the algal cells. Ammonium nitrogen was high in both digestates, far exceeding the 25–250 $mg{\cdot}L^{-1}$ range, which is deemed optimal for the cultivation of *C. vulgaris* [55]. These characteristics necessitated pre-treatment of digestate before its incorporation in algal growth media [56].

Digestate was centrifuged to reduce the TSS content and subsequently diluted in water to reduce the ammonium nitrogen concentration below toxic levels. This was then used to establish a concentration gradient of AD_1, ranging from 1 to 15 % *v*/v (Fig. 1), which corresponds to 36–540 $mg{\cdot}L^{-1}$ of ammonium nitrogen, and then used to determine the optimal digestate concentration for *C. vulgaris* growth. Over the six days of the experiment, *C. vulgaris* was able to grow at all concentrations, although maximal growth was achieved on 3 % *v*/v digestate, and the maximum carrying capacity achieved was greater than in control medium. A slight reduction in growth was observed after 4–6 days in some cultures, which might be induced by nutrient limitation, although this was not consistently observed. However, an extensive lag phase of 2–3 days was always seen for digestate concentrations above 10 % *v*/v.

Based on these encouraging laboratory-scale experiments, the ability of digestate to support *C. vulgaris* was tested in an outdoor glasshouse facility (the Algal Innovation Centre, Cambridge, UK). Cultures were first scaled gradually in TAP medium in the laboratory until sufficient inoculum had been generated to initiate cultures of 150 L in horizontal rotary photobioreactors at an initial density of $2–3{\cdot}10^6$ $cells{\cdot}L^{-1}$ (Fig. S1a). One culture was grown in TAP, whose nitrogen source is ammonium and contains organic carbon, unlike other conventional algal growth media used for *C. vulgaris* (e.g., Bold's Basal Medium); it thus acted as a benchmark to compare the growth of algae grown in digestate. The second experiment was carried out in digestate: the productivity of the anaerobic digesters at the time of this experiment provided sufficient material to allow 2.75 % *v*/v dilution of digestate in the final volume of culture. This dilution was in the range of the concentration of digestate found to be optimal for maximal *C. vulgaris* growth (Fig. 1) and corresponded to ammonium nitrogen concentration of ~90 $mg{\cdot}L^{-1}$, similar to that in TAP medium (100 $mg{\cdot}L^{-1}$).

After two cultivation cycles in the 150 L photobioreactors to acclimate the cultures to the environmental conditions in the glasshouse, *C. vulgaris* was grown in TAP for 13 days (between 27 September – 7 October 2021) and then in 2.75 % v/v digestate (AD_1; hereinafter referred to as digestate) for 13 days (between 7 and 20 October 2021). The cell concentration was measured throughout the experiments to assess productivity (Fig. 2a). After 2–3 days of cultivation, the growth rate in both media progressively decreased as the culture entered a stationary phase of growth, leading to a final cell concentration of $1.49{\cdot}10^7$ $cells{\cdot}ml^{-1}$ for digestate at day 13. The final algal cell concentration in TAP media was significantly higher ($p < 0.05$) than the digestate-grown cultures and was measured at $2.42{\cdot}10^7$ $cells{\cdot}mL^{-1}$. The corresponding dry cell biomass weight concentrations were 0.25 $g{\cdot}L^{-1}$ for digestate and 0.46 $g{\cdot}L^{-1}$ for cells grown in TAP media.

To identify the main factors underlying the 40 % lower algal biomass concentration in digestate, parameters that are likely to impact growth were analysed. Ambient light intensity and temperature heavily fluctuated throughout the experiments (Fig. 2d and e), with both parameters being at times suboptimal for *C. vulgaris* growth (e.g., for the control experiment, the temperature range was 9.3–32.2 °C while maximum light intensity was 403 $\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$; for the digestate experiment these values were 9.4–30.2 °C and 370 $\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$). However, the variation in average temperature and sunlight intensity between the two experiments was <10 %, suggesting that environmental conditions were not sufficient to explain the decrease in *C. vulgaris* growth on digestate. Given that the digestate was pasteurized and highly diluted, and because inhibition of *C. vulgaris* growth was evident from 1 day post-inoculation,

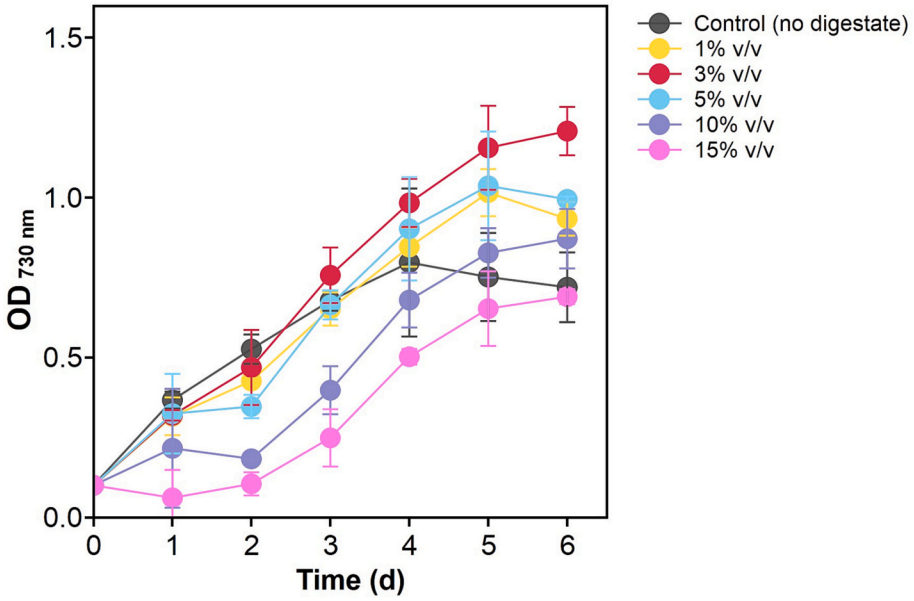

Fig. 1. Growth curves of *C. vulgaris* cultivated in different concentrations of anaerobic digestate (AD_1). Experiments were performed in 24-well plates (1 mL) under controlled light and temperature conditions (90 $\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$, 16:8 photoperiod, 25 °C). Cultures were inoculated with 20 % v/v of exponential-phase *C. vulgaris* grown in TAP. Mean values and standard deviations were calculated from four independent experimental replicates. The dilution range was selected so that 1 % is equivalent to 36 $mg{\cdot}L^{-1}$, 3 % is 108 $mg{\cdot}L^{-1}$, 5 % is 180 $mg{\cdot}L^{-1}$, 10 % is 360 $mg{\cdot}L^{-1}$, and 15 % is 540 $mg{\cdot}L^{-1}$ ammonium nitrogen. Cultures grown in the residual nutrients from the 20 % v/v inoculum, without addition of digestate, were used as controls.

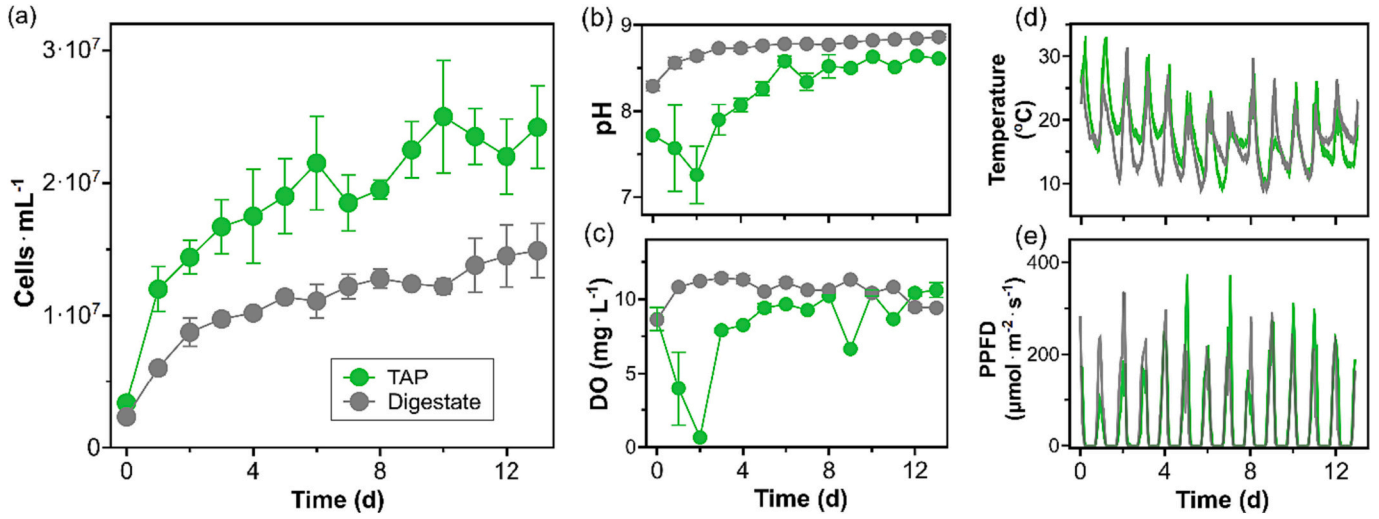

Fig. 2. Biotic and abiotic parameters measured during the cultivation of *C. vulgaris* in 150 L horizontal photobioreactors in TAP medium and in anaerobic digestate at a 2.75 % (*v*/v) concentration. (a) Cell count, (b) pH, (c) dissolved oxygen (DO) concentration, (d) ambient temperature, and (e) solar irradiance in the glasshouse interior. Ambient solar irradiance values are expressed as photosynthetic photon flux density (PPFD) measured in $\mu mol \cdot m^{-2} \cdot s^{-1}$ and visualized as hourly rolling averages of continuous readings to exclude outliers. Mean values and standard deviations were calculated from two independent experimental replicates.

we considered inhibition of growth by biological contaminants unlikely; cells were also visually inspected daily, and no significant bacterial contamination in the first 5 days of cultivation was observed (Fig. S2). Ammonium nitrogen and phosphate measured on day 13 were not limiting (Fig. S3), indicating that macronutrient depletion was also not responsible for the lower growth in digestate compared to TAP, particularly during the exponential growth phase.

During the first 5 days of cultivation when the most significant cell growth occurred, the pH in digestate-grown culture was 8.3 and reached a maximum of 8.8, while the pH of TAP-grown culture remained below 8 (Fig. 2b). At 16–17 °C (average temperature of the experiments), almost all N is present as ammonium at pH values below 8, while at higher values the ammonia:ammonium ratio sharply increases with the increase of pH [57]. Ammonia, rather than ammonium, is reported as the main chemical species responsible for toxicity in algae cells [58]. Therefore, ammonia toxicity might have been a significant inhibitor for algal growth in digestate. Dissolved oxygen concentrations were also significantly lower in TAP-grown culture during the first 4 days of the experiment, indicating heterotrophic growth in addition to phototrophic growth (Fig. 2c). Considering that the organic carbon in TAP is solely acetate, while digestate contains slowly biodegradable organic compounds such as humic acid, fulvic acid and lignocellulosic compounds [59], this suggests that lower bioavailability of organic carbon could also explain the decreased growth observed.

3.2. Optimization of algal productivity in anaerobic digestate

Further laboratory-scale experiments were conducted under controlled light and temperature conditions to test whether ammonia toxicity and decreased bioavailability of organic carbon were the main factors limiting growth in digestate. AD_2 was used as a growth medium in different dilutions (2, 5, 8, and 15 % *v*/v), corresponding to different NH_4^+ concentrations (36, 90, 145, and 270 mg $N \cdot L^{-1}$). For the 90 $mg \cdot L^{-1}$ experiment, additions of 2.4 $g \cdot L^{-1}$ of Tris buffer and 1 $g \cdot L^{-1}$ acetate were also tested to assess the influence of pH on ammonia toxicity and the presence of bioavailable organic carbon, respectively.

Increasing the concentration of digestate (and effectively ammonium concentration) correlated with lower cell density after 3 days of growth (Fig. 3a). The maximum cell concentration ($1.2 \cdot 10^7$ cells$\cdot mL^{-1}$) was recorded in the lowest digestate concentration tested (2 % *v*/v), and cell concentrations did not exceed $5 \cdot 10^6$ cells$\cdot mL^{-1}$ in higher digestate concentrations (5, 8, and 15 % v/v). Photosystem II maximum efficiency (F_v/F_m), which is a measure of the physiological state of the algal cells, was shown to follow a similar decreasing trend as the digestate concentration was increased (Fig. 3c) and was below the optimal range for *C. vulgaris* growth (0.70–0.78 [60,61]) indicating that the cultures were stressed. The pH significantly increased during the experiments, exceeding pH 9 in all dilutions tested (Fig. 3b).

The addition of Tris to 5 % v/v digestate maintained pH at 8.5, compared to 9.2 in the corresponding non-buffered experiment (Fig. 3b). As a result, cell concentration tripled (Fig. 3a) and F_v/F_m increased to 0.64, suggesting a recovery of photosynthetic efficiency (Fig. 3c). Addition of acetate (1 $g \cdot L^{-1}$) did not further enhance growth and photosynthesis (Fig. 3a & c). These findings strongly suggest that free ammonia formation due to high pH, rather than the absence of bioavailable organic carbon, is the main inhibitor in this system, and that limiting pH increase to <8.5 could significantly enhance productivity.

We selected the best conditions found in the laboratory (5 % v/v, 2.4 $g \cdot L^{-1}$ Tris buffer) and scaled them up in our outdoor facility to 5 L (Fig. S1b) under similar seasonal environmental conditions (Fig. S4). Cell concentrations over 9 days of cultivation in buffered digestate were compared to growth in non-buffered digestate and TAP (Fig. 3d). For the first 2 days of growth, cell densities were similar in all conditions, but cultures grown in non-buffered digestate entered the stationary phase from day 2 of the experiment ($8 \cdot 10^6$ cells$\cdot mL^{-1}$; 0.19 $g \cdot L^{-1}$). The cultures grown in buffered digestate and TAP, on the other hand, continued growth to a maximum cell concentration of $2.2 \cdot 10^7$ and $2.7 \cdot 10^7$ cells$\cdot mL^{-1}$, respectively (the corresponding biomass concrentrations of 0.44 and 0.56 $g \cdot L^{-1}$, translating to biomass productivities of 65 and 85 $mg \cdot L^{-1} \cdot d^{-1}$ until day 6, when cultures entered the stationary phase). F_v/F_m also significantly decreased in cultures grown in non-buffered digestate after 3 days of cultivation, compared to buffered digestate and TAP, where it remained above 0.6 (Fig. 3f). As observed in the lab-scale experiments, cell growth and photosynthetic efficiency were inversely correlated with pH (Fig. 3e). The pH in non-buffered digestate exceeded 8.6 after 3 days of growth, while in buffered digestate and TAP, it remained below 8.5 for the duration of the experiment.

3.3. Biochemical composition of C. vulgaris grown in different media

To assess the nutritional value of *C. vulgaris* grown in pilot-scale

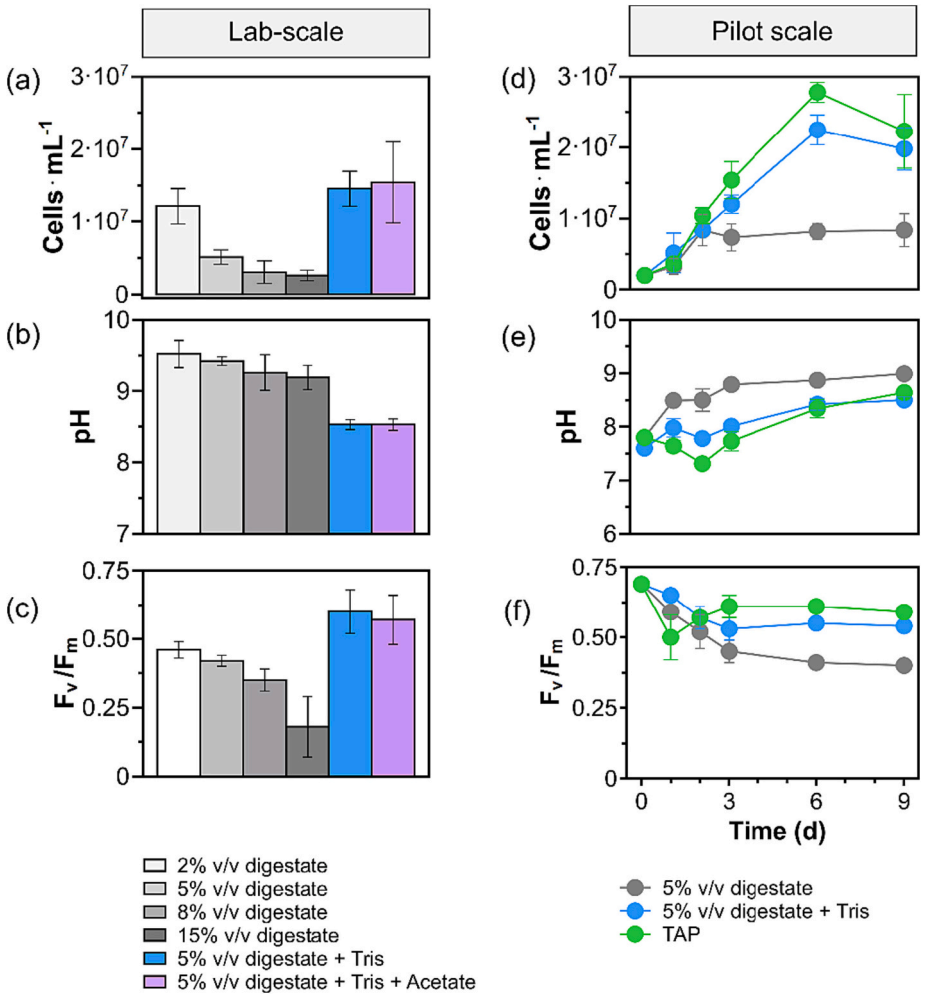

Fig. 3. Testing the impact of pH and ammonia on *C. vulgaris* growth at different scales. (a, d) Cell concentration, (b, e) pH, and (c, f) F_v/F_m measured after 3 days of cultivation at lab-scale (left) and during 9 days of cultivation at pilot scale (right). For lab-scale experiments (left), 150 mL flasks were incubated under controlled light and temperature conditions (90 μmol·m^{-2}·s^{-1}, 16:8 photoperiod, 25 °C), and different dilutions of digestate (2, 5, 8, and 15 % *v*/v), corresponding to different NH_4^+ concentrations (36, 90, 145, and 270 mg N·L^{-1}). For the 90 mg·L^{-1} experiment, additions of 2.4 g·L^{-1} of Tris buffer and 1 g·L^{-1} were also tested. Pilot-scale experiments (right) were performed in a glasshouse (Algal Innovation Centre, Cambridge, UK); cultures were grown in 5 L hanging bags, in 5 % *v*/v anaerobic digestate, 5 % v/v anaerobic digestate with 2.4 g·L^{-1} Tris added, and TAP. Mean values and standard deviations were calculated from three independent experimental replicates.

reactors on digestate and TAP, biomass was harvested at 13 days post-inoculation and the biochemical composition was characterised (Table 2). Differences in the main macromolecules (proteins, carbohydrates, lipids) were not statistically significant between the different growth conditions. The dominant group was proteins, accounting for 50–55 % of the total dry biomass weight in both media, while carbohydrates and lipids were ~ 31 % and ~ 5.5 % of dry biomass weight, respectively. These figures are similar to those previously reported for commercially produced *C. vulgaris* [62,63].

Total chlorophyll content was similar in the two samples, ranging between 15.8 and 19.7 mg·g^{-1}. Total carotenoid content was 2.6 mg·g^{-1} in the case of digestate-grown *C. vulgaris*, which is significantly higher ($p < 0.05$) than the corresponding carotenoid content of TAP-grown *C. vulgaris* (1.97 mg·g^{-1}). In terms of fatty acid methyl ester (FAME) composition *C. vulgaris* cultivated in digestate showed a similar profile to that grown in TAP. One exception was a greater abundance of polyunsaturated fatty acids in digestate-grown algal biomass (13 % increase), with alpha-linolenic acid (ALA, C18:3) being the dominant FAME. *C. vulgaris* is known to be an abundant source of omega-3 and omega-6 fatty acids, and alpha-linolenic acid is often dominant, ranging from 12.9 to 45.8 % of its total fatty acid content [64,65]. Overall, even though productivity was lower in digestate in the first pilot experiment, the resulting algae biomass had a similar macronutrient composition regardless of the medium, indicating that growth in digestate did not compromise the biochemical qualities of the biomass, and indeed may have resulted in enhanced unsaturated fatty acid content.

Cells grown on digestate had a mineral content in the same order of magnitude as cells grown on TAP (Table 2), even though the TAP medium had higher nutrient concentrations than digestate (Fig. S5). Digestate-grown *C. vulgaris* was even richer in a few elements, i.e., K, Fe and Co. Regarding potentially toxic elements, Ni, Cd, Pb, Al and Cr were measured in both algal samples (Fig. S6). Al, Ni, and Cr were detectable at low concentrations, within the permissible levels for animal feed [66].

3.4. Determination of B_{12} abundance during the bioremediation processes

Anaerobic digestates were previously shown to contain several bioactive metabolites (e.g., vitamins, hormones, humic acids, amino acids, etc.), generated by the microbial communities that facilitate the digestion of organic matter [17–19]. To investigate whether *C. vulgaris* grown in digestate can bioaccumulate digestate-derived micronutrients, vitamin B_{12} (cobalamin) was chosen as a case study. Analysis of the AD_1

Table 2
Biochemical composition of *C. vulgaris* grown at pilot-scale. Protein, carbohydrate, lipid, pigment, fatty acid methyl ester (FAME), and elemental composition of 13 days post inoculation *C. vulgaris* biomass grown in TAP and digestate in 150 L horizontal photobioreactors. Data are expressed as mean ± SD of three independent technical replicates. Significant differences ($p < 0.05$) between groups are indicated by the '*' symbol.

Component	TAP	Digestate
Biomass composition (% *w*/w)		
Proteins	52.5 ± 7.1	54.0 ± 9.0
Carbohydrates	31.1 ± 4.1	32.0 ± 5.3
Lipids	5.3 ± 0.7	5.5 ± 0.9
Pigments (mg·g^{-1} dry weight)		
Total chlorophyll	15.8 ± 0.5	19.0 ± 3.5
Total carotenoids*	2.0 ± 0.1	2.6 ± 0.4
Fatty acid methyl ester composition (%)		
C12:0*	2.7 ± 0.4	1.9 ± 0.1
C14:0	0.3 ± 0.1	0.3 ± 0.1
C16:0	21.9 ± 1.0	21.8 ± 0.4
C16:1*	5.7 ± 0.4	1.5 ± 0.3
C16:2*	7.8 ± 0.1	3.7 ± 0.2
C16:3*	8.6 ± 0.4	13.7 ± 0.1
C17:0	0.9 ± 0.2	0.7 ± 0.0
C17:1*	0.5 ± 0.0	1.0 ± 0.1
C18:0	4.7 ± 1.0	3.3 ± 0.2
C18:1*	4.3 ± 0.4	2.3 ± 0.6
C18:2*	13.9 ± 1.0	7.8 ± 0.1
C18:3*	26.4 ± 1.7	41.8 ± 0.4
Saturated FAMEs	30.5 ± 1.9	28.0 ± 0.6
Poly-unsaturated FAMEs	56.7 ± 2.1	67.1 ± 1.8
Mono-unsaturated FAMEs*	10.4 ± 0.9	4.2 ± 0.2
Elemental composition (mg·g^{-1} dry biomass)		
Ca*	3.78 ± 0.05	2.71 ± 0.14
Mg*	4.21 ± 0.03	2.92 ± 0.15
K*	5.31 ± 0.09	7.03 ± 0.36
Fe*	0.93 ± 0.02	1.41 ± 0.08
Na	0.91 ± 0.02	0.89 ± 0.14
Mn	0.09 ± 0.00	0.08 ± 0.00
Cu*	0.28 ± 0.00	0.17 ± 0.00
Zn*	0.11 ± 0.02	0.05 ± 0.00
Co*	0.002 ± 0.00	0.009 ± 0.001

showed that it contained 0.32 μg·mL^{-1} B_{12} (Fig. 4a). Because our analysis of the fruits and vegetables that were used as feed showed a negligible amount of B_{12} (<0.002 μg·g^{-1}), as expected, the B_{12} in the digestate can confidently be attributed to *de novo* biosynthesis by bacteria in the anaerobic digesters.

Analysis of B_{12} abundance in *C. vulgaris* grown in the scale-up experiment using TAP and digestate as media (see Section 3.1) showed that digestate-grown biomass contained 10.6 μg B_{12} per g dry weight at day 13. This amount was significantly higher ($p < 0.001$) than the TAP-grown *C. vulgaris* biomass, which contained 4.1 μg B_{12} per g dry weight (Fig. 4c) at day 13. The value recorded for digestate-grown *C. vulgaris* was also higher than those reported for commercial *C. vulgaris* powders, which range between 0 and 4.5 μg·g^{-1} dry weight [67,68].

There was no B_{12} in the TAP medium, which is evident from the near-zero extracellular B_{12} concentration at inoculation (day 0) (Fig. 4b). As algae cannot synthesize B_{12}, the uptake of this vitamin in this non-axenic experiment is attributed to biosynthesis by B_{12}-producing bacteria [69]. In the case of cultivation on digestate, this medium was a rich source of extracellular B_{12}, on day 0 containing 13.8 μg·L^{-1} of this vitamin. During the 13-day experiment, *C. vulgaris* cells accumulated 26 % of the B_{12} provided by the digestate medium, indicating that cells reached carrying capacity for this vitamin.

To determine if the abundance of B_{12} in digestate is a widely occurring phenomenon, additional digestate samples from two independent commercially operated anaerobic digesters were obtained and analysed. All digestates were found to be abundant sources of B_{12}, ranging between 0.06 and 1.16 mg·L^{-1} (Table 3). This result further validates the likely ubiquity of B_{12} biosynthesis in anaerobic digestion and provides a new means of valorising this by-product.

3.5. Microbial community structure and B_{12} biosynthetic capacity distribution in anaerobic digesters

Microbial consortia in anaerobic digesters are known to be complex communities, and it has been suggested that secondary consumer populations act as producers of essential nutrients and form a pool of metabolites of high energy demand (i.e., vitamins, amino acids) in the digestate [70]. To understand the structure of the microbial community in the anaerobic digesters of this study, taxonomic profiles of AD_1 and AD_2 were analysed at the phylum and genus level (Fig. 5a). >50 % of the anaerobic digestate microbiota comprised Proteobacteria (4–46 %) and Firmicutes (25–44 %). Including Bacteriodota (25–44 %), species of these three phyla were the core microbiome of the digestate samples. With increasing time of anaerobic digestion (i.e., after 4 weeks in the case of AD_2), an increase in the relative abundance of Proteobacteria (4–46 %) was observed.

To study the phylogenetic relationships of the microbial community further, the top 100 genera were selected, and an evolutionary tree was drawn using the aligned representative sequences (Fig. 5b). The relative abundance of each genus was also determined and the capacity of the genera to synthesize cobamides (term refers to B_{12} family of cofactors) was predicted according to Shelton et al. (2019) [48]. Both digestates largely comprised *Fastidiosipila,* a genus of Bacillota associated with proteolysis and cellulosic fibre degradation, which is often found in anaerobic digesters [71,72]. *Pseudomonas* spp. (γ-proteobacterium) also had a high relative abundance in the digestate samples and is likely one of the key contributors to B_{12} production, as nearly all species of this genus so far experimentally or computationally analysed are B_{12} producers [48]. Overall, out of the top 100 most abundant genera identified in the samples, we were able to predict the B_{12} biosynthetic capacity of 64 (the database did not include data for the other 36 genera). This indicated that ~35 % of the bacterial genera were likely B_{12} producers, ~20 % were likely cobamide or tetrapyrrole precursor salvagers, and ~45 % of the bacterial community were not associated with de novo B_{12} synthesis.

These results demonstrate the complex population structure within the digestate and provide supporting evidence that the B_{12} accumulation in the anaerobic digestate is likely due to presence of a sizable population of B_{12} producing bacteria in the reactors.

4. Discussion

In this study, we tested the capacity of algae to grow on digestate generated from fruit and vegetable waste and to bioaccumulate vitamin B_{12} from the digestate. Indeed, recent untargeted metabolomics studies of anaerobic digestate have revealed the complex nature of the digestate as a substrate rich in bioactive components [17–19]. Nonetheless, digestate is still almost exclusively approached as a conventional fertilizer, and little is known about its micronutrient qualities. In our case study, we found that fruit and vegetable waste with negligible B_{12} content could be anaerobically digested to produce digestate significantly enriched with this vitamin (0.32 μg B_{12}·g^{-1}, as shown in Fig. 4). Microbial composition analysis of the digestates showed that *Proteobacteria*, *Firmicutes* and *Bacteriodota* were the dominant bacterial phyla. This microbial community structure was similar to other microbiomes in mesophilic anaerobic digesters treating food and agricultural waste [73–76], and the dominant bacterial genera (*Fastidiosipila*, *Pseudomonas*) are associated with hydrolysis of carbohydrates [71,77]. Our bioinformatic analysis of the B_{12} biosynthesis capacity distribution for species within each genus revealed another layer of complexity and provided evidence that a sizeable population (~35 % of the 100 most

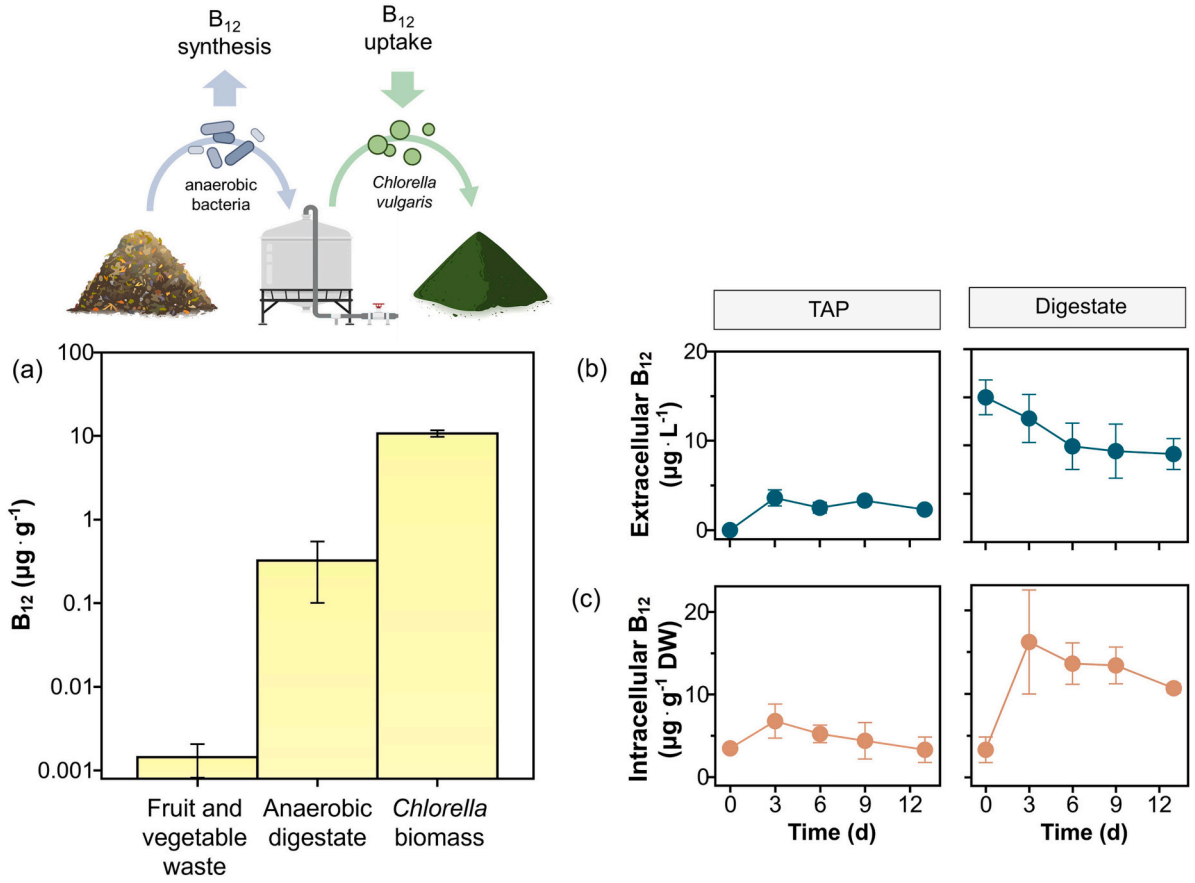

Fig. 4. B_{12} concentration throughout the different steps of the bioremediation process. (a) Schematic showing the process of B_{12} metabolism during anaerobic digestion of fruit and vegetable waste followed by algal cultivation. The fruit and vegetable waste (<0.002 µg $B_{12}{\cdot}g^{-1}$) is digested by anaerobic bacteria, which simultaneously synthesize large amounts of B_{12} (0.32 µg $B_{12}{\cdot}g^{-1}$), that are then further bioaccumulated in *C. vulgaris* biomass (10.6 µg $B_{12}{\cdot}g^{-1}$) (y-axis in logarithmic scale). (b) Extracellular B_{12} concentration, and (c) B_{12} content of *C. vulgaris* during cultivation on TAP medium and 2.75 % (*v*/v) anaerobic digestate. Data are expressed as mean +/− SD of technical replicates ($n = 3$).

Table 3
B_{12} content of digestate samples from different anaerobic digesters. In addition to samples from the anaerobic digesters operated as part of this study (AD_1, AD_2), samples from independent anaerobic digesters operated commercially were also analysed for their B_{12} content. AD_3 stems from 5 L anaerobic digesters operated for R&D purposes fed with food waste, and AD_4, AD_5 stem the digester compartment (AD_4) and the storage tank (AD_5) of a large-scale commercial anaerobic digestion facility in the UK, which processes food waste.

Digestate (derived from)	B_{12} (mg·L^{-1})
AD_1 (fruit & vegetable waste)	0.32
AD_2 (fruit & vegetable waste)	0.06
AD_3 (food waste)	0.74
AD_4 (food waste)	0.23
AD_5 (food waste)	1.16

abundant genera) of bacteria in the reactors were capable of *de novo* B_{12} biosynthesis. This is not unexpected given the requirement of enzymes of methanogenesis for a B_{12} cofactor [78]. B_{12} was also found to be abundant (0.05–1.16 mg $B_{12}{\cdot}L^{-1}$) in samples from other digesters using feedstocks of predominantly plant origin. Production of *C. vulgaris*, a facultative B_{12}-utilizing alga, on this digestate resulted in a substantial increase in concentration to 10–16 µg $B_{12}{\cdot}g^{-1}$ dry weight (Fig. 4c). Considering that eukaryotic algae lack the genes to synthesize B_{12} and acquire this vitamin exclusively from external sources [26,27], this finding translates to the attribution of additional value to an otherwise low-value product (digestate as fertilizer) and has implications for the utility of digestate for downstream applications (e.g., to meet B_{12} sufficiency in aquaculture). Recent research suggests that vitamin deficiency can have a significant impact on fish health and productivity, with optimal productivity and product quality requiring a dietary surplus of folate, vitamin B_6, and B_{12} above current recommendations [79,80]. This is particularly relevant because over the last 20 years, aquaculture feed composition has shifted to predominantly plant-based ingredients with an expected resulting decrease in B vitamin content (in 2020–60 % of the feed used for Atlantic salmon farming was plant-based, compared to 22 % in 2000) [81,82].

In addition to B_{12}, we were able to demonstrate the capacity for enrichment of other compounds in the algal biomass. The biochemical composition of *C. vulgaris* grown in digestate was similar in terms of protein content compared to that grown in control media (54 % vs 52 %), and to be enriched in ALA, C18:3, relative to the control, although with no change in total lipid (Table 2). Polyunsaturated fatty acids in feed have been determined as important for fish health [83], making this result valuable when considering digestate-grown algae biomass for such end use. Until recently, despite its nutritional qualities, digestate-grown algal biomass could not be considered as feed because of the lack of a clear legal framework [84]. However, as described in a recent policy report published by the ALG-AD European Project digestate derived from purely plant materials like crop or vegetable waste, and category 3 animal by-products (which are fit for human consumption) excluding catering waste, is permitted for use in feed according to EU regulation [85]. Consequently, the results of this study significantly expand the potential applications of digestate-grown *C. vulgaris* and

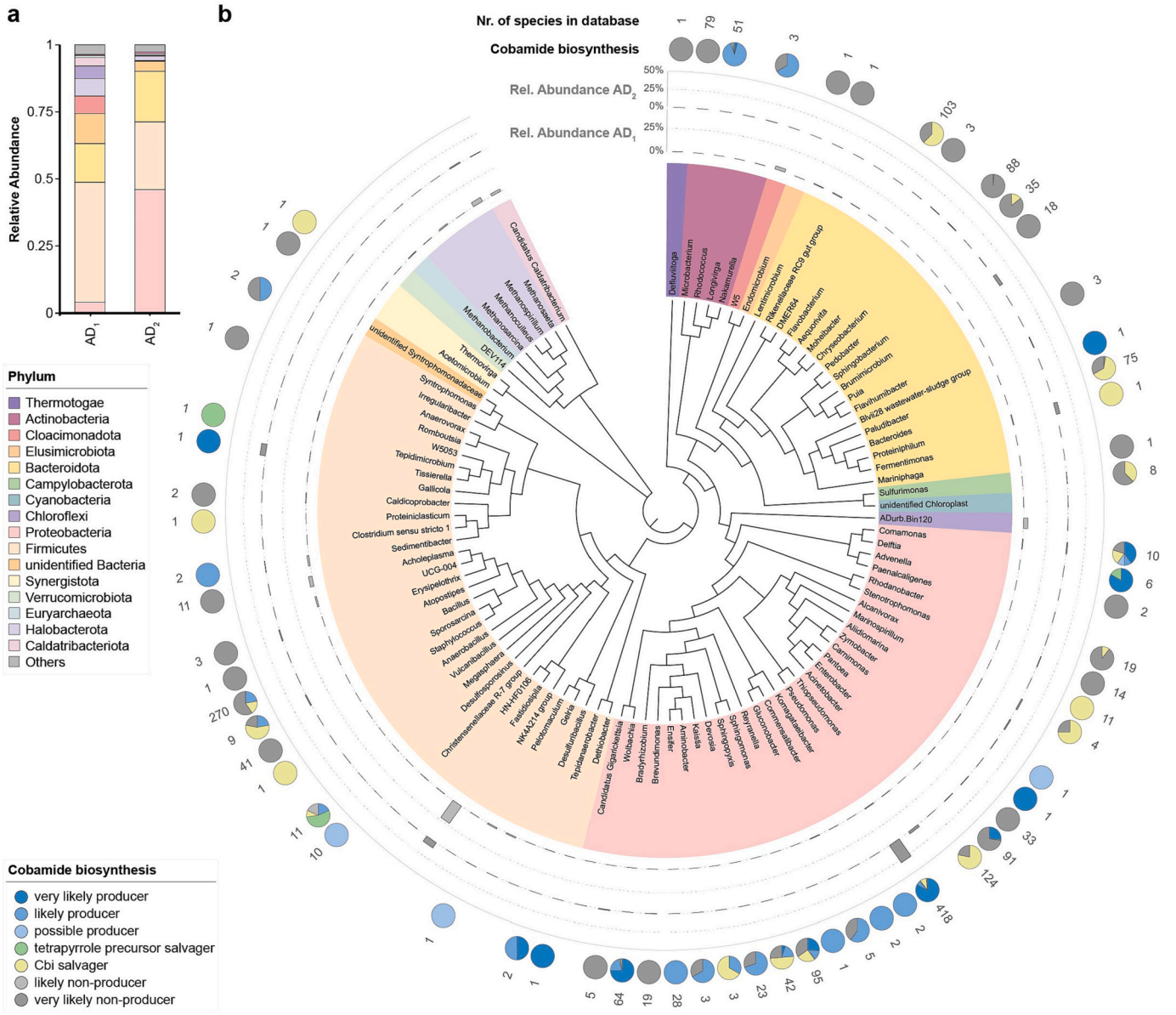

Fig. 5. Microbial composition and B_{12} biosynthesis trends in anaerobic digestate. (a) Fractions of the ten most abundant bacterial phyla in the anaerobic digestate samples AD_1 and AD_2. (b) Phylogenetic tree of the top 100 most abundant genera observed in both samples (multiple sequence alignments were conducted using the aligned representative sequences to calculate the phylogenetic relationship of OTUs employing MUSCLE (v3.8.31, −maxiters 2 [46]). The bacterial phyla are colour-coded and cobamide biosynthesis capacity distribution for species within each genus is shown as pie charts. We differentiate between complete biosynthesis (blues), partial biosynthesis (greens), and no biosynthesis (greys) capacity according to predictions from Shelton et al. [48]. Alongside, the number of species for which cobamide biosynthesis information was present is shown. Bar charts display the relative abundance of reads associated with each genus in AD_1 (inner circle) and AD_2 (outer circle).

imply that it could be of high value as a feed ingredient, especially in the aquaculture industry.

In terms of efficiency and productivity of algae production on digestate, in our experiments *C. vulgaris* could grow most effectively at both lab- and pilot-scale when the fruit and vegetable digestate was diluted to ~3 % *v*/v. This said, algal biomass concentration (as a measure of growth) was initially found to be 0.25 $g{\cdot}L^{-1}$, lower than that achieved in the control medium (TAP; 0.46 $g{\cdot}L^{-1}$) (Fig. 2) and the range reported by other scale-up studies (0.5–2.3 $g{\cdot}L^{-1}$) [86,87]. By recording chemical, biological, and environmental parameters when cultures were cultivated at scale, we were able to assess the direct impact of each parameter, in isolation, on *C. vulgaris* growth (i.e., at lab scale under controlled light and temperature conditions). Limitations of digestate as a growth medium were identified as pH-induced ammonia toxicity. This knowledge allowed us to control the pH resulting in a doubling productivity at the pilot scale (Fig. 2) and also to use higher amounts of digestate. This is significant because dilution, a common approach to eliminate ammonia toxicity in digestate, leads to a deficiency of other nutrients, ultimately limiting productivity [15]. Indeed, in this study, even when pH was maintained below 8.5, algal cell density in digestate was still 19 % lower than in TAP, likely because of micronutrient limitation induced by the 5 % v/v dilution used (Fig. 2). Observations such as this provide insights and highlight areas for further improvement to efficiently couple algae cultivation to waste-remediation by way of anaerobic digestion.

Currently, biogas is considered the main high-value product of the anaerobic digestion process; however it is not yet able to fully meet the profitability margins of biogas plants [88]. Expanding the example of

algal bioaccumulation of vitamin B_{12} to other bioactive compounds and other applications could signal a paradigm shift in the waste management industry. Realizing the true potential of anaerobic digestate can contribute to the development of higher-value products further down the supply chain, ensuring the financial viability of a circular economy. To do so, it is necessary to identify and characterize bioactive molecules in digestate, and to elucidate the underlying mechanisms of micronutrient transfer in microbes grown for biotechnological applications.

CRediT authorship contribution statement

Konstantinos P. Papadopoulos: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Marcella Fernandes de Souza:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision, Funding acquisition. **Lorraine Archer:** Methodology, Validation, Investigation, Resources, Writing – review & editing. **Ana Camila Zenteno Illanes:** Methodology, Validation, Formal analysis, Investigation, Writing – review & editing, Funding acquisition. **Ellen L. Harrison:** Methodology, Validation, Investigation, Resources, Writing – review & editing, Supervision. **Fiona Taylor:** Methodology, Validation, Investigation, Writing – review & editing. **Matthew P. Davey:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Daniela Ahuatzin Gallardo:** Investigation, Writing – review & editing, Funding acquisition. **Allan J. Komakech:** Investigation, Writing – review & editing. **Shahla Radmehr:** Methodology, Investigation, Writing – review & editing, Funding acquisition. **Andre Holzer:** Conceptualization, Methodology, Software, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **Erik Meers:** Resources, Writing – review & editing, Supervision. **Alison G. Smith:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Payam Mehrshahi:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2023.103290.

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