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Insect frass as a fertilizer for the cultivation of protein-rich *Chlorella vulgaris*

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A R T I C L E I N F O

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A B S T R A C T

Replacing the large amounts of chemical fertilizer with nutrients from waste or residual streams is an important factor to make microalgal production more sustainable, cost-effective, and part of a circular bioeconomy. This is the first study to investigate insect frass as a potential nutrient source for microalgal cultivation, and its effect on the protein content of the microalgae *Chlorella vulgaris*. When grown on nutrients extracted from insect frass, *C. vulgaris* grew equally well and showed similar high protein content (40 % of the dry weight) as when grown in a control medium based on commercial fertilizers. The nitrogen in the frass media consisted predominantly of organic nitrogen compounds, of which 71–78 % could be consumed by the microalgae. While the presence of dissolved organic carbon in the insect frass promoted the growth of algae-associated bacteria, microalgal performance was not affected.

1. Introduction

With the continuous increase in world population and growing societal awareness of diet, health, and nutrition, the demand for proteins and protein-rich dietaries is increasing (Amorim et al., 2021; Wang et al., 2021). Due to limited resources of arable land and freshwater, novel and more sustainable protein sources are required in addition to conventional animal- and plant-based production. Many microalgae have high protein contents (30–80 % of DW, depending on species and production conditions) and an amino acid profile that matches the requirements for food and feed (Janssen et al., 2022). They have high areal productivities, can be cultivated on non-arable land and have low water requirements, and can thus provide a valuable and sustainable protein feedstock for commercial food and feed products in the form of whole biomass or purified proteins (Wang et al., 2021). So far, implementing microalgal proteins into food and feed products has remained challenging, mainly due to their cost-intensive and small-scale production (Fernández et al., 2021; Wang et al., 2021). Creating cost-effective and environmentally responsible large-scale microalgal production technologies is essential to increase the production and use of microalgae as a protein source for future food and feed.

The basic nutritional requirements for microalgal production are carbon (C), nitrogen (N), phosphorous (P), and additional micronutrients. Under photoautotrophic conditions, carbon is usually supplied as CO_2-gas, whereas N and P are supplied from chemical fertilizers, mostly in the form of nitrate (NO_3), and phosphate (PO_4) salts. Both substrates are produced under high energy demands (Acién Fernández et al., 2018) and phosphate is mined and deposits are depleted at very high rates (Chavez and Uchanski, 2021). To produce 100 t microalgae biomass, approximately 200 t of CO_2, 10 t of N, and 1 t of P are required (Acién Fernández et al., 2018; Barbera et al., 2018). Many waste streams from industry, agriculture, or municipality contain large amounts of N and P and can therefore provide an alternative source for these nutrients in microalgal production. This would lead to more sustainable and resource-efficient microalgal production and simultaneous waste treatment, and can also contribute to reducing the high microalgal production costs (Acién Fernández et al., 2018; Wollmann et al., 2019).

Waste streams can also contain different dissolved organic carbon (DOC) fractions. Those can potentially be used as a carbon source by mixotrophic microalgae, which can in addition to photoautotrophic growth also grow heterotrophically on organic carbon. Mixotrophic cultivation reduces the microalgal dependency on light, as growth can proceed when dense cultures become light-limited. This leads to higher biomass concentrations and productivity compared to strict photoautotrophic cultivation and consequently further reduces overall production and energy costs (Abreu et al., 2012; Xia and Murphy, 2016). However, the presence of organic carbon sources also increases the risk of contamination of the microalgal culture, as other microorganisms such as bacteria, which naturally co-occur in most microalgal cultures can grow on the organic carbon substrate and compete for other

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nutrients (Pang et al., 2019).

Microalgal biomass production and simultaneous nutrient removal have been studied on a variety of different waste and residual streams (Ferreira et al., 2018), including, livestock (Chen et al., 2020), dairy (Daneshvar et al., 2019; Kiani et al., 2023), domestic (Ramanna et al., 2014) or urban wastewaters (Gouveia et al., 2016), aquaculture effluents (Daneshvar et al., 2018; Villar-Navarro et al., 2022), bio sludge from pulp and paper industry (Tao et al., 2017), or food waste (Kumar et al., 2022). Another residual stream that has been shown to contain large amounts of fertilizing nutrients is insect frass. Insect frass is an animal by-product (ABP) of insect production, consisting of solid excreta and exoskeletons from the insects (it may also include feeding substrate, dead eggs, and a limited content of dead farmed insects, according to Regulation (EU) 2021/1925), and can be up to 10 times more than the actual animal biomass production (Salomone et al., 2017). Mass breeding of insects as an alternative to conventional protein production for food and feed has been developing rapidly in recent years and is foreseen to further increase in the future (Barragán-Fonseca et al., 2022; Poveda, 2021). The large amount of insect frass that is produced along with the insects does not yet have a widespread application area but it is considered and used at a small scale as a sustainable organic fertilizer to enhance crop production and circular agriculture (Barragán-Fonseca et al., 2022; Carroll et al., 2023; Chavez and Uchanski, 2021), as it contains macro- and micronutrients with a similar range as commercial organic fertilizers (Gärttling and Schulz, 2022). However, insect frass as fertilizer for microalgal cultivation has, to our knowledge, not yet been explored.

In this study, we aimed to examine the efficacy of insect frass as a nutrient source for the cultivation of the microalgae *Chlorella vulgaris*. *C. vulgaris* is known for its high protein content and has been shown to grow mixotrophically on different carbon sources. We analyzed the nutritional composition of insect frass extracts upon two different sterilization techniques and performed a batch experiment in bubble columns to compare the growth and protein content of *C. vulgaris* cultures grown on insect frass medium to those grown in conventional growth medium. We also analyzed media nutrient concentrations and utilization (N, P, DOC) and the bacterial abundance and community composition.

2. Material and methods

2.1. Preparation of growth media

A package with 2 kg insect frass powder was obtained from Larveriet in Voss (Invertapro AS). The company produces mealworms (*Tenebrio molitor*) grown on organic waste for protein-rich feed and food. The obtained insect frass was sieved but not treated any further (when sold as plant fertilizer, the frass is heat-treated at 70 °C for 1 h, following legislation (Mattilsynet, 2020)). To make the nutrients available for microalgal cultivation, the frass was mixed with water to extract the nutrients, the remaining insoluble parts were removed from the liquid, and the solution was sterilized to avoid bacterial contamination. Two stock solutions were prepared to test the effect of two different sterilization techniques: autoclaving and sterile filtration (Fig. 1 A). For each stock solution 40 g insect frass was blended with 1 L reverse osmosis (RO) water, followed by 2 h incubation at room temperature. For the first stock solution (AC) the frass-water suspension was autoclaved, and insoluble particles were removed afterward by centrifugation (15 min,

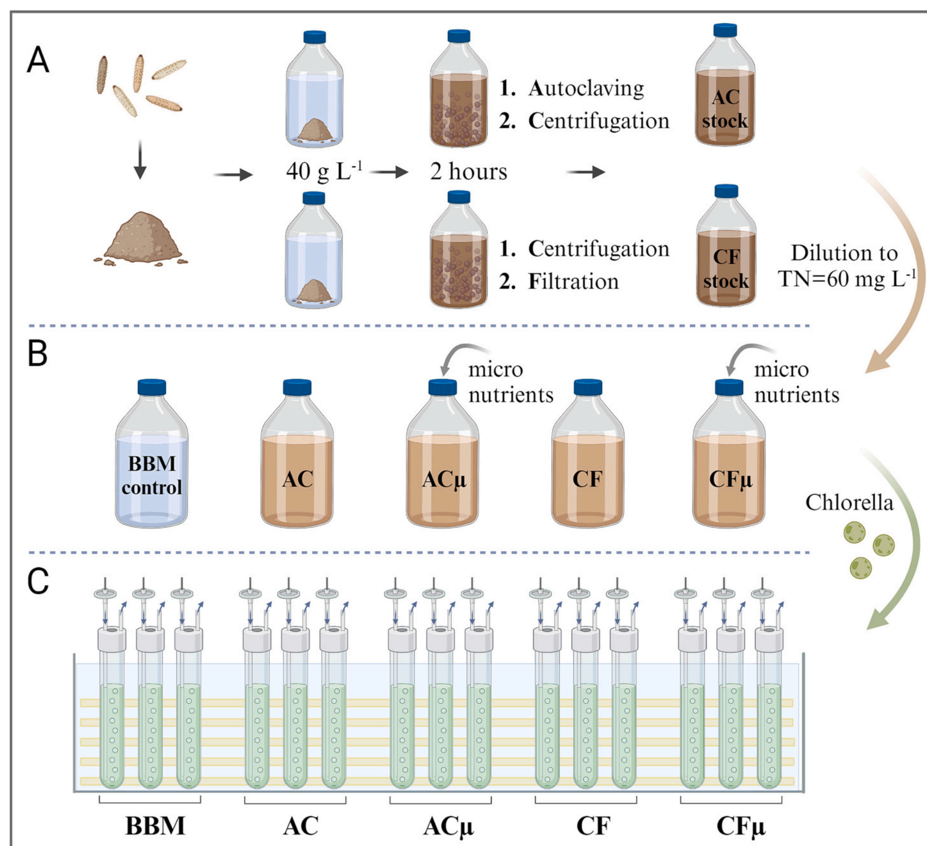

Fig. 1. Experimental setup for growth experiment of *Chlorella vulgaris* on insect frass. A: Preparation of two insect frass stock solutions. B: Preparation of five growth media with total dissolved nitrogen (TN) concentration of 60 mg L^{-1}. Two growth media were prepared from each frass stock solution, one only diluted (AC and CF) and one diluted and with micronutrient addition (ACμ and CFμ), and a control medium (BBM). C: Batch cultivation in bubble columns with three biological replicates for each growth medium. Created with BioRender.com.

5445 *g*), and the supernatant was collected in a sterile glass bottle. For the second stock solution (CF), insoluble particles were first removed by centrifugation, and the supernatant was collected and sterile-filtered (0.2 μm) into a sterile bottle. From each stock solution, dissolved organic carbon (DOC), total dissolved nitrogen (TN), and phosphate-phosphorous (PO_4-P) concentrations were measured.

For the following cultivation experiment, five different growth media of 1.2 L were prepared including one control medium and four insect frass media (Fig. 1 B). Each medium was prepared to have a TN concentration of 60 mg L^{-1} (4.4 mM). This concentration was chosen to allow the microalgae to grow dense enough to follow their growth, but to become N-limited before light limitation would occur, to assess the amount of TN that is bioavailable for the microalgae. As control medium, modified BBM medium was used, containing (in mM, unless otherwise stated): $NaNO_3$ 4.4; $CaCl_2{\cdot}2H_2O$ 0.17; $MgSO_4{\cdot}7H_2O$ 0.3; K_2HPO_4 0.43; KH_2PO_4 1.29; NaCl 0.43; Thiamine 0,297 μM; vitamin B12 0,0037 μM, Biotin 0,021 μM. Micronutrients were provided through a commercial trace metal mix with an end concentration of 0.04 g L^{-1} (YaraTera Rexolin APN, Boron 1.1 %, Copper 0.25 %, Iron 6 %, Manganese 2.4 %, Molybdenum 0.25 %, Zink 1.3 %, chelated with DTPA). From each insect frass stock solution (AC and CF) two frass media were prepared by blending the frass stock solution with RO water in a proportion to reach the desired TN concentration (water: stock ratio of 0.1 and 0.16 for AC and CF media, respectively). One of the two AC and CF media was supplemented with the trace metal mix APN, (0.04 g L^{-1} final concentration, as in the BBM control medium) to evaluate if micronutrients are limiting in the frass media (ACμ and CFμ). From each 1.2 L medium, 200 mL was removed to analyze nutrient concentrations (DOC, PO_4-P, TN, NH_4-N, NO_3-N, and Urea-N), and bacterial concentration and composition, and the remaining 1 L was used for the batch culture experiment.

2.2. Batch culture experiment

C. vulgaris strain Agf-ext-435 was provided by the Allmicroalgae's production unity, Pataias, Portugal, and stock cultures were kept in 50 mL Erlenmeyer flasks in sterile modified BBM medium, at 15 °C and an irradiance of 50 μmol m^{-2} s^{-1} (L:D cycle of 16:8 h). For inoculum production, biomass was transferred to two 300 mL glass tubes with fresh BBM medium, sealed with a rubber top, and placed in a temperature-controlled water bath (24 °C) with LED lights in the back (100 μmol m^{-2} s^{-1}, 24 h). For carbon supply and culture mixing, 0.2 μm filtered air, enriched with 1 % CO_2, was infused through glass tubing into the bottom of the glass tubes. The cultures were grown until an optical density (OD 750) of 0.7. The biomass was collected by centrifugation (5 min 1620 *g*) and resolved in 30 mL autoclaved RO water. Approximately 10 mL inoculum was added to each 1 L medium (BBM, AC, ACμ, CF, and CFμ) resulting in a starting OD750 of between 0.05 and 0.1.

Each 1 L starting culture was evenly distributed over three 300 mL glass tubes (three biological replicates for each growth medium) which were sealed with a rubber top and placed in a temperature-controlled water bath (24 °C) and aerated with 0.2 μm filtered air, enriched with 1 % CO_2, as described for inoculum production (Fig. 1 C). Irradiance (24 h) was increased accordingly with the increase in biomass concentration from 10 to 400 μmol m^{-2} s^{-1} (Table A.1) to avoid photoinhibition at the beginning of the cultivation and light limitation when the cultures became denser. Cultures were grown until three days of stationary phase were reached (10 days in total). The optical density and quantum yield (QY) of the cultures were monitored daily to follow microalgal growth. Samples for dry weight (DW) and protein analysis of the microalgal biomass were taken in exponential growth phase (Day 5). Samples for TN, NO_3-N, NH_4-N, and PO_4-P analysis were taken in exponential phase (Day 5), at the onset of stationary phase (Day 7), and after three days into stationary phase (Day 10). Samples for DOC analyses were taken in exponential phase and at the end of cultivation (Day 10). Samples for the enumeration of microalgae and bacteria and microbiome analysis were taken at the beginning of the cultivation (Day 0) and in exponential and stationary phases (Days 5 and 10 respectively). An overview of the sampling time points of the different analyses is shown in Table 1.

2.3. Analytical procedures

2.3.1. Daily measurements – OD and QY

Optical densities were measured on a spectrophotometer (V-1200, VWR®) at 750 nm, diluted to give an attenuation below 0.2. A blank was performed before each measurement with the respective medium and dilution. Maximum quantum yield (QY) was measured on the same sample with AquaPen (AquaPen-C, AP-C 100, Photon System Instruments, Brno, Czech Republic) after an initial 2-min dark incubation. Measurements were performed in duplicates.

2.3.2. Nutrient analysis

Nutrients analyzed from growth media and culture broth during the experiment included dissolved nitrogen (TN, NO_3-N, NH_4-N, and urea-N), phosphate-phosphorous (PO_4-P), and dissolved organic carbon (DOC). Before analysis, all samples were centrifuged (15 min, 5445 *g*) and filtered (0.2 μm, Puradisc™, Whatman™ 30 mm), and either measured instantly or stored at −20 °C until analysis. Samples with nutrient concentrations that were assumed or confirmed to be outside of the given measurement range were diluted with Milli Q water. NANOCOLOR® tube tests were used for TN and NH_4-N analysis and VISOCOLOR ECO® tube tests for NO_3-N and PO_4-P analysis. Samples were combined with the test-specific reagents in a 10 mL glass vial, as described in the respective kit instructions (https://www.mn-net.com/water-analysis/photometric-tests), and absorbance measured on a photometer PF-12 Plus (Macherey-Nagel, Switzerland). For urea-N analysis, the Invitrogen™ Urea Nitrogen (BUN) Colorimetric Detection Kit was used. Samples (technical duplicates) and reagents were combined in wells of a 96-well plate following the manufacturer's instructions, absorbance was measured at 450 nm on a Hidex Sense Microplate Reader, and concentrations were calculated using a standard curve. DOC concentrations were analyzed by high-temperature combustion using a TOC analyzer (Vario TOC cube) with an autosampler. Volumes of 8 mL (three technical replicates per sample) were transferred into a glass vial and placed in the autosampler of the TOC analyzer. Volumes of 50 mL hydrochloric acid (HCL 37 %) were added automatically to each sample while bubbling with synthetic air to remove dissolved inorganic carbon. Thereafter the samples were transferred into a high-temperature furnace where the organic carbon was converted into CO_2. A non-dispersive infrared (NDIR) detector was used to detect CO_2 and concentrations were quantified based on a standard (potassium

Table 1
Overview of the analysis parameters and sampling time points during the cultivation experiment.

Measurement	Growth medium	Start (Day 0)	Exp. phase (Day 5)	Stat. phase (Day 7)	Stat. phase (Day 10)
TN	x		x	x	x
NO_3-N	x		x	x	x
NH_4-N	x		x	x	x
Urea-N	x		x	x	x
PO_4-P	x		x	x	x
DOC	x		x		x
Microalgal cell concentration		x	x		x
Bacterial cell concentration	x	x	x		x
Bacterial community composition	x	x	x		x
Protein analysis			x		

hydrogen phthalate) that was measured at the beginning and the end of each sequence run. A summary of the nutrient analysis kits and instruments is available in the Appendix (Table A.2).

2.3.3. Bacterial and microalgal cell concentrations

For sample preparation, volumes of 1 mL (growth media) and 0.5 mL (cultures) were transferred into 2 mL cryotubes, fixated with glutaraldehyde (0.5 % final conc.) at 4 °C for a minimum of 30 min, flash frozen in liquid nitrogen and stored at −80 °C until analysis. For microalgal counts, thawed samples were diluted × 10–10,000 (dependent on culture density) with RO water. For bacterial counts, thawed culture samples were diluted × 10–50,000 (dependent on culture density) with RO water, while medium samples were measured undiluted and with × 10 dilutions, and cells were stained with a green fluorescent nucleic acid dye (SYBR Green I; Molecular Probes, Eugene, Oregon, United States) and incubated for 10 min in the dark. All samples were measured on a Guava EasyCyte™ Flow Cytometer (Cytek Biosciences) for 60 s. All measurements were performed with technical duplicates per biological replicate.

2.3.4. Microbiome analysis

Samples for microbiome analysis were stored in falcon tubes at −20 °C. DNA extraction, amplification, preparation for Illumina sequencing, and bioinformatic sequence analysis were performed as described in Steinrücken et al. (2023). In brief, volumes between 0.25 and 50 mL (depending on culture density) of thawed samples were filtered onto a 0.22 μm Durapore® Membrane Filter (Ø 47 mm, Merck-Millipore) and the DNeasy Power Soil Kit (Qiagen) was used for DNA extraction. One single extraction was performed for each biological replicate, as high similarity between technical replicates has been shown (Marotz et al., 2019; Wen et al., 2017). However, four samples were analyzed in duplicate for quality control of the sample preparation and sequencing. Furthermore, two control samples (blank filters) were included. The bacterial 16S rRNA gene V5-V7 region was amplified with a two-step nested PCR approach, using the chloroplast-excluding primers 799F and 1193R (Beckers et al., 2016) for the first PCR step. For the second PCR step, a specific forward and reverse primer combination with a distinct eight-nucleotide barcode was designated to each sample. Sequencing of the final PCR products was performed at the Norwegian Sequencing Center (Oslo, Norway) using the MiSeq platform (MiSeq Reagent Kit v3, Illumina, CA, United States) and illumina sequencing data are available at the European nucleotide archive (ENA) under study accession number PRJEB64266.

Obtained sequencing reads from the batch experiment were on average 12,167 ± 3992 (after quality filtering, merging, and excluding chimeric sequences), showing that high-quality sequencing was accomplished. The two control samples (blank filters) and the analyses from the five starting media had only 5–87 reads indicating that no bacteria or very low quantities were present in the starting media. The four duplicate analyses produced very similar bacterial communities, suggesting consistent sample preparation and sequencing results. Bacteria were identified to the genus level and reads of different OTUs assigned to the same bacterial genus were merged.

2.3.5. Protein Analysis

Culture volumes of 90 mL were centrifuged (5 min, 1620 *g*), the supernatant discarded, and the pellet frozen at −20 °C and freeze-dried (Edwards Freeze Dryer Modulyo). For protein extraction, approximately 10 mg freeze-dried biomass was transferred to 2 mL screw-cap tubes containing 0.5 mm grinding beads (Lysing Matrix Y, MP Biomedicals) and 1 mL lysis buffer (60 mM Tris pH 9, 2 % SDS) was added. Tubes were placed on a bead beater for 3 x 60s, then incubated for 30 min at 100 °C and centrifuged for 10 min (1807 *g*). The supernatant was diluted 4× with lysis buffer and the total protein concentration was determined according to the manufacturer's instructions in 96 well plates using the BioRad DC Protein Assay kit. Absorbance was measured at 750 nm on a Hidex Sense Microplate Reader, and protein concentrations were calculated using a standard curve of bovine serum albumin (BSA) with a concentration range between 0 and 1.4 mg mL^{-1}.

2.4. Statistics

The growth experiment was performed with three biological replicates for each growth medium. Two-way ANOVA was performed using GraphPad Prism 9 (RRID: SCR_002798), to test for significant differences in OD, QY, nutrient concentration, and microalgal and bacterial cell concentrations between the five media and between the different sampling time points. One-way ANOVA was performed to test for significant differences between the protein content of the cultures grown in different media.

3. Results and discussion

3.1. Nutrient recovery from insect frass

Two insect frass stock solutions (AC and CF) were prepared (40 g L^{-1}) to assess the effect of autoclavation (AC) and sterile filtration (CF) on nutrient recovery from the insect frass. After centrifugation, approximately 40 % of the water remained in the separated wet frass fraction, resulting in frass stock solutions (supernatant) of approximately 0.6 L. Both stock solutions were brownish in color (Fig. 2 A). The CF stock was clear, while the AC stock was slightly turbid, as centrifugation alone did not completely remove all solid particles. The nutrient concentrations of the stock solutions are presented in Fig. 2 B. The AC-stock had a 26 % higher DOC and 31 % higher TN concentration (2535 and 645 mg L^{-1}, respectively) compared to CF-stock (1884 and 445 mg L^{-1}, respectively), but the PO_4-P concentration was very similar for the two stock solutions (320 and 300 mg L^{-1} for AC and CF respectively). This indicates that autoclaving can recover additional dissolved carbon and nitrogen-containing molecules compared to incubation only.

The DOC, TN and PO_4-P content in the two frass extracts represent 6.3 and 4.7 % (DOC), 1.6 and 1.1 % (TN), and 0.8 and 0.8 % (PO_4-P) of the frass DW used for extraction, for the AC and CF treatments respectively (Fig. 2 B). Frass from yellow mealworm (*T. molitor*) larvae has been shown to have C, N, and P contents in the range of 39–42, 2.7–7.7, and 1–1.5 % of frass DW, respectively, depending on the diet provided (Poveda et al., 2019). The TN and P values of the two frass extracts were slightly below this range. This indicates that a great part of N and P could already be extracted from the frass by simply blending it with water, but that higher extraction efficiencies potentially could be achieved by applying additional extraction techniques. Prolonging the incubation time from 2 to 24 h did not increase the nutrient concentration in the frass stock culture (data not shown). Other pre-treatments like chemical, biological, or mechanical handlings could help make more N and P bioavailable for microalgal cultivation (Kumar et al., 2022). However, insect frass is not a uniform product, and its nutrient content and composition are strongly impacted by the insect species and the feeding substrates (Beesigamukama et al., 2022; Gärttling and Schulz, 2022).

3.2. Growth of *C. vulgaris* during the batch experiment

C. vulgaris was grown as batch culture in five different growth media with an initial TN concentration of approximately 60 mg L^{-1}. The media included the control medium (BBM), and the four frass-media AC and CF (diluted stock solution) and ACμ and CFμ (diluted stock solution supplemented with micronutrients). The four frass media were slightly darker in color compared to the control medium and both AC media were slightly turbid. Microalgal growth curves (based on OD 750) and maximum quantum yield during cultivation are shown in Fig. 3 A and B, respectively, and microalgal cell concentrations at the start and in exponential and late stationary phase (Days 0, 5 and 10, respectively) are shown in Fig. 3 C.

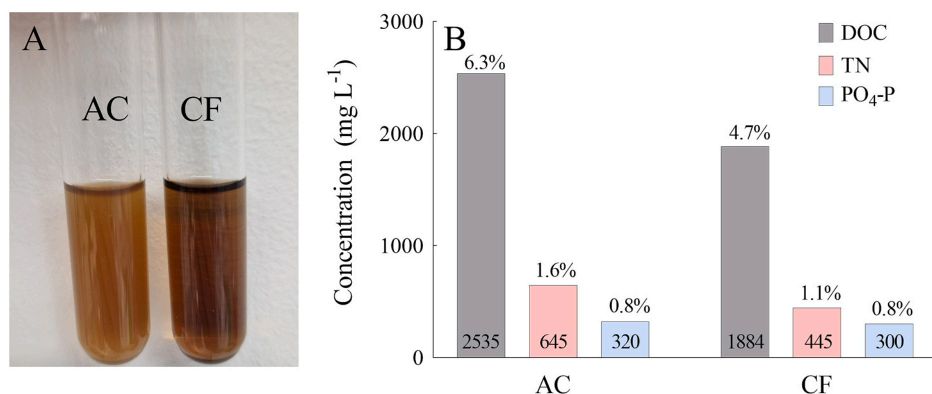

Fig. 2. Two insect frass stock solutions prepared with autoclavation and centrifugation (AC) or centrifugation and sterile filtration (CF). A: Picture of the two stock solutions. B: Dissolved organic carbon (DOC), total dissolved nitrogen (TN), and phosphate-phosphorous (PO_4-P) concentrations. Values at the bottom of the bars are concentrations in mg L^{-1} and values above the bars are percentages of the insect frass dry weight.

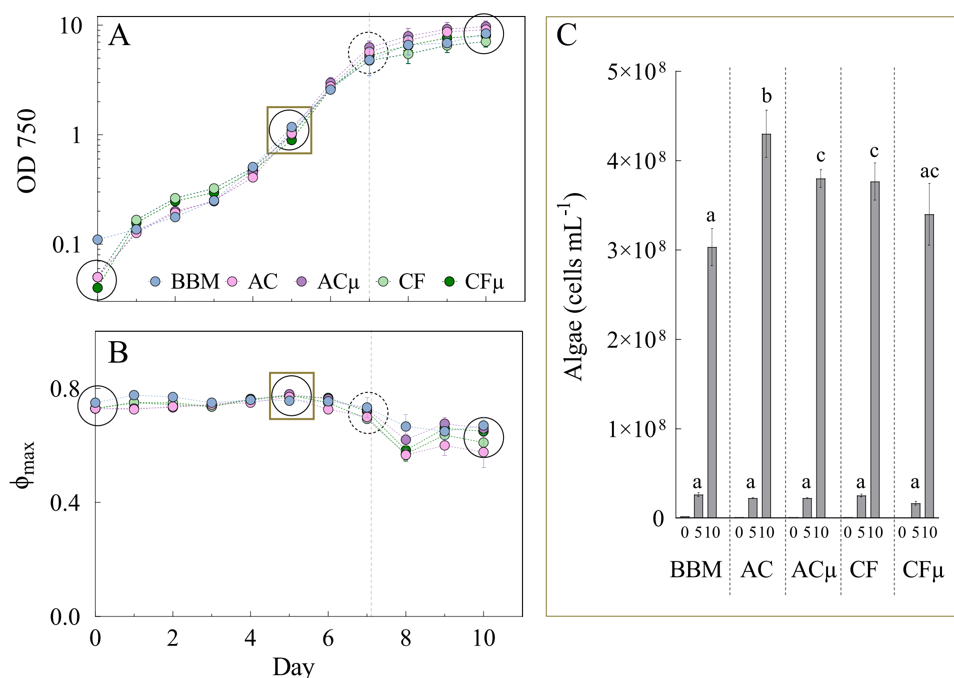

Fig. 3. Batch cultivation of *Chlorella vulgaris* in five different growth media. BBM = control medium, AC = medium prepared with autoclaved and centrifuged insect frass stock solution, CF = medium prepared with centrifuged and sterile filtrated insect frass stock solution, μ = micronutrient addition. Values show the average and standard deviation of three biological replicates. A: OD-based growth curves (logarithmic scale on Y-axis). B: maximum quantum yield. In A and B, black full circles indicate sampling time points for nutrient analysis (DOC, TN, NH_4-N, NO_3-N, Urea-N, PO_4-P), cell count, and microbiome analysis. Dashed black circles indicates additional nutrient analysis for TN, NH_4-N, NO_3-N, Urea-N, and PO_4-P. Squares indicate the sampling time point for microalgal protein analysis. Dashed grey lines indicate the start of stationary phase. C: *C. vulgaris* cell concentration for Days 0, 5 and 10. Same letters above bars indicate no significant differences ($p > 0.05$) between the growth media at the respective day.

Apart from the starting value, which was slightly higher for BBM cultures, the OD based growth curves were very similar for all five cultures (Fig. 3 A). After an initial lag phase and following exponential growth phase (Day 4–6), all cultures entered stationary phase on Day 7, after which the OD only slightly increased further until the end of cultivation on Day 10. Significant differences in OD values between the cultures were only observed after stationary phase was reached (from Day 7) but were inconsistent. No persistent significant differences were observed between the control and frass media (BBM, AC, CF) or between micronutrient treatments (AC vs ACμ or CF vs CFμ). The maximum quantum yield (QY) reflects the photosynthetic performance of photosystem II and decreasing values are associated with stressful growth conditions. The QY values during the experiment were also very similar for the five cultures with average values of 0.76 during the start of cultivation and exponential phase (Fig. 3 B). As cultures entered stationary phase (Day 7), the QY dropped and reached final values of 0.58–0.67 on Day 10. From Day 8 to 10, the QY was significantly lower for AC cultures compared to ACμ cultures, but no significant differences were observed between CF and CFμ cultures. With progressing stationary phase, the QY of AC cultures was also significantly lower compared to BBM and CFμ cultures, but not CF. The microalgal cell concentrations at the start of the experiment were between $8.2*10^5$ and $2.0*10^6$ cells mL^{-1}. Cell concentrations increased in exponential phase to values between $1.7*10^7$ and $2.6*10^7$ cells mL^{-1} with no significant difference between the media (Fig. 3 C). At the end of cultivation after three days of stationary phase, cell concentrations had reached values between $3.0*10^8$ and $4.3*10^8$ cells mL^{-1}. Here, significant differences in cell concentrations between the media were observed but with no consistent

pattern. Algae cell concentrations in the AC culture were significantly higher compared to the other cultures and cell concentrations in BBM cultures were significantly lower compared to AC, ACμ, and CF but not to CFμ.

For all three growth parameters, OD, QY, and algal cell concentration, no consistent significant differences between media groups (AC vs CF vs BBM) or between micronutrient treatments (AC and CF vs ACμ and CFμ) were found. Hence, the microalgae grew equally well in all growth media in this experimental setup. Since no consistent effect of micronutrient addition was observed on growth or QY, micronutrients did not seem to become limiting during batch cultivation in the frass media. However, to assess if all micronutrients were present in sufficient amounts in the frass extracts their actual concentrations need to be determined. A limitation of micronutrients might not have become apparent within the short time span of the experiment, as small amounts present within the microalgae cells, or the inoculum could have sustained the growth during the experiment. Also, the color of the frass media and the light turbidity of the AC media did not seem to have had a negative effect on the growth rates of the microalgae. In fact, from Day 0 to Day 1, the OD increased to a much greater extent for frass medium cultures compared to the BBM medium cultures.

3.3. Protein content of *C. vulgaris* during the batch experiment

The protein contents of *C. vulgaris* cultures during exponential phase (Day 5) are shown in Fig. 4. The values were 39.8 (±8.7), 38.9 (±3.2), 43.2 (±1.0), 38.1 (±1.4) and 44.3 (±0.3) % of DW for BBM, AC, ACμ, CF, and CFμ, respectively, and showed no significant differences ($p > 0.05$). Several studies have shown that the growth medium and the nutrient source and concentration can affect the biochemical composition and therewith the protein content of different microalgae. The protein content of *Dunaliella salina* was two times higher when grown on NH_4 compared to when grown on NO_3, while no impact on the growth rate was observed (Norici et al., 2002). Ferreira et al. (2018) found protein contents in *Scenedesmus obliquus* to vary (31–53 % DW) when grown in different wastewaters. However, the insect frass media did not affect protein content in *C. vulgaris* in this experiment and is therefore a promising growth medium with regards to microalgal protein production.

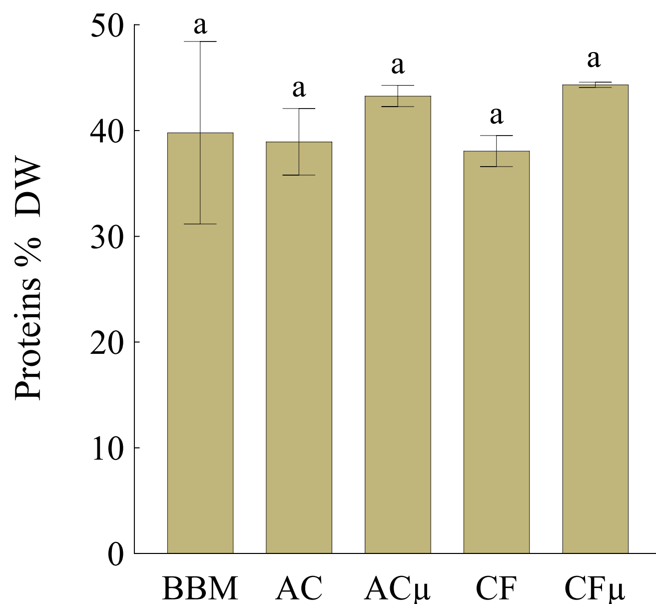

Fig. 4. Protein content of *Chlorella vulgaris* in % of dry weight (DW) during exponential phase. Values show the average and standard deviation of three biological replicates. Same letters above bars indicate no significant differences ($p > 0.05$).

3.4. Nutrient concentrations during the batch experiment

Fig. 5 shows the dissolved nitrogen (TN, NO_3-N, NH_4-N, Urea-N),

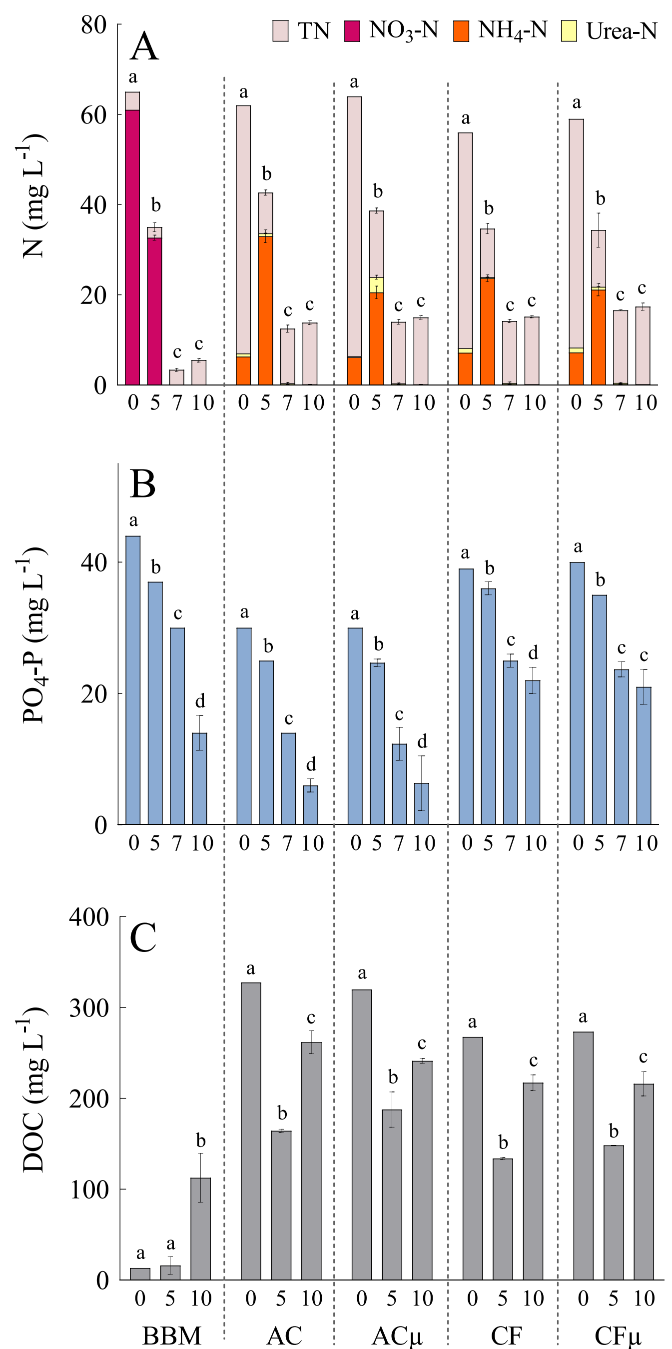

Fig. 5. Nutrient concentrations during batch cultivation of *Chlorella vulgaris* in five different growth media. A: Total nitrogen (TN) concentrations, superimposed with stacked values of ammonium-N (NH_4-N), nitrate-N (NO_3-N), and urea-N. B: Phosphate-phosphorous (PO_4-P) concentrations. C: Dissolved organic carbon (DOC) concentration. BBM = control medium, AC = medium prepared with autoclaved and centrifuged insect frass stock solution. CF = medium prepared with centrifuged and sterile filtrated insect frass stock solution, μ = addition of micronutrients. Values are the average and standard deviation of three biological replicates and are shown for Days 0, 5, 7, and 10 (A, B) or Days 0,5 and 10 (C). For each growth medium, concentrations with the same letter are not significantly different ($p > 0.05$). In A. significances are shown for TN and NO_3-N (BBM) and TN and NH_4-N (frass media).

phosphorous (PO_4-P), and DOC concentrations in the five different growth media during the batch experiment for the starting media (Day 0), exponential phase (Day 5) and after three days of stationary phase (Day 10). Nitrogen and phosphorus concentrations were additionally measured when cultures entered the stationary phase (Day 7).

3.4.1. Nitrogen

The TN starting concentrations were aimed at 60 mg L^{-1} for all media and were measured to be 65, 62, 64, 56, and 59 mg L^{-1}, for BBM, AC, ACμ, CF, and CFμ media, respectively (Fig. 5 A). In exponential phase, the TN concentrations had decreased significantly ($p < 0.05$) in all cultures to values between 30 and 43 mg L^{-1}. With the onset of stationary phase (Day 7), TN concentrations had further decreased to 3.4, 13, 14, 14, and 17 mg L^{-1} for BBM, AC, ACμ, CF, and CFμ media, respectively, and remained at this level until Day 10 for all cultures. Nitrate was the nitrogen source of the control medium (BBM) and accounted for 94 % of TN in both, starting medium and exponential phase cultures, and was below the detection limit in stationary phase (Days 7 and 10). The remaining TN fraction of 5–8 % in the control medium is most likely derived from the added trace metal mix, which is chelated with pentetic acid (DTPA, $C_{14}H_{23}N_3O_{10}$), accounting for approximately 6 % of TN. TN of the four insect frass media consisted at the start of the experiment of 10–13 % NH_4-N (6–7 mg L^{-1}) and 0.3–1.9 % urea-N (0.2–1 mg L^{-1}), while NO_3-N could not be detected (< 1 mg L^{-1}). Ammonium concentrations increased significantly in exponential phase in all four frass media, with values between 20 and 35 mg L^{-1}, accounting for 55–78 % of the TN. Urea concentration remained at the same levels but increased to 9 % of TN in ACμ cultures. In stationary phase (Days 7 and 10) both ammonium and urea were depleted (< 0.2 mg L^{-1}) in all frass media.

From the initial TN in the frass media, 71–78 % was consumed after the cultures reached stationary phase, while 22–29 % remained in the culture media also after three days of stationary phase. This indicates that a large fraction of the TN in the frass media could be consumed, while a smaller fraction was not bioavailable for the microalgae, and that the cultures were hence nitrogen limited from Day 7. Total nitrogen (TN) is the sum of NO_3, NO_2 (nitrite), $NH4^{+,}$ and organic nitrogen compounds. At the start of cultivation in the frass media, 10–15 % of the TN was present in the form of ammonium and urea, while no NO_3 (or NO_2-) could be measured. Hence, the majority of TN in the frass media appears to consist of organic nitrogen compounds such as proteins, peptides, or amino acids. Proteins have been shown to constitute a large part of insect frass. Wedwitschka et al. (2023) characterized six different insect frass samples and found the protein content in the frass to be between 5 and 29 % of the dry mass, depending on the feeding substrate. The increase in NH_4-N in exponential phase indicates the degradation of the organic nitrogen components in the frass media, releasing ammonium as a degradation product (Wedwitschka et al., 2023) that became available for microalgal consumption.

Another potential source of TN in the insect frass media could be chitin. Chitin is a major component of the exoskeletons of insects. During larval development, the exoskeletons are shed several times and become a component of the insect frass (Chavez and Uchanski, 2021; Hahn et al., 2022; Quilliam et al., 2020). So far, chitin-catabolizing microorganisms have been known to be heterotrophic bacteria and archaea. However, Blank and Hinman (2016) could show that different axenic strains of eukaryotic microalgae including *C. vulgaris* were capable of growing on chitin (and/or chitosan), showing comparable growth rates to conventional nitrogen sources. They also showed that chitin utilization is ecologically widespread in phototrophic organisms. However, chitin is also known to be highly insoluble in water and other organic solutions, which makes it unlikely that it was present in the frass extracts.

3.4.2. Phosphorous

The PO_4-P concentrations during the experiment are shown in Fig. 5 B. The starting PO_4-P concentrations differed between the different growth media with the highest values for BBM (44 mg L^{-1}), followed by CF media (39 and 40 mg L^{-1} for CF and CFμ, respectively) and AC media (30 mg L^{-1} for both, AC and ACμ). The different starting PO_4-P concentrations between the two insect frass media (AC and CF) are due to the different volumes of stock solution that were used. Phosphate-P concentrations decreased continuously in all media from the start to exponential phase, and further to the end of cultivation on Day 10, but was not consumed completely. Phosphorous concentrations continued to decrease in stationary phase from Day 7 to Day 10, although microalgal growth had stopped and nitrogen was depleted. This can most probably be linked to a process called luxury uptake, where microalgae take up more phosphorous than necessary for immediate growth and accumulate it within their cells as polyphosphate (Brown and Shilton, 2014; Solovchenko et al., 2019). Interestingly the decrease in PO_4-P from Day 7 to Day 10 was much more distinct in BBM and AC media compared to CF media. In total, PO_4 uptake was highest in the AC and ACμ media with 80 and 78 % after 10 days, respectively, compared to BBM (68 %) and CF and CFμ cultures (44 and 48 %, respectively).

3.4.3. DOC

The DOC concentration during the experiment is shown in Fig. 5 C. Low DOC amounts were measured in the control starting medium (BBM, 13 mg L^{-1}) where no organic carbon was added. This DOC fraction is most probably derived from the pentetic acid (DTPA, $C_{14}H_{23}N_3O_{10}$) from the trace metal mix, with calculated DTPA-C concentrations of approximately 15 mg L^{-1}. DOC concentration remained at the same level in exponential phase (Day 5) but increased significantly to 113 mg L^{-1} in stationary phase (Day 10). In the insect frass media, DOC was present in the starting media and was higher in the AC media (327 and 320 mg L^{-1} for AC and ACμ, respectively) than in the CF media (268 and 273 mg L^{-1} for CF and CFμ, respectively). A significant decrease in DOC concentration of 50, 41, 50, and 46 % was observed from the start to exponential phase (Day 5), followed by a subsequent significant increase to stationary phase (Day 10) of 37, 22, 38, and 31 % for AC, ACμ, CF and CFμ cultures, respectively.

The strong decrease in DOC concentration from the cultivation start to exponential phase in the frass media indicates that DOC was consumed during microalgae cultivation. However, if the DOC was consumed by the microalgae cannot be determined from this study. The microalgae were N-depleted from Day 7 before light limitation could occur. If the microalgae were able to utilize the DOC in the media an effect would most probably become apparent once the microalgae become light-limited. Although mixotrophic growth of *Chlorella* has been reported, it has been demonstrated mostly on low molecular weight molecules (Li et al., 2013). The strong increase in bacterial abundance in the frass media (Fig. 6) suggests that DOC was consumed by the associated bacteria in the culture. A following increase in DOC concentration from exponential phase to Day 10 of stationary phase occurred in all cultures, including the control (BBM). This is most probably attributed to the onset of cell senescence and death due to nitrogen depletion, leading to a release of organic carbon compounds from the microalgal cells back into the solution.

3.5. Bacterial cell concentration and community composition during cultivation

Bacterial cell concentrations and community composition were analyzed from the starting growth media, and during the experiment from the start (Day 0), exponential phase (Day 5), and after three days of stationary phase (Day 10; Fig. 6). In the starting growth media, bacteria were absent or below the detection limit for both methods, cell count and microbiome analysis, indicating that bacteria derived from the *C. vulgaris* culture inoculum.

At the start of the experiment, the bacterial cell concentrations in the five different cultures were between $6.0*10^4$ and $1.4*10^5$ cells mL^{-1}

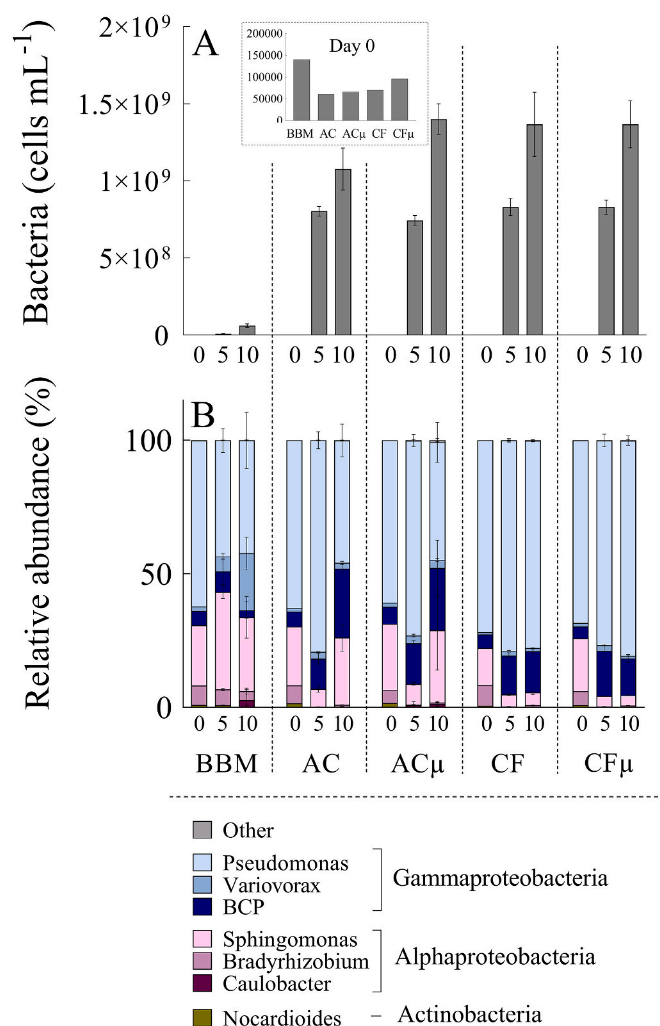

Fig. 6. Associated bacteria during batch cultivation of *Chlorella vulgaris* in five different growth media for Days 0, 5 and 10. A: Bacterial cell concentrations at the respective sampling time points. B: Taxonomic profiles at the genus level, genera with <1 % abundance were merged into "others". BBM = control medium, AC = medium prepared with autoclaved and centrifuged insect frass stock solution. CF = medium prepared with centrifuged and sterile filtrated insect frass stock solution, μ = micronutrient addition. On Day 0 samples were taken from the starting culture (one biological replicate per media). Values for Days 5 and 10 show the average and standard deviation of three biological replicates.

(Fig. 6 A). The bacterial abundance progressively increased in exponential and stationary phases in all media, but the increase was significantly lower for BBM cultures (9.0*10^6 and 6.1*10^7 cells mL^{-1} in exponential and stationary phase, respectively) compared to the frass media (7.4–8.3*10^8 and 1.1–1.4*10^9 cells mL^{-1} in exponential and stationary phase, respectively). In the control medium, no organic carbon was supplemented and the only available organic carbon source for bacteria was DOC released by the microalgae (Le Chevanton et al., 2013), which naturally sets a limit to their development. The presence of DOC in the four frass media promoted bacterial growth, leading to high bacterial concentrations. However, the high bacterial abundance did not seem to have any impact on microalgal growth.

To identify if specific bacteria would benefit from the DOC in the frass media, the bacterial community composition was analyzed. The analyses revealed a total of 38 bacterial OTUs representing 16 different bacterial genera. Most bacteria (> 98 %) were represented by 7 bacterial genera, belonging to the classes *Alphaproteobacteria*, *Gammaproteobacteria*, and *Actinobacteria* (Fig. 6 B). The bacterial community composition at the start of cultivation (Day 0) was very similar in all five media, with *Pseudomonas* representing the most dominant fraction (61–72 %) followed by *Sphingomonas* (14–25 %), *Bradyrhizobium* (5–8 %) and *Burkholderia-Caballeronia-Paraburkholderia* (*BCP*, 4–7 %) and low concentrations of *Variovorax* and *Nocardioides* (both 1–2 %). In the following exponential and stationary phases (Days 5 and 10, respectively), the bacterial community remained very similar between the different media, but the relative bacterial abundances changed slightly and to different degrees. In BBM medium, the relative abundance of *Variovorax* increased gradually until stationary phase, while it remained at low levels in the frass media. In all frass media, the relative abundance of *BCP* increased towards stationary phase while it did not in the control BBM medium. In the CF media, *Pseudomonas* relative abundance remained at the same levels while relative abundances decreased in the control medium and the AC media. The increase in relative abundance of *BCP* in the frass media suggests that especially this genus could benefit from the DOC in the frass media.

3.6. Future perspectives

Based on the experimental set-up of this study, insect frass appears to be a suitable nutrient source for microalgal cultivation. However, to further evaluate its actual applicability, especially in terms of larger-scale cultivation and high biomass production, more detailed investigations are necessary. While N and P were monitored during the experiment, the micronutrient concentrations and utilization were not determined. Although, micronutrient supplementation (APN) did not affect microalgal growth, and literature shows that insect frass has adequate concentrations of micronutrients (Beesigamukama et al., 2022), an exact analysis of the content and concentration of the essential micronutrient in the extracts is important to determine whether they can sustain microalgal growth also at higher densities and over a prolonged cultivation period. Small deficiencies might not directly affect the growth in a short batch experiment but could become evident when cultivated continuously for a longer time.

Under production conditions, the nitrogen concentrations in the growth media would need to be increased by 3–5 times to allow for high cell concentrations and avoid nitrogen limitation during the cultivation process. Hence, larger volumes of stock solutions would need to be added for media preparation which would result in a darker color (and increased turbidity for AC) of the growth media, which could decrease the light availability for the microalgae and reduce microalgal growth (Wang et al., 2010; Xia and Murphy, 2016). At the same time, this would also increase the DOC concentration in the medium which could affect the bacterial concentrations and their effect on the microalgal culture. Although the bacteria did not seem to have affected microalgal growth in this experiment, under longer cultivation conditions and higher DOC concentrations, bacterial concentrations could significantly increase further and potentially outcompete the microalgae or even cause a collapse of the culture. Whether the DOC in the frass medium can also be used by the microalgae and thus support mixotrophic growth is another aspect that needs to be investigated under actual production and light-limiting conditions. Finally, one major aspect that needs to be evaluated is the impact of insect frass on the sustainability and economy of microalgal production and its contribution to a circular bioeconomy approach. Great amounts of insect frass would be necessary for large-scale microalgal production requiring previous processing for nutrient extraction and sterilization. The solid part of the insect frass remains as a waste product after nutrient extraction, therefore other application areas for this waste product would need to be established.

4. Conclusion

Our study is the first to show that insect frass is suitable as a basis for a growth medium for microalgal cultivation. At the tested conditions, *C. vulgaris* was able to utilize most of the provided nutrients from the

insect frass extracts while showing the same growth pattern and protein content as when grown in a standard control medium. It is important to further evaluate the feasibility of insect frass as a nutrient supplement at larger and continuous cultivation conditions and to identify its impact on the sustainability and economy of microalgal production.

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CRediT authorship contribution statement

Pia Steinrücken: Conceptualization, Investigation, Formal analysis, Methodology, Visualization, Writing - original draft preparation. **Oliver Müller**: Formal analysis, Methodology, Writing - review & editing. **Hanna Böpple**: Conceptualization, Methodology, Writing - review & editing. **Dorinde Kleinegris**: Conceptualization Writing - review & editing.

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

Illumina sequencing data are available at the European nucleotide archive (ENA) under study accession number PRJEB64266.

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Appendix A

Table A.1
Irradiance (μmol photons m^{-2} s^{-1}) and OD 750 values during the batch experiment.

Day	0	1	2	3	4	5	6	7	8	9	10
Irradiance	10	10	20	40	80	160	320	400	400	400	400
OD_{750} BBM	0.11	0.13	0.18	0.25	0.51	1.17	2.62	4.80	6.59	6.86	8.39
OD_{750} AC	0.05	0.13	0.19	0.25	0.41	1.02	2.75	5.70	7.28	8.66	9.10
OD_{750} ACμ	0.05	0.13	0.20	0.25	0.45	1.09	2.97	6.27	7.93	9.23	9.70
OD_{750} CF	0.05	0.17	0.27	0.32	0.49	1.00	2.68	4.76	5.46	6.49	7.08
OD_{750} CFμ	0.04	0.16	0.25	0.30	0.46	0.89	2.63	5.25	6.53	7.63	8.03

Table A.2
Test kits or instrument used for different nutrient analysis. Instructions for the different test kits can be found on the manufacturers or distributers website.

Test kit / instrument	Parameter	Range (mg L^{-1})	Manufacturer
NANOCOLOR total-Nitrogen TN_b	TN	5–220	MACHEREY-NAGEL
NANOCOLOR total-Nitrogen TN_b	TN	0.5–22	MACHEREY-NAGEL
NANOCOLOR Ammonium 3	NH_4-N	0.04–2.3	MACHEREY-NAGEL
Invitrogen™ Urea Nitrogen (BUN)	Urea-N	0.07–23	Invitrogen™ EIABUN
VISOCOLOR ECO Nitrate	NO_3-N	1–14	MACHEREY-NAGEL
VISOCOLOR ECO Phosphate	PO_4-P	0.2–5	MACHEREY-NAGEL
TOC analyzer Vario TOC cube	TOC	1–10	Elementar

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