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From waste to products: microalgal cultivation in insect frass to obtain valuable biomass

Alessandra Petrucciani [a,b], Lorenzo Mollo [a,*], Simona Anna Siena [a], Behixhe Ajdini [a], Irene Biancarosa [c], Cristina Truzzi [a], Alessandra Norici [a,b]

[a] Department of Life and Environmental Sciences, Università Politecnica delle Marche, Via Brecce Bianche, 60131, Ancona, Italy
[b] CIRCC, consorzio interuniversitario reattività chimica e catalisi, Italy
[c] Nutrinsect SRL, Via Villa Enrico Mattei, 65, 62010, Montecassiano, MC, Italy

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ABSTRACT

Insect frass, a residual by-product of insect farming, is increasingly considered for circular-bioeconomy applications. Here we quantitatively evaluated *Acheta domesticus* frass as the sole nutrient source for *Chlorella vulgaris*, *Nannochloropsis salina* and *Phaeodactylum tricornutum*, using frass loadings equivalent to control nitrogen supplies (4.12 g L^{-1} for freshwater species; 250 mg L^{-1} for marine species) under both autoclaved and non-autoclaved conditions. *P. tricornutum* did not survive acclimation. In non-autoclaved frass, *C. vulgaris* reached a μmax of 0.62 d^{-1} and final cell density of 1.1 × 10^7 cells·mL^{-1}, similar to control, while *N. salina* attained 6.5 × 10^6 cells·mL^{-1} but with growth rate reduced by ~50 %. Autoclaving reduced maximum biomass yields by 30–40 % relative to non-autoclaved cultures, although growth rates remained similar. Growth in frass shifted algal biochemical composition toward lower protein content, whereas lipid fractions remained similar to controls. Fatty acid profiles showed increased saturated fatty acids (16:0, 18:0) and reduced levels of 18:2n-6 and 18:3n-3, with an overall decline in PUFA content. In summary, insect frass supported algal growth but yielded lipid characteristics more suitable for biodiesel than for PUFA-rich feed.

1. Introduction

The 17 Sustainable Development Goals (SDGs) set by the United Nations identify key global challenges that demand immediate and coordinated efforts to ensure the health of both humanity and the environment. Among these challenges, the sustainable production of food, feed, and energy together with the fight against environmental pollution are major objectives of scientific research. To move toward these goals, two strategies have gained increasing attention: the cultivation of microalgae and the farming of insects. Microalgae are investigated as sustainable feedstocks for multiple applications, including biofuels (D'Ippolito et al., 2015; Hildebrand et al., 2012; Watson et al., 2021), food or feed supplements (Amorim et al., 2021; Dineshbabu et al., 2019; Lamminen et al., 2019; Pudney et al., 2019), cosmetic ingredients (Mourelle et al., 2017), fertilisers and biostimulants (Braun and Colla, 2023; Mollo et al., 2025; Mollo and Norici, 2025), and bioremediation (Hedayatkhah et al., 2018; Mojiri et al., 2020). Despite their versatility, algal cultivation is still cost-intensive and limited to small-scale production (Fernández et al., 2021; Vázquez-Romero et al., 2022). At the same time, insect farming has rapidly expanded in Europe and elsewhere as a sustainable source of protein for food and feed (Barragán-Fonseca et al., 2022; Poveda, 2021), but it also generates large amounts of residual streams, particularly frass — typically three times the mass of the insects produced (pers. comm. Nutrinsect SRL). Frass consists of insect excreta mixed with substrate residues, cuticle fragments and a small fraction of dead insects (Commission Regulation (EU) 2021/1925; Salomone et al., 2017; Steinrücken et al., 2024), and its reuse has mainly been explored as a fertiliser (Poveda, 2021).

Efforts to reduce algal cultivation costs and to integrate circular economy principles have led to the use of waste streams as alternative nutrient sources. Residual matrices tested include domestic or urban wastewaters (Chieti et al., 2024), pulp and paper biosludge (Silva et al., 2021), aquaculture sludge (Daneshvar et al., 2018; Villar-Navarro et al., 2022), agrifood residues (Daneshvar et al., 2019; Kiani et al., 2023; Kumar et al., 2022; Mollo et al., 2023), and livestock waste (Blanco-Vieites et al., 2024). These studies consistently reported dual benefits:

* Corresponding author.
E-mail addresses: a.petrucciani@staff.univpm.it (A. Petrucciani), l.mollo@staff.univpm.it (L. Mollo), S1105578@studenti.univpm.it (S.A. Siena), b.ajdini@staff.univpm.it (B. Ajdini), biancarosairene@gmail.com (I. Biancarosa), c.truzzi@staff.univpm.it (C. Truzzi), a.norici@staff.univpm.it (A. Norici).

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waste remediation and algal biomass production. Within this framework, frass has recently been investigated as a nutrient source for *Chlorella vulgaris* (Steinrücken et al., 2024), showing maximum cell densities comparable to those in standard medium when frass was simply added to water.

Nonetheless, regulatory standards for food and feed applications remain particularly stringent. Therefore, the most realistic sectors for valorising algal biomass grown on waste-derived substrates include biodiesel, aquaculture feed, and agricultural inputs (A Talip et al., 2019; Apandi et al., 2019; Chen et al., 2018; Lima e Silva et al., 2024; Viegas et al., 2021). The lipid composition of algae is central in this respect, as it underpins both the caloric performance of biodiesel (Monirul et al., 2015; Ruiz-Marin et al., 2018; Sharma et al., 2021) and the nutritional quality of animal feed (Bell et al., 1995; Calder, 2015; Tocher, 2010). Microalgae are also widely recognised as fertilisers or biostimulants, especially in circular economy projects that couple wastewater remediation with biomass reuse (Acién Fernández et al., 2018; Lage et al., 2018; Stiles et al., 2018). Their biomass can act as a slow-release nutrient source (Mulbry et al., 2005; Piltz and Melkonian, 2018) or as a biostimulant extract rich in carbohydrates, amino acids and phytohormones that enhance root architecture and stress tolerance (Chiaiese et al., 2018; Mollo and Norici, 2025; Ronga et al., 2019).

Against this background, the present work investigated the growth of the freshwater species *Chlorella vulgaris* and the marine species *Phaeodactylum tricornutum* and *Nannochloropsis salina* in frass-based media. These species were chosen because of their established industrial relevance and expanding markets: *C. vulgaris* is widely commercialised in food, nutraceutical and fuel sectors (Al-Hammadi and Güngörmüşler, 2024), while *N. salina* and *P. tricornutum* are oleaginous, EPA-rich microalgae of growing importance in aquaculture and biofuels (Borges et al., 2011; Mutanda et al., 2020; Seychelles et al., 2009; Sunil et al., 2024; Xin et al., 2024). We compared growth performance in frass-water media and standard controls, and analysed biomass composition with particular attention to lipids, a key determinant for both feed and biofuel applications.

2. Material and methods

2.1. Experimental design

The freshwater microalga *Chlorella vulgaris* (CCAP 211/11b) and the marine microalgae *Nannochloropsis salina* (CCAP 849/3) and *Phaeodactylum tricornutum* (DCG 0981) were chosen as experimental species and maintained in freshwater BG11 medium (Allen and Stanier, 1968) or marine AMCONA medium (Fanesi et al., 2014).

Insect frass was obtained from the insect rearing company Nutrinsect SRL (Montecassiano, Italy) and resulted from sub-adults of house crickets (*Acheta domesticus*). The frass was sieved to eliminate dead individuals and feed residues and stored at −80 °C until further use. According to frass composition (Table 1), proteins constituted 36 % of the frass dry weight: since 17 % of proteins is made of nitrogen (Mariotti et al., 2008; Sosulski and Imafidon, 1990), the nitrogen content in the frass was calculated to be 6 % (w/w). We normalized frass additions to this value of protein-derived nitrogen and no external nutrients were supplemented in line with a circular approach aimed at testing frass as a stand-alone resource. Moreover, since the silicon (Si) content was found in frass, no extra silicon was added to meet the diatom requirement for growth.

2.1.1. Microalgae grown in frass-water medium

A frass-based growth medium was prepared by adding a specific amount of thawed, non-sterile frass to either autoclaved deionized water (freshwater mimic) or autoclaved 0.5 M NaCl solution (seawater mimic). The frass was stored frozen prior to use. The amount of frass was calculated to match the nitrogen concentration of standard control media, based on a measured N content of 6 % in the frass (see Section 2.1). For the freshwater medium, 4.12 g L^{-1} of frass was added to match the 250 mg L^{-1} of nitrogen in the BG11 control medium. For the marine medium, the target was 7.7 mg L^{-1} of nitrogen, equivalent to the AMCONA medium. However, to account for the potentially low bioavailability of nitrogen from frass proteins, twice the theoretically required amount was used, resulting in the addition of 250 mg L^{-1} of frass. After preparing the media, the inorganic N composition and the overall microelement composition was assessed.

Table 1
Frass composition according to the analysis provided by Nutrinsect SRL.

Crude proteins	36 %
Crude lipids	0.83 %
Calcium (Ca)	2.537 %
Phosphorous (P)	1.510 %
Magnesium (Mg)	0.762 %
Potassium (K)	3.137 %
Chlorine (Cl)	1.169 %
Sodium (Na)	0.822 %
Sulphur (S)	0.635 %
Silicon (Si)	1.51 %
Aluminium (Al)	658 mg kg^{-1}
Zinc (Zn)	576 mg kg^{-1}
Cupper (Cu)	50 mg kg^{-1}
Iron (Fe)	902 mg kg^{-1}
Manganese (Mn)	480 mg kg^{-1}
Chromium (Cr)	27 mg kg^{-1}
Lead (Pb)	5 mg kg^{-1}
Titanium (Ti)	42 mg kg^{-1}
Bromine (Br)	10 mg kg^{-1}
Arsenic (As)	0.85 mg kg^{-1}
Iodine (I)	1.4 mg kg^{-1}

Nutrient levels in media were determined using an EasyChem Plus automated discrete analyser (SYSTEA s.p.a., Frosinone, Italy) equipped with standard colorimetric methods. Quantification was performed using external calibration curves. Nitrate ($N–NO_3$) was measured following the National Environmental Methods Index 9171, which involves the reduction of nitrate to nitrite by an acid solution of vanadium (III) chloride solution. The nitrite fraction present in the sample is subtracted automatically through the use of a sample blank, allowing for direct measurement of nitrate. Ammonium concentrations were determined based on a modified version of EPA Method 350.1. Under strongly alkaline conditions (pH 12.6), ammonium ions react with sodium salicylate and hypochlorite in the presence of sodium nitroprusside, yielding a chromophore with a characteristic emerald-green colour. Absorbance was measured at 670 nm using a spectrophotometric analyser (Fanelli et al., 2022). Trace element analysis was performed using a Total Reflection X-ray Fluorescence spectrometer (S2 Picofox, Bruker AXS Microanalysis GmbH, Berlin, Germany), following the protocol described by Petrucciani et al. (2022). Media characterization was implemented, determining pH and water clarity spectrophotometrically (UV-1900i, SHIMADZU CORP.), recording the absorbance at 440 nm (Ask et al., 2009; Pace et al., 2012); finally, results were compared to the composition of the respective control medium (Table 2).

Triplicates of algal cultures were established in 150 mL Erlenmeyer flasks filled with 50 mL of control or frass - water saline or non-saline media to measure growth; they were maintained in climatic chamber with controlled temperature of 20 °C and illuminated with fluorescent lamps (100 μmol photons m^{-2} s^{-1}, 24 h light). Microalgae were acclimated to these media for at least 10 generations before the growth analysis.

2.1.2. Microalgae grown in autoclaved frass-water medium

C. vulgaris (CCAP[1] 211/11b) and *N. salina* (CCAP 849/3) were then

[1] https://www.ccap.ac.uk/.

Table 2
Nutrient concentrations measured in frass–water media compared to the inorganic composition of the control media (as per recipe). Values lower than those in the corresponding control are shown in bold. Heavy metals are indicated with an asterisk (*).

	 Frass – water non-saline medium	BG11	 Frass – water saline medium	AMCONA
Nitrogen as NO_3	**0.005 ± 0.001 mg·L^{-1}**	250 mg·L^{-1}	**0.013 ± 0.002 mg·L^{-1}**	7.7 mg·L^{-1}
Nitrogen as NH_4	7.8 ± 0.2 mg·L^{-1}	/	0.9 ± 0.4 mg·L^{-1}	/
Phosphorous	30 ± 0.3 mg·L^{-1}	8.9 mg·L^{-1}	5 ± 1 mg·L^{-1}	0.62 mg·L^{-1}
Sulphur	21 ± 0.01 mg·L^{-1}	9.79 mg·L^{-1}	**11 ± 3 mg·L^{-1}**	80.087 mg·L^{-1}
Bromide	0.135 ± 0.001 mg·L^{-1}	/	**6 ± 0.006 mg·L^{-1}**	54 mg·L^{-1}
Magnesium	9 ± 0 mg·L^{-1}	7.4 mg·L^{-1}	**Not detectable**	1.002 g·L^{-1}
Boron	Not detectable	0.5 mg·L^{-1}	Not detectable	4 mg·L^{-1}
Fluorine	/	/	Not detectable	1 mg·L^{-1}
Potassium	98 ± 1 mg·L^{-1}	18 mg·L^{-1}	**6 ± 0.006 mg·L^{-1}**	0.338 g·L^{-1}
Silicon	/	/	**Not detected**	5.74 mg·L^{-1}
Copper	0.28 ± 0.03 mg·L^{-1}	0.02 mg·L^{-1}	0.026 ± 0.002 mg·L^{-1}	2.54 µg·L^{-1}
Manganese	**0.27 ± 0.005 mg·L^{-1}**	0.502 mg·L^{-1}	**0.013 ± 0 mg·L^{-1}**	0.13 mg·L^{-1}
Zinc	0.34 ± 0.007 mg·L^{-1}	0.02 mg·L^{-1}	0.061 ± 0.01 mg·L^{-1}	0.016 mg·L^{-1}
Calcium	17 ± 0.8 mg·L^{-1}	9.6 mg·L^{-1}	**3 ± 0.03 mg·L^{-1}**	0.367 g·L^{-1}
Iron	**0.32 ± 0.02 mg·L^{-1}**	1.2 mg·L^{-1}	**0.08 ± 0.02 mg·L^{-1}**	0.366 mg·L^{-1}
Nichel	0.036 ± 0 mg·L^{-1}	/	0.02 ± 0.01 mg·L^{-1}	0.37 µg·L^{-1}
Cobalt	**Not detected**	9.9 µg·L^{-1}	**Not detected**	0.33 µg·L^{-1}
*Chromium**	**0.01 ± 0.0007 mg·L^{-1}**	/	Not detected	/
*Arsenic**	**0.002 ± 0 mg·L^{-1}**	/	Not detected	/
*Lead**	Not detected	/	**0.02 ± 0.01 mg·L^{-1}**	/
pH	7.08	7.6 *(buffered with Tris HCl)*	6.87	8.3 *(buffered with Tris HCl)*
Water clarity (Abs 440 nm)	0.31	0	0.02	0

cultivated in batch cultures in the second phase of the experiment. The media, unlike the initial phase, were prepared by autoclaving frass together with deionized water (again, 4.12 g L^{-1} and 250 mg L^{-1} of frass for non-saline and saline medium, respectively), hereafter called autoclaved frass – water media. Autoclaving was chosen because during the initial acclimation phase non-autoclaved frass media developed contaminating protist-like organisms, likely originating from resistant spores, which compromised culture stability. Thus, sterilization was applied to ensure reproducible and uncontaminated algal growth, from which biomass could reliably be harvested for characterization.

After preparing the media, they were characterized as described in Section 2.1.1 and results were compared to the composition of their respective control media (Table 3).

Triplicates of algal cultures were established in 150 mL Erlenmeyer flasks filled with 50 mL of control or autoclaved frass - water media, maintained at temperature ranging between 19 and 23 °C and illuminated with fluorescent lamps (250 µmol photons m^{-2} s^{-1}, 24 h light). Microalgae were acclimated to these media for at least 10 generations before the growth analysis.

Triplicates of 1 L cultures of *C. vulgaris* and *N. salina* were established and maintained as previously mentioned for biomass characterization. Cells were harvested in late exponential phase, filtered with mesh size of 150 µm to eliminate frass residual and centrifugated for 15 min 6000 rpm. Biomass was washed with deionized water to remove the growth salts before further analysis.

2.2. Algal growth

A fixed number of 1 × 10^5 exponentially growing cells was inoculated in control and frass - water media; growth was followed by daily cell count. Each culture was allowed to grow until it reached the stationary phase, and the experimental timeline was adjusted accordingly. Cell density and size distribution were assessed using a CASY TT Cell Counter (Innovatis AG, Reutlingen, Germany). Samples (80 µL) were diluted in 8 mL of CASY TON electrolyte solution and processed through a 150 µm capillary under constant flow. Cell counts were based on conductivity changes induced by particle passage. Simultaneously, cell volume was determined as the displaced electrolyte volume during transit through the measurement aperture, as outlined by Palmucci et al. (2011).

To determine maximum growth rate (μ_{max}) and maximum cell density (Nt_e) the β-function (Yin et al., 2003) was used. β-function is a non-linear regression model and it was already applied to algal growth (Mollo et al., 2023; Petrucciani et al., 2023),

2.3. C and N analysis

Isotopic composition analysis of C and N was conducted to evaluate the assimilation of frass-derived nutrients by the microalgae. Washed microalgal biomass and frass samples were dried at 80 °C till a stable dry weight was attained. Dried samples of 0.5–1 mg were analysed by an elemental analyser (ECS 4010, Costech Italy) connected to the ID Micro EA isotope ratio mass spectrometer (Compact Science Systems, Lymedale Business Centre, Newcastle-Under-Lyme, United Kingdom) to obtain carbon and nitrogen stable isotope ($\delta^{13}C$ and $\delta^{15}N$) ratios, following the procedure illustrated by Petrucciani et al. (2022). Data acquisition and analysis were performed with the software EA IsoDelta (Compact Science Systems, Lymedale Business Centre, Newcastle-Under-Lyme, United Kingdom). All the measurements were carried out on three biological replicas of control and frass growing species.

2.4. Macronutrient composition

Washed algal biomass was lysed using a N_2 cell disruption bomb (4639 Cell Disruption Vessel, Parr Instrument Company). Cell suspensions were deposited on silicon windows and dried at 80 °C prior to infrared spectroscopic analysis, in accordance with the methodology described by Domenighini and Giordano (2009). Fourier Transform Infrared (FTIR) spectra were acquired using a Tensor 27 spectrometer (Bruker Optics, Ettlingen, Germany). FTIR spectra were normalized on the protein peak (1640 cm^{-1}) and the relative abundance of lipids (1730 ± 5 cm^{-1} corresponding C═O of ester functional groups) and carbohydrates (1200–900 cm^{-1} corresponding to C—O—C of etheric groups) were derived and thus used to compare biochemical composition of algae grown in control or in frass medium. A fixed amount of the lysed cellular suspension was then used for protein quantification using the

Table 3
Nutrient concentrations measured in autoclaved frass–water media compared to the inorganic composition of the control media (as per recipe). Values lower than those in the corresponding control are shown in bold. Heavy metals are indicated with an asterisk (*).

	 Autoclaved non saline frass – water medium -	BG11	 Autoclaved saline frass – water medium	AMCONA
N - NO_3	**0.015 ± 0.001 mg·L^{-1}**	250 mg·L^{-1}	**0.008 ± 0.001 mg·L^{-1}**	7.7 mg·L^{-1}
N - NH_4	2.8 ± 0.003 mg·L^{-1}	/	0.4 ± 0.05 mg·L^{-1}	/
Phosphorous	30 ± 4 mg·L^{-1}	8.9 mg·L^{-1}	2 ± 0.1 mg·L^{-1}	0.62 mg·L^{-1}
Sulphur	18 ± 3 mg·L^{-1}	9.79 mg·L^{-1}	**1.6 ± 0.01 mg·L^{-1}**	80.087 mg·L^{-1}
Bromide	0.121 ± 0.004 mg·L^{-1}	/	**0.01 ± 0.001 mg·L^{-1}**	54 mg·L^{-1}
Magnesium	11 ± 1 mg·L^{-1}	7.4 mg·L^{-1}	**Not detectable**	1.002 g·L^{-1}
Boron	Not detectable	0.5 mg·L^{-1}	Not detectable	4 mg·L^{-1}
Fluorine	/	/	Not detectable	1 mg·L^{-1}
Potassium	89 ± 8 mg·L^{-1}	0.018 g·L^{-1}	**5 ± 0.02 mg·L^{-1}**	0.338 g·L^{-1}
Silicium	/	/	/	5.74 mg·L^{-1}
Copper	0.2 ± 0.01 mg·L^{-1}	0.02 mg·L^{-1}	0.012 ± 0 mg·L^{-1}	2.54 µg·L^{-1}
Manganese	**0.3 ± 0.04 mg·L^{-1}**	0.502 mg·L^{-1}	**0.03 ± 0.001 mg·L^{-1}**	0.13 mg·L^{-1}
Zinc	0.4 ± 0.06 mg·L^{-1}	0.02 mg·L^{-1}	0.04 ± 0.001 mg·L^{-1}	0.016 mg·L^{-1}
Calcium	21 ± 3 mg·L^{-1}	9.6 mg·L^{-1}	**2.6 ± 0.005 mg·L^{-1}**	0.367 g·L^{-1}
Iron	**0.3 ± 0.06 mg·L^{-1}**	1.2 mg·L^{-1}	**0.026 ± 0.003 mg·L^{-1}**	0.366 mg·L^{-1}
Nichel	0.03 ± 0.001 mg·L^{-1}	/	0.002 ± 0.001 mg·L^{-1}	0.37 µg·L^{-1}
Cobalt	Not detected	9.9 µg·L^{-1}	/	0.33 µg·L^{-1}
*Chromium**	Not detected	/	**0.006 ± 0.0007 mg·L^{-1}**	/
*Arsenic**	**0.002 ± 0 mg·L^{-1}**	/	Not detected	/
*Lead**	Not detected	/	Not detected	/
pH	6.83	7.6 *(buffered with Tris HCl)*	6.09	8.3 *(buffered with Tris HCl)*
Water clarity (Abs 440 nm)	0.336	0	0.005	0

Lowry colorimetric method as reported by Peterson (1977).

2.5. Lipid extraction and fatty acid determination

Samples of *C. vulgaris* and *N. salina* were analysed for their fatty acid (FA) composition. Aliquots of 500 mg (three aliquots per sample) were freeze-dried at −20 °C in a vacuum (0.2–0.01 mBar) (BUCHI Lyovapor L-200, BUCHI s.r.l, Milan, Italy) and then weighed (±0.2 mg) (analytical balance AT261 DeltaRange, Mettler Toledo, Greifensee, Switzerland).

Total lipid extraction was conducted following the procedure originally established by Folch et al. (1957), with minor adaptations. Lyophilised biomass was extracted overnight using 6 mL of chloroform, 3 mL of methanol, and 2 mL of water, spiked with 100 µL of internal standard (methyl nonadecanoate, 99.6 %, Dr. Ehrenstorfer GmbH, Germany), and vortexed for 1 min. The mixture was filtered through GF/C glass fiber filters (Ø 90 mm, GE Healthcare Life Sciences, UK), pre-filled with anhydrous sodium sulphate (Carlo Erba, Milan, Italy) and rinsed with additional chloroform/methanol solution (Truzzi et al., 2020). The collected extracts were evaporated under a nitrogen stream until constant weight, then redissolved in 0.5 mL of n-heptane (Truzzi et al., 2018; Zarantoniello et al., 2021).

Total lipids were quantified gravimetrically (g kg^{-1} dry mass). Fatty acid methyl esters (FAMEs) were prepared by transesterification using sodium methylate as the catalyst (Canonico et al., 2016). FAMEs were analysed using an Agilent 6890 gas chromatograph coupled with an Agilent 5973 N quadrupole mass selective detector. Separation was achieved on a DB-WAX-UI capillary column (30 m × 0.25 mm, 0.25 µm film thickness; D.T.O. srl, Spinea, Italy). Manual injections of 1 µL were performed in split mode (1:5) using a splitless double taper liner (CPS Analitica, Milan, Italy). Chromatographic conditions were optimized following Truzzi et al. (2018). Data acquisition, peak identification, and quantification of fatty acids were performed following the procedures described in Truzzi et al. (2023).

FAMEs were identified and quantified using standard retention times and mass spectra from a 37-component FAME reference mix (≥99 %, Supelco, Bellefonte, PA, USA) and validated against the NIST spectral database. Detector response factors (Rf) were calculated for each FAME relative to the internal standard (Johnson et al., 2009). All analyses were performed in triplicate. Limits of detection (LOD) and quantification (LOQ) ranged from ~4 to 22 µg mL^{-1} and from 13 to 66 µg m L^{-1}, respectively, as reported by Truzzi et al. (2017).

2.6. Biodiesel property prediction models

2.6.1. Cetane number

The cetane number is a dimensionless measure of a fuel's ignition quality, indicating how readily it ignites in a diesel engine; a value of 100 corresponds to pure cetane, and higher numbers result in quicker, smoother, and more efficient combustion. The cetane number of a biodiesel depend on the fatty acid composition and it can be predicted using Eq. 1 as reported by Bamgboye and Hansen (2008):

$$CN = 61.1 + 0.088x_2 + 0.133x_3 + 0.152x_4 + 0.101x_5 + 0.039x_6 + 0.243x_7 + 0.395x_8 \quad (1)$$

where x_2 to x_8 are the weight percentages of methyl myristate (14:0), palmitate (16:0), palmitoleate (16:1), stearate (18:0), oleate (18:1), linoleate (18:2), and linolenate (18:3) in biodiesels, respectively (Bamgboye and Hansen, 2008).

2.6.2. Cold filter plugging point

The cold filter plugging point (CFPP) is the lowest temperature at which a fuel can still pass through a standard filter without clogging; lower CFPP values indicate better low-temperature flow properties and improved performance in cold conditions. The CFPP of the biodiesel was calculated using Eq. 2 by Ramos et al. (2009):

$$\text{CFPP}\ (^{\circ}\text{C}) = 3.1417\ \text{LCSF} - 16.477 \quad (2)$$

where LCSF is the Long Chain Saturated Factor. The parameter was obtained taking into account the composition of saturated fatty acids and lending more weight to the composition of fatty acids with a long chain (Ramos et al., 2009):

$$LCSF = 0.1\ \text{C16(wt\%)} + 0.5\ \text{C18(wt\%)} + 1\ \text{C20(wt\%)} + 1.5\ \text{C22(wt\%)} + 2\ \text{C24(wt\%)} \quad (3)$$

2.7. Statistical analysis

Each experiment was conducted with a minimum of three independent biological replicates. Results are expressed as the mean accompanied by the standard deviation (±SD). Principal Component Analysis (PCA) was performed with Quasar 1.9.1 (Toplak et al., 2021) focusing on the 1800–800 cm^{-1} spectral region of the FTIR data (dependent variable) to compare algal biomass grown under different culture conditions (independent variables).

GraphPad Prism 9.5.0 (GraphPad Software) was used to perform statistical analysis. Student's *t*-test was used to analyse parameters between growth conditions (CTR and Frass – water medium/autoclaved frass - water medium) of the same algal species (*C. vulgaris* or *N. salina*). All statistical tests were carried out using a significance threshold of $\alpha = 0.05$. Asterisk (*) were used in figures to distinguish significantly different groups (ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

3. Results and discussion

3.1. Microalgae grown in frass - water medium

Table 2 compares frass - water saline and non-saline media to the respective control media. In this experiment, frass was not autoclaved to avoid the disruption of thermolabile molecules important for algal growth (e.g. vitamins). However, this approach reduced the overall sterility of the medium (data not shown).

Nutrient concentration, chemical form and relative abundance in the algal growth medium can strictly constrain algal growth (Norici et al., 2022). Nitrogen is a crucial macronutrient in algal cells and is mainly located into proteins (Templeton and Laurens, 2015). In addition to the organic nitrogen supplied during medium preparation (Table 1), inorganic nitrogen was found in frass – water media, even though mainly in the reduced form of ammonia (Table 2). This form may be preferentially up taken by microalgae and energetically cheaper to assimilate as compared to other oxidised or organic forms of nitrogen; however, it is known that high ammonia concentration could be potentially toxic to algal cells, interfering with autotrophic metabolism (Allqvist and Svenson, 2003; Britto and Kronzucker, 2002; Esteban et al., 2016; Gutierrez et al., 2016; Mollo et al., 2024). As already reported, *Chlorella* and *Nannochloropsis* species can tolerate concentrations between 3.4 – 339 mg L^{-1} of ammonia (Gutierrez et al., 2016) while diatoms growth is inhibited at concentrations of ammonia of about 119 mg·L^{-1} (Collos and Harrison, 2014): the concentrations here found (Table 2) were quite below the toxic contents, suggesting that microalgae can use the amount of ammonia present in the medium for their metabolism. Other crucial nutrients for algal growth include phosphorus and iron; in the frass-based media, phosphorus levels exceeded the replete range of standard media, while iron levels are significantly reduced. What interestingly emerged was that some heavy metals present in the frass were also found in the media (Cr, As, Pb) and their presence could produce oxidative damage to algal cells (Danouche et al., 2022). Nonetheless, their concentrations in the frass-water media (Table 2) were far below most IC_{50} values reported for microalgae (e.g., ~0.08–2.00 mg L^{-1} for Cr; ~5 mg L^{-1} for As and Pb) (Munagamage et al., 2020; Pereira et al., 2013; Rodgher et al., 2012; Satoh et al., 2005). Although interactions with other micronutrients or environmental parameters (e.g., pH shifts or autoclaving) could modulate toxicity thresholds (Rathnayake et al., 2021), the measured values are still too low to be considered a major source of toxicity for the tested species.

Regarding the physical properties, the frass – water non-saline medium showed a lower pH and less water clarity than BG11. Since light penetration was limited, treated algae may have experienced a lower irradiance available for photosynthesis (Jeon et al., 2005; Singh and Singh, 2015) The frass - water saline medium showed again lower pH but similar water clarity compared to control condition. A more acidic growth medium may have influenced the overall inorganic carbon availability and species equilibrium (Boyd, 2020; Butler, 2019).

When grown in frass-based media, microalgae showed a species-specific growth behaviour (Fig. 1). Indeed, while the growth of *C. vulgaris* was not statistically affected by the change of growth medium (Fig. 1A, Fig. 2), seawater species showed a limited or hindered growth (Table 2). In particular, *N. salina* cultures reached the same maximal cell density in frass-based medium as in control medium but at nearly half the rate (Fig. 1C and Fig. 2) while *P. tricornutum* could not survive after the initial acclimation period (Fig. 1B and Fig. 2). These results may be attributed to an imbalance and/or scarcity in nutrient availability and to pH alterations compared to control conditions (Table 2). Furthermore, as shown in Table 2, silicon was not detected in the frass–water medium, although present in the frass itself (Table 1). This suggests that silicon may have been in a chemical form that was neither soluble nor bioavailable. Although diatoms may be strongly affected by silicon concentration, it has already been reported that many strains of *P. tricornutum* do not require Si for growth, conversely to the majority of diatom species (De Martino et al., 2007; Zhao et al., 2014). However, we cannot rule out that the unavailability of silicon contributed to hinder *P. tricornutum* growth; this species was indeed excluded from further experiments.

Although frass was more suitable for freshwater algal growth, our findings suggest that this waste could be a promising nutrient source for microalgal cultivation, confirming observations made by (Steinrücken et al., 2024), who found that *C. vulgaris* can grow equally well in a medium composed by insect frass as compared to a control medium, without any enrichment.

3.2. Microalgae grown in autoclaved frass - water medium

In the second experiment, the frass was sterilized together with the water using an autoclave; however, this process affected the bioavailability of nutrients altering the chemical composition of the medium. Indeed, as shown in Table 3, ammonium availability decreased in media after the sterilization treatment as compared to the amount of ammonium present in frass-water media (Table 2); autoclaving may have degraded the nitrogen-containing molecules and volatilized the nitrogen, releasing it into the atmosphere (F. Montes et al., 2009). As discussed in the previous section, traces of heavy metals were also found in these media and constituted possible inhibiting factors of algal growth. Furthermore, the medium prepared following this protocol exhibited lower pH values (Table 3) than those recorded with both the initial protocol and the standard formulation (Table 2).

Using autoclaved frass – water media, maximal cell density of both algal species was significantly lower than those attained in control condition (Fig. 3A and C) and in the first experiment with frass-water media (Fig. 2B). Although growth rates were not statistically affected (Fig. 3B), growth seemed in general less favoured than in the first experiment, where nutrient source substitution led to nearly identical growth to control conditions in the two selected species (Fig. 1). A different chemistry of the autoclaved media (Tables 2 and 3) could have contributed to the growth limitation. Indeed, the autoclaving process is known to be responsible of nutrient precipitation and degradation as well as ammonium evaporation (Boyer et al., 2024; F. Montes et al.,

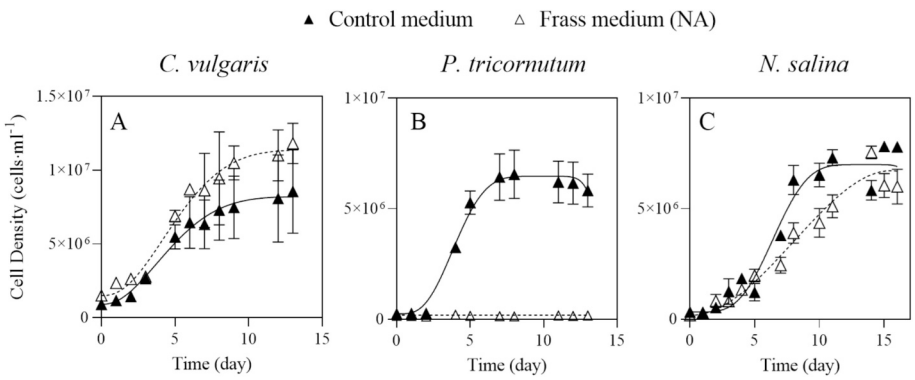

Fig. 1. Growth curves of microalgae grown in control medium (black triangle) and frass medium (not autoclaved, NA) (white triangle). Data are expressed as means of at least 3 biological replicates ± SD.

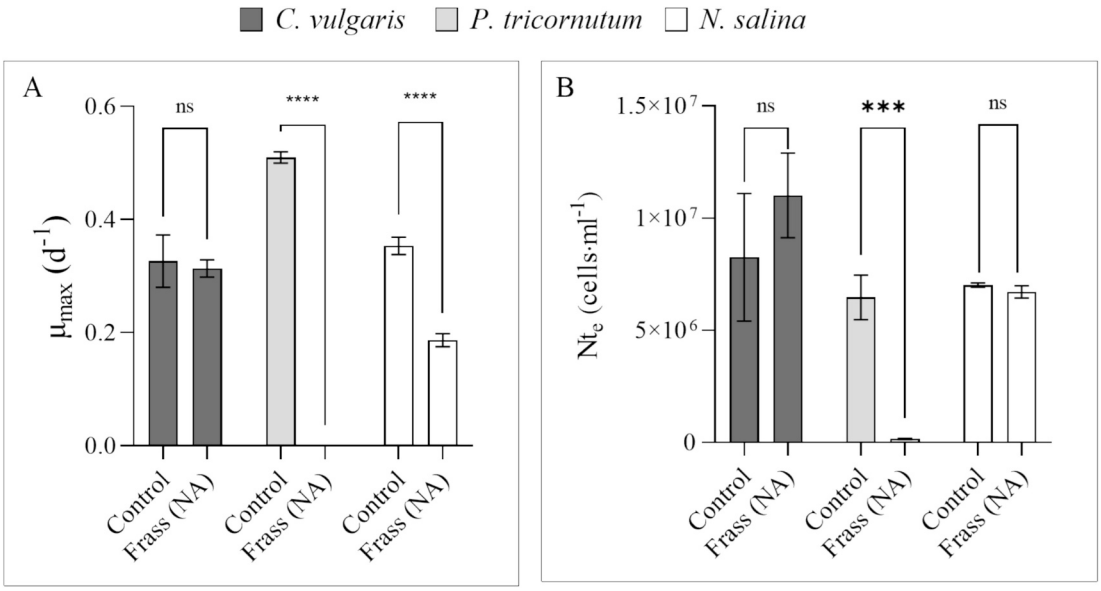

Fig. 2. Maximum growth rates and Nt_e of microalgae grown in control medium and frass medium (not autoclaved, NA). Data are expressed as means of at least 3 biological replicates ± SD. Asterisks represent significant differences between means ($p < 0.05$).

2009; Filip and Joe Middlebrooks, 1975). A degradation of the vitamin content can be ruled out, since both tested algae are known to be prototrophic and therefore capable of synthesizing these vitamins (Jinkerson et al., 2013). This result contrasts with the findings of Steinrücken et al. (2024), who observed no difference in *C. vulgaris* growth when using either autoclaved or filtered frass. They reported higher levels of dissolved organic carbon (DOC) and total nitrogen (Total N) following high-temperature and high-pressure treatment of the frass, suggesting that such processing may enhance nutrient recovery. Nonetheless, differences in the particle size and solubility of the frass, together with a different origin (i.e., *Tenebrio molitor in* Steinrücken et al., 2024) could explain the divergent outcomes between studies. Moreover, the lower pH observed compared to the frass–water and control media, likely resulting from the sterilization process, could have compromised cellular homeostasis and photosynthesis. pH strongly influences both the growth and biochemical composition of microalgae, even in wastewater-based media where maintaining stable pH can be particularly challenging (Wang et al., 2010; Yu et al., 2022).

Microalgae rely on pH gradients across cellular membranes to maintain ion balance and drive ATP synthesis; deviations from optimal pH can therefore disrupt membrane potential and enzyme function, leading to oxidative stress and impaired growth (Raven, 2011). Additionally, the enzymatic activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), critical for carbon fixation, is pH-sensitive and may be impaired under acidic conditions (Badger et al., 1980).

The evaluation of growth performance was followed this time by the analysis of biomass quality. Microalgae grown in autoclaved frass-water media showed similar or slightly less percentage of assimilated C per dry biomass (Fig. 4A) as compared to the percentage in their counterparts; on the other hand, assimilated N per dry biomass was significantly lower (Fig. 4B). C isotopic fractionation (Fig. 4C) varied in both the species acclimated to autoclaved frass-water medium as compared to that at the control condition, but in opposite directions depending on the species: *C. vulgaris* decreased fractionation (more positive $\delta^{13}C$) while *N. salina* increased fractionation (more negative $\delta^{13}C$). As each C source is characterized by a stable isotope signature, the recorded variation was consistent with a species-specific change in the C source assimilated by microalgae ($\delta^{13}C$ values in frass are -24.2 ± 0.33, similar to those found in algal biomass). Indeed, both species are known to implement a mixotrophic metabolism (Poddar et al., 2018; Xu et al., 2004) concurrently assimilating CO_2 and organic carbon. Nevertheless, we may assume that when algae fixed carbon via C_3 metabolism, the introduction of mixotrophy reduced fractionation due to decreased involvement of Rubisco (e.g., in *C. vulgaris*). Conversely, when algae acquired carbon via CO_2 concentrating mechanism and subsequently fixed it through C_3

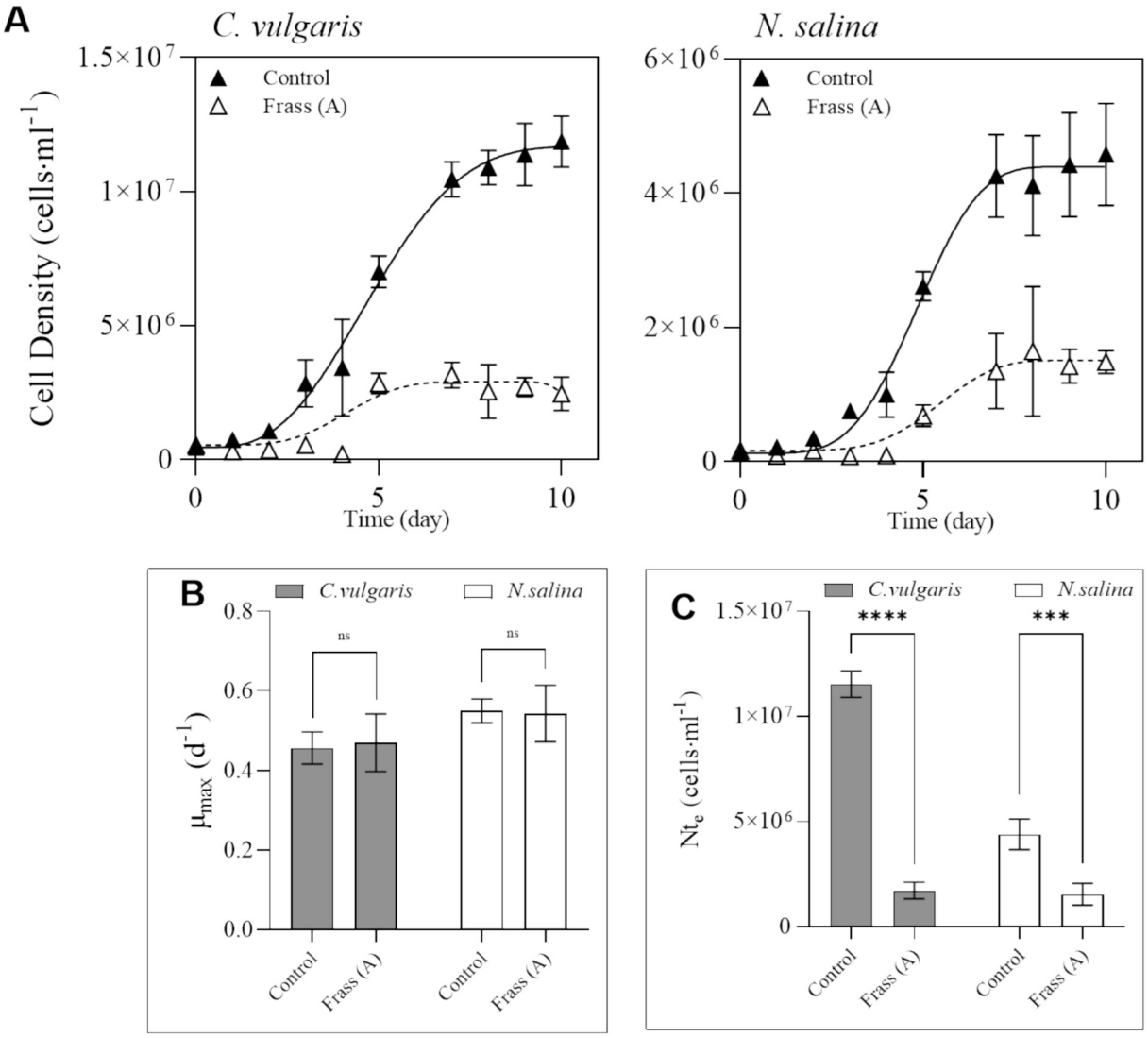

Fig. 3. Growth analysis of *C. vulgaris* and *N. salina* grown in control and frass media (autoclaved, A). (A) Growth curves (B) Growth rates (C) Nt_e. Data are expressed as means of at least 3 biological replicates ± SD. Asterisks represent significant differences between means ($p < 0.05$).

metabolism, the introduction of mixotrophy with frass supplementation (derived from the waste of vegetarian dietary insects) increased fractionation (more negative $\delta^{13}C$), as observed in *N. salina* (Fig. 4C) (Brandenburg et al., 2022; Petrucciani et al., 2023, 2024). However, mixotrophy typically results in a higher biomass yield (Castillo et al., 2021; Poddar et al., 2018), contrary to the observations in the present experiment (Fig. 3).

Regarding the nitrogen content per dry biomass, although the organic nitrogen concentration in the autoclaved frass-water medium was kept equal to or double that of the control medium, the assimilated quota was significantly lower in algal biomass (Fig. 4B), Moreover, both species exhibited a variation in N isotopic fractionation when acclimated to the frass medium. The $\delta^{15}N$ value significantly decreased in the biomass, indicating increased fractionation of the isotope and meaning cells assimilated more N forms that were deficient in the heavier N^{15} isotope. The result can be explained by a shift toward the assimilation of more reduced N species, which were available in the alternative nutrient source to which the microalgae were acclimated (Needoba et al., 2003; Needoba and Harrison, 2004).

While many microalgae can utilize both inorganic and organic nitrogen sources, the ability to assimilate organic nitrogen is species dependent. Some microalgal taxa rely predominantly on inorganic nitrogen forms such as nitrate (NO_3^-), nitrite (NO_2^-), and ammonium (NH_4^+), and lack the enzymatic machinery to uptake and metabolize organic nitrogen compounds (Berges and Mulholland, 2008; Flynn and Fasham, 1997). For instance, certain diatoms and green algae exhibit limited uptake of organic nitrogen such as urea or amino acids, either due to the absence of specific transporters or key enzymes like urease or amino acid oxidases (Antia et al., 1991). Notably, both *Nannochloropsis salina* and *Chlorella vulgaris* have been shown to efficiently utilize organic nitrogen to grow, including urea and free amino acids, in addition to inorganic forms like nitrate and ammonium (Fathy et al., 2023; Hodson et al., 1975; Sauer, 1984; Syrett and Leftley, 1976), as observed in frass - water growth (Fig. 1). In *Chlorella vulgaris*, uptake of amino acids has been linked to improved growth and biomass productivity under nitrogen-limited conditions (Markou et al., 2014). Similarly, *Nannochloropsis salina* can assimilate urea via urease activity, contributing to sustained growth even in the absence of inorganic nitrogen (Loira et al., 2017). Thus, we cannot rule out that the reduced assimilation of N found in the biomass was caused by an altered chemistry of the autoclaved frass - water medium due to the sterilization process hindering algal global physiological performance.

Due to the different nutrient source and the metabolic shift, remodulation of the C pools occurred in both the species, as shown by

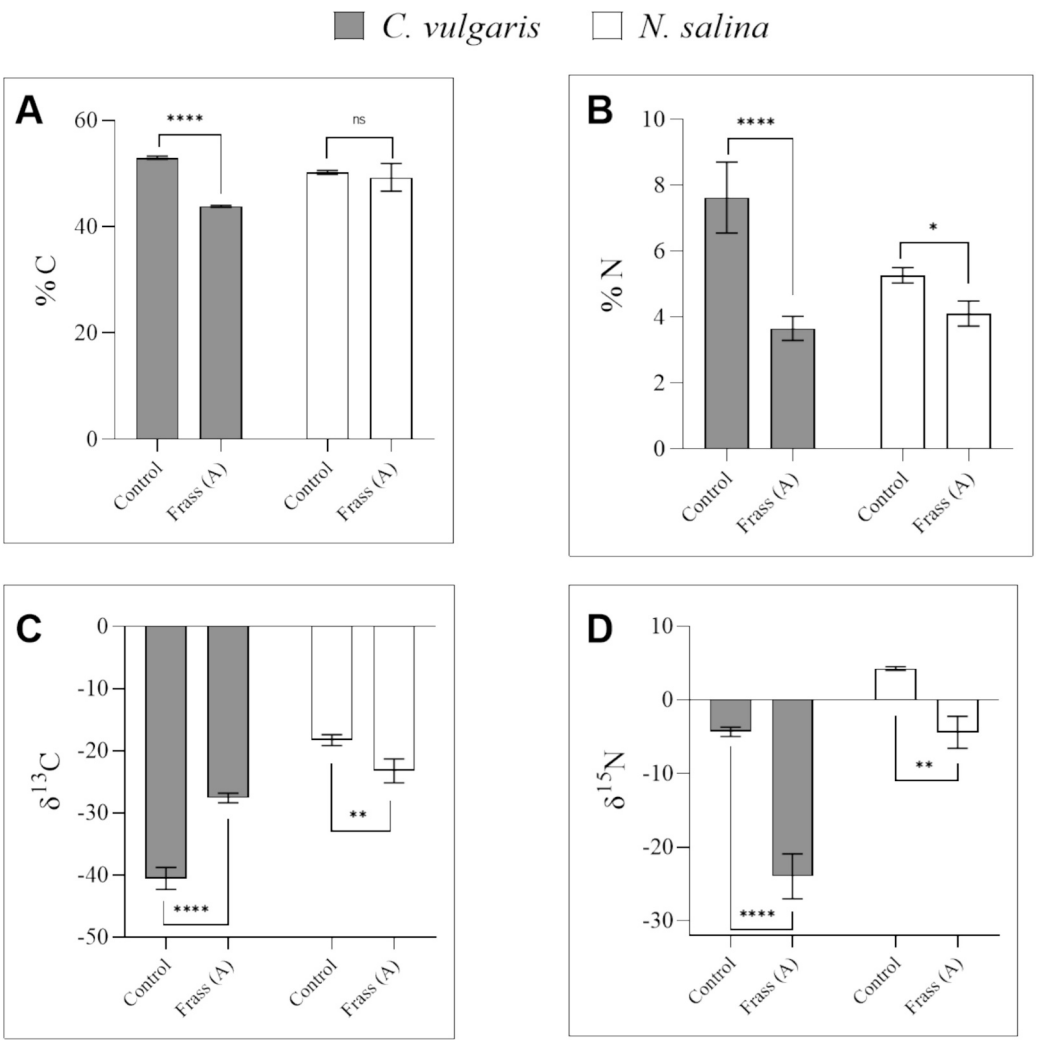

Fig. 4. (A) Carbon percentage on dry weight, (B) Nitrogen percentage on dry weight, (C) $\delta^{13}C$ isotopic fractionation and (D) $\delta^{15}N$ isotopic fractionation measured in *C. vulgaris* and *N. salina* grown in control and frass media (autoclaved, A). Data are expressed as means of at least 3 biological replicates ± SD. Asterisks represent significant differences between means ($p < 0.05$).

the FTIR results pictured in Fig. 5. When macromolecular composition of algae grown in different media (Fig. 5A), cells of each species grown in autoclaved frass-water medium accumulated more carbohydrates (in the 1200–1000 cm^{-1} range, according to Giordano et al. (2001)) than those grown under control conditions, for the same amount of proteins (spectra normalized to the 1650 cm^{-1} peak) (Fig. 5A, C). In contrast, lipids, which were measured using the peak at 1730 cm^{-1} (Giordano et al., 2001), did not show any relevant variation between the different growth media (Fig. 5A, C).

PCA carried out on the range of FTIR spectra associated to biomolecules (1800–800 cm^{-1}, Fig. 5B, D) showed that there was a clear separation between control and frass grown cells, again proving that changes in the nutrient source led to a distinct quality of the biomass. Moreover, the quantified protein pool (Fig. 6) was significantly less abundant in cells grown in frass based medium for both species compared to the pool in their respective controls. The finding was consistent with the lower nitrogen percentage per dry biomass (Fig. 4B). In summary, overall data suggested the new media promoted C allocation into carbohydrates more than into proteins and lipids.

These results once again did not confirm the findings of Steinrücken et al. (2024), who reported equal protein content in *C. vulgaris* cultivated with frass and in control cells. In contrast, our observations suggest that the type of frass used plays a significant role in affecting algal performance. Specifically, the use of frass from *Acheta domesticus* yielded different results for the same algal species compared to studies employing frass derived from *Tenebrio molitor* (Steinrücken et al., 2024).

The macromolecular composition analysis was complemented by a characterization of the lipid pool. Quantification of lipid content per dry mass (DM) revealed no significant differences between the control condition and the autoclaved frass-water medium in *C. vulgaris* and *N. salina* (Fig. 7), as also reported in Fig. 5A and C. In general, the lipid content quantified in *C. vulgaris* in both treatments (6.7 g 100 g^{-1} DM for the control and 7.9 g 100 g^{-1} DM for the frass treatment) was lower than previously reported values (13–25 g 100 g^{-1} DM) (Aguoru and Okibe, 2015; Tokuşoglu and Ünal, 2003). Similarly, the lipid content of *N. salina* (14.9 g 100 g^{-1} DM for the control and 10.3 g 100 g^{-1} DM for the frass treatment) was considerably lower than in earlier reports, which ranged from 37 to 39 g 100 g^{-1} DM (Aguoru and Okibe, 2015; Tokuşoglu and Ünal, 2003). Such differences may be attributed not only to the different growth media but also to the variation in the growth conditions, e.g. temperature, light intensity, CO_2, which are known to affect lipid content in microalgae (Aguoru and Okibe, 2015). Indeed, the lipid values reported by Tokuşoglu and Ünal (2003) refer to *C. vulgaris* grown semi-continuously and blown with air, and not in static batch culture as done in this study. Moreover, lipid content in microalgae is often influenced by macronutrient limitations, particularly nitrogen (Illman et al., 2000; Norici et al., 2022; Rodolfi et al., 2009; Shifrin and Chisholm, 1981). Rodolfi et al. (2009) observed an increase in lipid

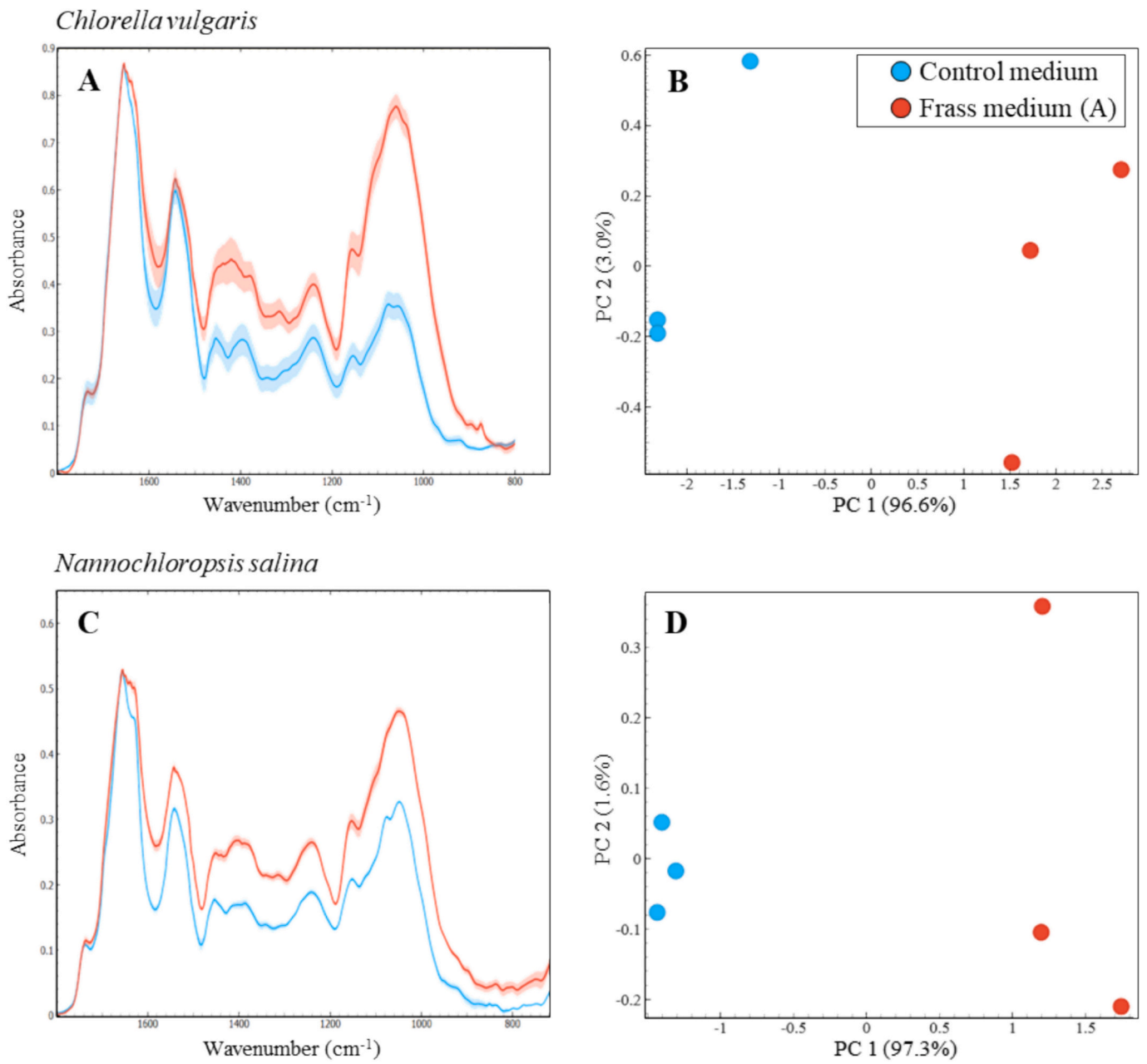

Fig. 5. FTIR spectra of *C. vulgaris* (A) and *N. salina* (C) grown in control (blue line) and frass media (autoclaved, A) (red line). Spectra are normalized in the common peak at 1650 cm^{-1}; spectra are represented by mean (line) and standard deviation (shadow). PCA analysis on FTIR spectra of *C. vulgaris* (B) and *N. salina* (D) grown in control (blue dots) and frass media (red dots).

content from 15 % to 60 % in *Nannochloropsis* sp. after a three-day nitrogen deprivation period. Similarly, Converti et al. (2009) observed that reducing nitrate concentration in the growth medium by 75 % resulted in a two-fold increase of lipid content in *Nannochloropsis* sp. and a three-fold increase in *C. vulgaris*. The unchanged lipid content may provide further evidence algae did not experienced a different nitrogen availability between growth media.

The fatty acid (FA) composition analysis conducted in this study demonstrated that altering the growth medium resulted in significant differences ($p < 0.05$) between different treatment groups for both algal species, as illustrated in the figures and tables below. Fig. 8 presents the FA class distribution of *C. vulgaris* cultivated in control and autoclaved frass-water media, with detailed FA composition (g 100 g^{-1} FAs) provided in Table 4. Similarly, Fig. 9 displays the FA class distribution of *N. salina* under the same conditions, with the corresponding detailed composition listed in Table 5.

Indeed, as was observed here, the addition of cricket frass to the growth medium of the studied microalgae led to a significant increase of SFAs in both the microalgal species investigated, primarily due to the increased levels of palmitic acid (16:0) and stearic acid (18:0), while the content of MUFAs was significantly higher only in *C. vulgaris* (Figs. 8 and 9). On the contrary, in both *C. vulgaris* and *N. salina* grown in frass based medium, a decrease of PUFA and n-3 was observed compared to microalgae grown in control medium, mainly due to the reduction (about 30 %) of the essential FAs such as linoleic acid (18:2n-6) and alpha-linolenic acid (18:3n-3) (Figs. 8 and 9, Tables 4 and 5). Nonetheless, a significantly higher proportion (roughly 4 times) of docosahexaenoic acid (DHA, 22:6n-3) was found in the *C. vulgaris* biomass (Table 5), a well-known lipid for its benefits and essential roles in animal health and growth (Bell et al., 1995; Calder, 2015; Tocher, 2010).

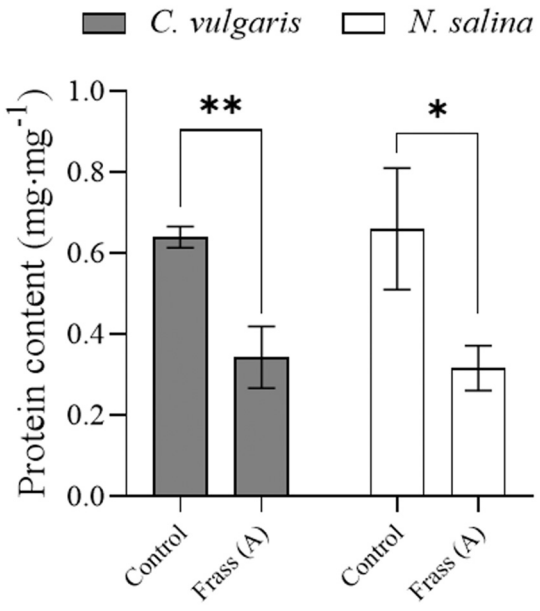

Fig. 6. Protein content (mg·mg^{-1}) measured in *C. vulgaris* and *N. salina* grown in control and frass media (autoclaved, A). Data are expressed as means of at least 3 biological replicates ± SD. Asterisks represent significant differences between means ($p < 0.05$).

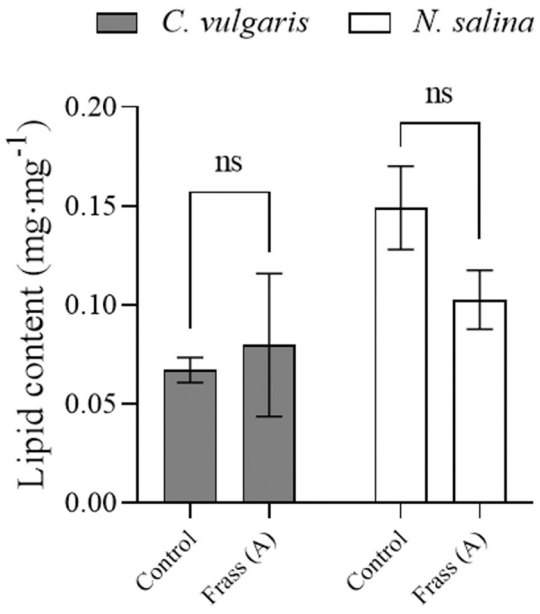

Fig. 7. Lipid content (g 100 g^{-1} DM) measured in *C. vulgaris* and *N. salina* grown in control and frass media (autoclaved, A). Data are expressed as means of at least 3 biological replicates ± SD. Asterisks represent significant differences between means ($p < 0.05$).

Despite this increase, the total PUFA content in *C. vulgaris* cultivated in the frass medium remained lower than in the control condition. The most representative FAs in *C. vulgaris* were 16:0, 17:1n-7, 18:0, 18:1n-9, 18:2n-6, and 18:3n-3, while in *N. salina*, the dominant ones were 16:0, 18:0, 18:1n-9, 18:2n-6, 18:3n-3, and 20:5n-3. Together, these fatty acids accounted for more than 90 % of the total fatty acids (Tables 4 and 5). The addition of cricket frass to the growth medium of the studied microalgae led to similar changes in the major fatty acids of both microalgal species, with a significant increase in 16:0 and 18:0, and a notable decrease in 18:1n-9, 18:2n-6, and 18:3n-3 (Tables 4 and 5). The

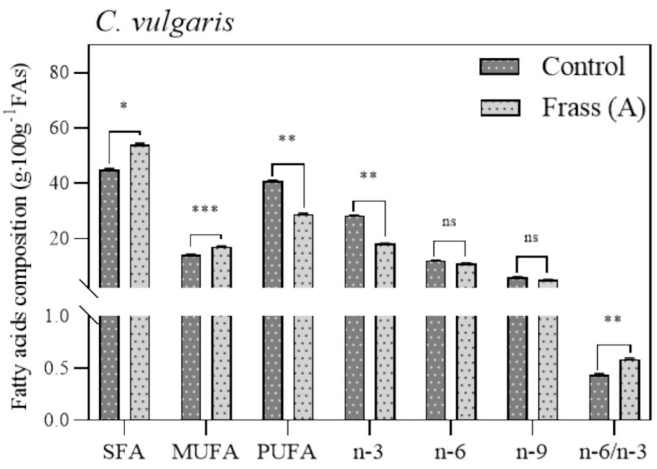

Fig. 8. Fatty acid composition of *C. vulgaris* grown in control and frass- media (autoclaved, A). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; n-3, omega-3 polyunsaturated fatty acids; n-6, omega-6 polyunsaturated fatty acids; n-9, omega-9 polyunsaturated fatty acids; n-6/n-3, omega-6/omega-3 ratio. Asterisks indicate statistically significant differences between experimental groups compared within the same fatty acid class ($p < 0.05$). Values are presented as mean ± SD ($n = 3$).

Table 4
Fatty acid composition (g 100 g^{-1} FAs) of *C. vulgaris*.

FAs	Ctrl *C. vulgaris*	Frass *C. vulgaris*
8:0	0.0123 ± 0.0001	0.015 ± 0.001
10:0	0.0097 ± 0.0002	0.0123 ± 0.0002
11:0	0.00110 ± 0.00004	0.003 ± 0.001
12:0	0.039 ± 0.002^{b}	0.10 ± 0.01^{a}
13:0	0.00273 ± 0.00001	0.021 ± 0.001
14:0	0.49 ± 0.03^{b}	0.82 ± 0.01^{a}
15:0	0.098 ± 0.007^{b}	0.318 ± 0.006^{a}
16:0	31.1 ± 0.7^{b}	33.1 ± 2.0^{a}
16:1n-7	2.89 ± 0.03^{b}	7 ± 1^{a}
17:0	0.088 ± 0.003^{b}	0.200 ± 0.007^{a}
17:1n-7	5.3 ± 0.2^{a}	5.06 ± 0.02^{b}
18:0	12.9 ± 0.2^{b}	19.0 ± 1.7^{a}
18:1n-9	5.9 ± 0.5^{a}	4.7 ± 0.6^{b}
18:2n-6	12.4 ± 0.4^{a}	10.7 ± 0.9^{b}
18:3n-3	28.2 ± 1.3^{a}	18.0 ± 2.3^{b}
20:0	0.130 ± 0.003^{b}	0.22 ± 0.01^{a}
20:1n-9	0.018 ± 0.001	0.032 ± 0.001
20:2n-6	0.0202 ± 0.0004^{b}	0.051 ± 0.004^{a}
20:3n-6	0.029 ± 0.002	0.018 ± 0.001
21:0	0.023 ± 0.001^{b}	0.040 ± 0.003^{a}
20:3n-3	0.027 ± 0.002^{b}	0.049 ± 0.001^{a}
22:0	0.025 ± 0.002^{b}	0.067 ± 0.004^{a}
22:1n-9	Nd	0.31 ± 0.02
22:2n-6	0.017 ± 0.007	nd
23:0	0.013 ± 0.001	0.014 ± 0.001
24:0	0.054 ± 0.002^{b}	0.085 ± 0.005^{a}
22:6n-3	0.07 ± 0.01^{b}	0.29 ± 0.05^{a}

Ctrl *C. vulgaris*, *Chlorella vulgaris* grown in control media; Frass *C. vulgaris*, *Chlorella vulgaris* grown in frass-media. Values are reported as mean ± SD (n = 3). Mean within rows bearing different letters are significantly different ($p < 0.05$). FAs content <0.02 g 100 g^{-1} FAs were excluded from any statistical analysis because their concentrations were close to the limit of detection (LOD). nd, not detected.

outcomes of the present study align with previous research indicating that nutrient modulation (such as N availability and chemical form) in growth medium play a key role in determining the FAs profile of microalgae (Gouveia and Oliveira, 2009; Jahromi et al., 2022; Qiang et al., 1997; Rodolfi et al., 2009; Tran et al., 2009). For instance, Rodolfi et al. (2009) observed that in *Nannochloropsis* sp., high nitrogen availability is associated with an increase in PUFAs, whereas nitrogen

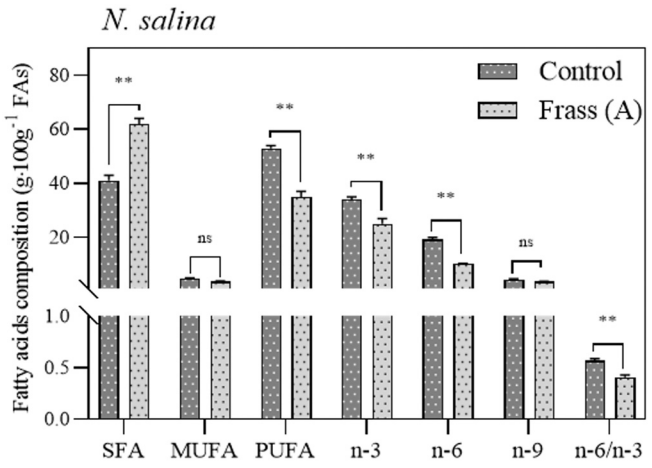

Fig. 9. Fatty acid composition of *N. salina* grown in control and frass-media (autoclaved, A). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; n-3, omega-3 polyunsaturated fatty acids; n-6, omega-6 polyunsaturated fatty acids; n-9, omega-9 polyunsaturated fatty acids; n-6/n-3, omega-6/omega-3 ratio. Asterisks indicate statistically significant differences between experimental groups compared within the same fatty acid class ($p < 0.05$). Values are presented as mean ± SD (n = 3).

Table 5
Fatty acid composition (g 100 g^{-1} FAs) of *N. salina*.

FAs	Ctrl *N. salina*	Frass *N. salina*
8:0	0.02 ± 0.01	0.019 ± 0.003
10:0	0.009 ± 0.003	0.015 ± 0.001
11:0	0.005 ± 0.001	0.006 ± 0.001
12:0	0.194 ± 0.005^{b}	0.21 ± 0.01^{a}
13:0	0.016 ± 0.001	0.015 ± 0.001
14:0	1.343 ± 0.001^{a}	1.10 ± 0.05^{b}
14:1n-5	0.019 ± 0.002	0.006 ± 0.001
15:0	0.41 ± 0.02	0.38 ± 0.03
16:0	29 ± 5^{b}	36 ± 3^{a}
17:0	0.23 ± 0.02^{a}	0.19 ± 0.02^{b}
17:1n-7	0.4 ± 0.1^{a}	0.14 ± 0.02^{b}
18:0	9 ± 2^{b}	23 ± 2^{a}
18:1n-9	4.2 ± 1.0^{a}	3.3 ± 0.4^{b}
18:2n-6	18 ± 4^{a}	10 ± 2^{b}
18:3n-3	28 ± 2^{a}	19 ± 2^{b}
20:0	0.14 ± 0.04^{b}	0.27 ± 0.01^{a}
20:1n-9	0.034 ± 0.004	0.039 ± 0.000
20:2n-6	0.12 ± 0.05^{a}	0.05 ± 0.01^{b}
20:3n-6	0.4 ± 0.1	0.3 ± 0.14
21:0	0.03 ± 0.01	0.04 ± 0.01
20:4n-6	0.21 ± 0.07^{a}	0.09 ± 0.03^{b}
20:3n-3	0.53 ± 0.15^{a}	0.2 ± 0.1^{b}
20:5n-3	5.4 ± 1.5	5.7 ± 2.0
22:0	0.13 ± 0.01	0.16 ± 0.04
22:1n-9	0.08 ± 0.02	0.14 ± 0.01
22:2n-6	0.31 ± 0.02^{a}	0.11 ± 0.09^{b}
23:0	0.193 ± 0.045^{a}	0.05 ± 0.05^{b}
24:0	0.5 ± 0.1^{a}	0.4 ± 0.1^{b}
22:6n-3	0.3 ± 0.1	0.3 ± 0.2
24:1n-9	0.07 ± 0.01	0.10 ± 0.05

Ctrl *N. salina*, *N. salina* grown in control media; Frass *N. salina*, *N. salina* grown in frass- media. Values are reported as mean ± SD (n = 3). Mean within rows bearing different letters are significantly different ($p < 0.05$). FAs content <0.02 g 100 g^{-1} FAs were excluded from any statistical analysis because their concentrations were close to the limit of detection (LOD). nd, not detected.

limitation leads to the increase of SFAs and MUFAs. Similarly, Converti et al. (2009) reported that under nitrogen-starved conditions *C. vulgaris* produces higher amounts of SFA at the expense of PUFAs. The fatty acid profile shifts observed in this study (increased SFA, decreased PUFA; Figs. 8 and 9) are consistent with the established paradigm that both nitrogen and carbon availability are key regulators of lipid composition (Rodolfi et al., 2009; Shifrin and Chisholm, 1981). Notably, frass-grown algae exhibited an higher C:N ratio as compared to control condition, which was concomitant with an increased SFA:PUFA ratio (Fig. 4).

3.3. Potential use of the derived biomass – toward a circular approach

Biochemical analysis revealed significant changes in the properties of biomass cultivated in media enriched with an alternative nutrient source. These findings not only enhance our understanding of the metabolic versatility of the microorganisms studied but also offer valuable insights for the potential reuse of this biomass within a circular economy framework.

FTIR analysis indicated that the biomass was notably enriched in carbohydrates (Fig. 5). This is particularly relevant, as algal-derived carbohydrates are known to function as effective biostimulants in agriculture. They promote root development by enhancing root elongation and branching (Kapoore et al., 2021), which in turn improves the plant's ability to absorb water and nutrients. Such improved root architecture is essential for optimal water uptake and nutrient efficiency, ultimately fostering robust plant growth (Ali et al., 2020; Calvo et al., 2014). Additionally, carbohydrates can stimulate soil microbial activity, thereby enhance nutrient cycling and further increase nutrient availability for plants (Alvarez et al., 2021). Hence, a carbohydrate-enriched biomass holds strong potential for agricultural applications.

Regarding the lipid profile, a decrease in polyunsaturated fatty acids (PUFAs) and a corresponding increase in saturated fatty acids (SFAs) were observed in both *C. vulgaris* and *N. salina* when grown in autoclaved frass-based media (Figs. 8 and 9). From an animal nutrition perspective, PUFAs are critical for promoting growth, brain development, immune function, and stress resistance in fish, crustaceans, and terrestrial animals (Bell et al., 1995; Calder, 2015; Tocher, 2010). In contrast, high SFA levels are generally less desirable, as they can impair lipid digestibility and negatively impact animal health (Halpern et al., 2015). Consequently, the lipid composition of algae grown in frass-based media may limit their suitability for use as animal feed (Hawrot-Paw et al., 2021; Sia et al., 2020).

By contrast, the lipid profile shaped by frass supplementation is more compatible with biodiesel feedstock requirements. Higher SFA and limited PUFA contents generally improve oxidative stability and increase cetane number (CN), both desirable biodiesel attributes, while lower PUFA content reduces tendencies toward fuel polymerisation (Bamgboye and Hansen, 2008; Knothe, 2005; Lin and Wu, 2022). Using the FAME profiles obtained in this study and the prediction models (Bamgboye and Hansen, 2008; Ramos et al., 2009), we estimated the CN and cold filter plugging point (CFPP) of putative biodiesel from harvested biomass (Table 6). The estimated CN values ranged from 51 to 60: the lowest was observed for control *N. salina* and the highest for *N. salina* grown in frass media. *C. vulgaris* similarly showed an increase from approximately 53 in the control to about 58 in frass-treated biomass. These ignition-quality estimates meet commonly applied regulatory thresholds (e.g. ASTM D6751 and EN 14214) (ASTM International, 2008; CEN, 2023), indicating satisfactory ignition behaviour for biodiesel from these biomasses.

However, estimated CFPP increased with frass supplementation, reflecting worsened low-temperature operability. For *N. salina* CFPP rose from around 11 °C (control) to roughly 35 °C (frass-treated), while

Table 6
Predicted biodiesel properties calculated from FAME profiles (estimated CN and CFPP).

Species	Condition	Cetane number	Cold filter plugging point (°C)
N. salina	Control	51	11
	Frass	60	35
C. vulgaris	Control	53	14
	Frass	58	25

for *C. vulgaris* it increased from about 14 °C to approximately 25 °C (Table 6). The higher CFPP values are consistent with the increased proportion of long-chain saturated FAMEs (Tables 3 and 4) and point to potential cold-flow limitations in temperate and cold climates (Bouaid et al., 2024; Monirul et al., 2015). This combination of improved ignition quality and reduced cold-flow performance highlights a practical trade-off that should guide downstream decisions: in warm climates, neat biodiesel from frass-grown biomass could be a feasible option with minimal conditioning, whereas in colder regions the biodiesel would likely require blending with lower-CFPP fuels, cold-flow additives, winterization, or fractionation to remove high-melting-point components (Bouaid et al., 2024; Monirul et al., 2015; Yuan et al., 2022).

A biorefinery perspective may reconcile these trade-offs by valorising biomass fractions selectively. Carbohydrate-rich streams could be directed to agricultural biostimulants, soil amendments, or fermentation feedstocks, while the lipid fraction could be conditioned and allocated to biodiesel production with appropriate processing.

An additional consideration for downstream valorisation is metal content. Trace metals (e.g. Cu, Zn, Ni, Cr, As, Pb) were detected at low dissolved concentrations in the frass-derived media (Tables 2, 3; e.g. Cu $\approx$ 0.20–0.28 mg L^{-1}, Zn $\approx$ 0.34–0.40 mg mg L^{-1}; Cr $\approx$ 0–0.01 mg L^{-1}; As $\approx$ 0.002 mg L^{-1}; Pb generally below detection). Although these medium concentrations are small, regulatory limits for finished fertilising products are expressed per dry product (Reg. (EU) 2019/1009) and, depending on the Product Function Category, set maximum contents (e.g. inorganic As $\approx$ 40 mg kg^{-1}, Pb $\approx$ 120 mg kg^{-1}; Cu and Zn limits vary but can be in the low hundreds mg kg^{-1}). In conservative scenarios of strong accumulation and low biomass yield some elements (notably Cu and Zn) could approach category limits, whereas As and Pb, under the non-saline conditions measured here, are unlikely to do so. Actual bioaccumulation is, however, strongly modulated by speciation, exposure time and processing (washing, separation from residual solids). Therefore, prior to any feed or fertiliser application, harvested biomass should be processed (washed and dried) and analysed for total metals (ICP-MS) and, when relevant, for speciation (e.g. inorganic As, Cr(VI)); these empirical analyses will determine the safest downstream use and any required mitigation (washing, blending, selective allocation to PFCs).

Beyond chemical analysis, the microbiological safety of the biomass is a critical consideration. Frass naturally hosts a microbial community that may include pathogens or toxin-producers. While pre-treatment like autoclaving significantly reduces the initial microbial load (Van Looveren et al., 2022), the final biomass must be tested to ensure it is free from harmful contaminants. Such analysis is essential to verify its safety and compliance with regulatory standards before any use in animal feed or agriculture (Amorim et al., 2024; Safitri et al., 2024).

Overall, these considerations suggest that frass-derived algal biomass has clear potential for agricultural and biofuel uses, but that targeted processing and metal testing are necessary steps to safely translate laboratory results into applied circular-economy solutions.

4. Conclusion

In conclusion, the results of this study highlight the potential of insect-derived frass as an effective growth medium for both marine and freshwater microalgae. Except for *P. tricornutum*, both *C. vulgaris* and *N. salina* successfully acclimated to a medium composed solely of water and frass, producing valuable biomass. This biomass was not only enriched in carbohydrates, making it suitable for use in agricultural applications, but also exhibited a fatty acid profile compatible with lipid feedstocks for biodiesel production. These findings support the sustainable reuse of nutrients present in frass, such as nitrogen, phosphorus, and micronutrients, to generate biomass with sustainable inputs for agriculture and biofuel industries.

CRediT authorship contribution statement

Alessandra Petrucciani: Visualization, Supervision, Investigation, Formal analysis, Conceptualization, Writing – review & editing, Writing – original draft. **Lorenzo Mollo:** Visualization, Supervision, Investigation, Formal analysis, Writing – review & editing, Writing – original draft. **Simona Anna Siena:** Investigation, Formal analysis. **Behixhe Ajdini:** Supervision, Investigation, Formal analysis, Writing – original draft. **Irene Biancarosa:** Supervision, Resources, Conceptualization, Writing – original draft. **Cristina Truzzi:** Resources, Project administration, Funding acquisition, Conceptualization, Writing – review & editing. **Alessandra Norici:** Supervision, Resources, Project administration, Funding acquisition, 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.

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Data availability

Data will be made available on request.

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