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Composition of microalgae produced using different types of water and nutrient sources

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ABSTRACT

Microalgae are commonly referred to as 'unicellular factories' due to their size and their capacity to produce and accumulate valuable biomolecules. The synthesis of a given biomolecule is generally triggered by external stimuli (e.g., light). This work evaluated the effect of different types of water and nutrient sources (freshwater, wastewater, fertilisers, or pig slurry) on the biochemical composition of the microalga *Tetradesmus almeriensis* produced on a pilot-scale. Overall, significant differences were observed in the biochemical composition of the biomasses ($p < 0.05$). The biomass produced using freshwater and fertilisers showed potential to be used as food given its high content of essential amino acids, carotenoids (mainly lutein and β-carotene), and PUFAs (C18:2 and C18:3). The microalgae produced using wastewater showed potential applications in the animal feed and agricultural industries. They showed a higher abundance of essential amino acids and carotenoids, including fucoxanthin and lycopene; the latter was only detected when the biomass was produced using secondary wastewater. The biomass produced using pig slurry as the source of nutrients showed potential for being used as animal feed or as an agricultural product given its high content in essential biomolecules and amino acids. The biochemical composition was not only a cause of different microbial diversities but also of the effect of the different composition of the culture medium that trigger different responses of the microalgal cells.

1. Introduction

Microalgae are photosynthetic organisms; they use sunlight, which is free and unlimited, to convert carbon dioxide and inorganic nutrients into oxygen and high-value biomass. Microalgae can be produced using freshwater, wastewater, or seawater and different nutrient sources such as commercial fertilisers or agricultural waste. For these reasons, industrial processes based on these microorganisms have been suggested as one of the keys to sustainable production. The key aspect of microalgae-based processes, especially of waste and wastewater treatment processes, is that a valuable product (microalgal biomass) can be obtained from waste while simultaneously obtaining recycled water. Microalgal biomass can be used for different applications ranging from biofertilisers to feed and food ingredients. Several products are currently commercially available [1] and the acceptance of these products is generally high [2].

The composition of the culture medium can significantly affect the biomass composition of the produced and, therefore, the quality of the end commercial product. For example, under nitrogen limitation, many microalgal species change their carbon storage patterns in favour of neutral lipids [3] or carbohydrates [4]. Similarly, microalgae tend to accumulate lipids and carbohydrates when produced in phosphorus-limited medium [5]. The abundance of nitrogen or phosphorus can affect not only the composition of microalgal biomass but also the source of nitrogen or phosphorus. For example, the use of urea instead of nitrate as the main nitrogen source increased the lipid content of *Chlorella zofingiensis* by approximately 30 % [6]. Similarly, the lipid productivity of *Mychonastes afer* was higher when produced using urea than $N\text{-}NO_3^-$

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and N-NH_4^+ [7]. Most of these studies were conducted on a laboratory scale under controlled conditions. However, when produced outdoors in industrial facilities, especially when produced using wastewater or other waste streams, both the composition of the media and the environmental conditions vary significantly between seasons and even within the same week. For example, the composition of animal feeds largely influences the nitrogen and phosphorus content of slurry and manure [8] and the chemical oxygen demand (COD) of wastewater can vary by up to 120 % within the same week [9]. In addition, wastewater and other wastewater streams generally contain other compounds that can affect microalgal growth and metabolism and a large microbial load that can affect the composition of the microalgal biomass.

It has been suggested that if reactors are managed properly, most of the biomass produced will be microalgae because of the presence of inorganic nutrient sources and because phototrophic growth is promoted under high-light availability conditions. However, depending on the composition of the culture medium and the environmental conditions, this value can vary and affect the composition of the biomass produced. The present work is the continuation of a previous work describing the production of *Tetradesmus almeriensis* using thin-layer cascade photobioreactors and different types of water [10]. The four different types of water used were: (i) freshwater supplemented with commercial fertilisers; (ii) primary urban wastewater; (iii) secondary urban wastewater; (iv) and diluted pig slurry. In that work, it was observed that the different types of water led to a significant variation in the microbial populations present in the biomass. The microalga *T. almeriensis*, which was the inoculated strain, only represented 1.6 and 4.9 % of the eukaryotic populations after 30 days of semi-continuous production. In the present work, the biochemical differences between the different biomasses will be described. *T. almeriensis* was selected based on its potential for implementation in agriculture [11], feed production [12], and food production [13].

2. Materials and methods

2.1. Production of microalgal biomass

The biomass production was carried out as described in a previous work [10]. The reactor was operated in semi-continuous mode at a dilution rate of 0.3 day^{-1} and using different types of water and nutrients: (i) freshwater and agricultural fertilisers, (ii) primary urban wastewater, (iii) secondary urban wastewater, and (iv) diluted fresh pig slurry. The freshwater-based medium contained 0.0, 200.6, 41.7, and 15.1 mg·L^{-1} of N-NH_4^+, N-NO_3^-, P-PO_4^{3-}, and COD, respectively. The concentrations of N-NH_4^+, N-NO_3^-, P-PO_4^{3-}, and COD in the primary and secondary treated wastewater were 81.1 and 12.1, 4.9 and 14.2, 16.1 and 3.2, and 500.1 and 106.6, respectively. Finally, the culture medium based on diluted pig slurry contained 120.8, 9.5, 21.1. and 415.3 mg·L^{-1} of N-NH_4^+, N-NO_3^-, P-PO_4^{3-}, and COD, respectively. The biomass was harvested by centrifugation, frozen, freeze-dried, vacuum sealed, and stored at −20 °C until further analysis.

2.2. Amino acid profile

Amino acid quantification was done following a methodology described in a previous work [14]. Briefly, 100 mg of dried biomass were hydrolysed using 6 N HCl under vacuum at 110 °C for 24 h; then, the samples were filtered (0.45 μm), washed and evaporated to dryness under nitrogen. The dry residue was resuspended in 2 mL of distilled water and the single amino acids were analysed using a Perkin Elmer Series 200 HPLC (MA, USA) coupled to a Perkin Elmer Altus A-10 fluorescence detector (MA, USA). The mobile phase A consisted of methanol: acetonitrile (12:1, v:v) and the mobile phase B was 23 mM sodium acetate (pH 5.95). After applying a linear gradient for 75 min at a flow rate of 1 mL min^{-1} from 0 to 53.5 % B, an equilibration step was performed with 100 % A for 20 min.

2.3. Fatty acid profile

The preparation of fatty acid methyl esters for the analysis of fatty acids was carried out following a previously described method [15] using a microwave MARS 6 Express 40 (CEM Corporation, NC, USA). Separation and quantification of the fatty acid methyl esters was carried out using a Clarus 580 gas chromatograph (Perkin Elmer, MA, USA) fitted with a flame ionisation detector and a CP-Sil 88 capillary column (100 m × 0.25 mm, 0.2 μm; Agilent, CA, USA). Hydrogen was used as the carrier gas at a flow rate of 1.25 mL·min^{-1}. The injection volume of 0.5 μL and the split ratio 10:1. The injector and detector temperatures were 250 and 270 °C, respectively. The oven temperature was first set at 80 °C and increased to 220 °C at an increase rate of 6.2 °C·min^{-1}, holding this temperature for 3.2 min. Then, the temperature increased to 240 °C at a rate of 6.3 °C·min^{-1}, holding this temperature for 6.5 min. The fatty acids were determined by comparing their retention times with those of a certified reference SupelcoTM FAME mix material (Sigma Aldrich, Arklow, Ireland). The peask integration was carried out using TotalChrom 6.3.2 (PerkinElmer, Waltham, MA, USA). The quantification of the fatty acids was carried out according to the internal standard [15].

2.4. Carotenoid profile

Carotenoids were determined following a previously reported methodology [16] using a Beckman Coulter GOLD HPLC system (CA, USA) equipped with an L-7420 TermoQuest diode-array detector (CA, USA) and a Merck LiChroCART RP-18 column (Darmstadt, Germany), 5 μm size, 250 × 4 mm. The mobile phase A was ethyl acetate and the mobile phase B was acetonitrile:water (9:1, v:v). The mobile phase gradient was as follows: 0–16 min, 0–60 % solvent A; 16–30 min, 60 % A; and 30–35 min, 100 % A. The flow rate was 1 mL·min^{-1}. The selected carotenoids were detected at a wavelength of 450 nm and quantified by comparing the peak areas obtained with those of commercial standards.

2.5. Volatile organic compounds

The volatile organic compounds were extracted by solid phase microextraction as described elsewhere [17] using a PAL RSI 85 autosampler (CTC Analytics, Zwingen, Sitzerland). Two different fibres were used: DVB/C-WR/PDMS and DVB/PDMS. The volatile compounds were analysed using a 7820 A gas chromatograph (Agilent Technologies, Madrid, Spain) coupled to a 5975 series mass spectrometer (Agilent Technologies, Madrid, Spain). The column used was a Supelcowax-10 (Supelco) fused silica capillary column (60 m long, 0.25 mm i.d., 25 μm film thickness). The mass spectrometer was operated in full scan mode (1.4 scans·s^{-1}, m/z range 26–350) at 230 °C with a total ion current of 70 eV. The data were analysed with MSD ChemStation Data Analysis version 5.52 (Agilent Technologies, Madrid, Spain).

2.6. Statistical analysis

Data were analysed using one-way analysis of variance and a Fishers' LSD post hoc test ($p < 0.05$) using Statgraphics Centurion v18 (Statgraphics Technologies Inc., VA, US).

3. Results and discussion

As mentioned above, the present work is a continuation of a previous study that evaluated the effect of different types of water on cultures of *T. almeriensis* [10]. In that study, the macromolecular composition of biomass varied as a function of the microbial populations that were most abundant in the biomass, being *Tetradesmus* strains when the biomass was produced using freshwater or diluted pig slurry, *Nitzchia* when the biomass was produced using secondary wastewater, and an undetermined eukaryote when the biomass was produced using primary wastewater [10]. In the present work, the goal was to fully characterise

Table 1
Amino acid profile of microalgal biomass produced using freshwater, wastewater, or diluted pig slurry. Values represent the means of three independent determinations ± SD. Different letters in the same row indicate statistical differences ($p < 0.05$).

Amino acid	I- Freshwater (g·100 g^{-1})*	II- Primary wastewater (g·100 g^{-1})*	III- Secondary wastewater (g·100 g^{-1})*	IV- Diluted pig slurry (g·100 g^{-1})*
Asp – D	3.36 ± 0.18 ^{B}	4.54 ± 0.16 ^{A}	4.58 ± 0.09 ^{A}	2.94 ± 0.02 ^{C}
Thr – T	1.61 ± 0.02 ^{B}	2.30 ± 0.05 ^{A}	2.16 ± 0.18 ^{A}	1.47 ± 0.07 ^{C}
Ser – S	1.44 ± 0.09 ^{B}	2.09 ± 0.04 ^{A}	1.93 ± 0.29 ^{A}	1.20 ± 0.15 ^{B}
Glu – Q	4.06 ± 0.24 ^{B}	5.36 ± 0.18 ^{A}	5.46 ± 0.11 ^{A}	3.96 ± 0.04 ^{B}
Gly – G	1.92 ± 0.16 ^{B}	2.66 ± 0.14 ^{A}	2.73 ± 0.02 ^{A}	1.65 ± 0.02 ^{C}
Ala – A	2.84 ± 0.15 ^{B}	3.72 ± 0.13 ^{A}	3.80 ± 0.05 ^{A}	2.46 ± 0.01 ^{C}
Cys – C	0.17 ± 0.01 ^{A}	0.16 ± 0.01 ^{A}	0.14 ± 0.02 ^{A}	0.11 ± 0.01 ^{B}
Val - V	1.89 ± 0.27 ^{B}	2.78 ± 0.26 ^{A}	2.79 ± 0.10 ^{A}	1.84 ± 0.05 ^{B}
Met – M	0.65 ± 0.05 ^{C}	0.83 ± 0.03 ^{B}	0.96 ± 0.00 ^{A}	0.53 ± 0.01 ^{D}
Ile – I	1.25 ± 0.19 ^{B}	1.91 ± 0.20 ^{A}	1.84 ± 0.07 ^{A}	1.17 ± 0.03 ^{B}
Leu – L	2.61 ± 0.17 ^{B}	3.77 ± 0.21 ^{A}	3.80 ± 0.04 ^{A}	2.39 ± 0.01 ^{C}
Tyr – Y	0.95 ± 0.09 ^{C}	2.28 ± 0.15 ^{A}	1.50 ± 0.18 ^{B}	0.77 ± 0.04 ^{C}
Phe – F	1.53 ± 0.06 ^{C}	2.95 ± 0.01 ^{A}	2.36 ± 0.05 ^{B}	1.38 ± 0.02 ^{D}
His – H	0.66 ± 0.05 ^{B}	0.82 ± 0.05 ^{A}	0.86 ± 0.01 ^{A}	0.49 ± 0.00 ^{C}
Lys – K	2.97 ± 0.20 ^{C}	3.81 ± 0.19 ^{B}	4.17 ± 0.06 ^{A}	2.43 ± 0.02 ^{D}
Arg – R	3.65 ± 0.16 ^{A}	2.88 ± 0.14 ^{B}	2.83 ± 0.07 ^{B}	2.81 ± 0.04 ^{B}
Pro - P	1.33 ± 0.06 ^{A}	1.05 ± 0.06 ^{B}	1.06 ± 0.02 ^{B}	0.63 ± 0.02 ^{C}

* Data is expressed as g of amino acid per 100 g of biomass. The protein content of the biomass produced using freshwater, primary urban wastewater, secondary urban wastewater, and diluted pig slurry was 35.5 ± 0.1, 52.9 ± 0.3, 48.0 ± 0.3, and 29.1 ± 0.2 %, respectively.

the biomasses produced to predict their potential uses as a function on the type of water used for their production.

3.1. Essential amino acids

The protein content of the biomass produced biomass was 35.5 ± 0.1, 52.9 ± 0.3, 48.0 ± 0.3, and 39.1 ± 0.2 g·100 g^{-1} when produced with freshwater supplemented with nutrients, primary wastewater, secondary wastewater, and pig slurry diluted with freshwater [10]. The protein content of the four biomasses was relatively high compared to other natural resources, especially when produced using wastewater. The high protein content of the biomasses suggested a potential use as animal feed. In fact, *Tetrademus* cultures demonstrated potential for being used as aquafeed ingredients in the past [18]. The protein content of the biomasses also suggested a potential use in agriculture, where amino acids and peptides can have biostimulant effects [19]. *Tetradesmus* strains have also been evaluated as biostimulants [20]. The protein content and the amino acid profile of the biomass are relevant when used in agriculture or as food or feed. The amino acid profile of the different biomasses is shown in Table 1. Essential amino acids for humans are histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. These cannot be synthesised by humans and must be obtained from the diet. When produced using freshwater, the biomass produced contained histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine, demonstrating its potential use as a food supplement for vegans. Tryptophan was not determined because it is degraded during acid hydrolysis [21]. One of the most positive aspects of incorporating essential amino acids from microalgae is that they are generally provided together with other nutritious compounds such as carotenoids, as will be seen in the next subsections. Other microalgae such as Spirulina are generally commercialised as a protein source rich in essential amino acids [22]. Due to their higher protein content, the content of all essential amino acids was higher in biomasses produced using primary and secondary wastewater ($p < 0.05$). However, these biomasses cannot be used as human food. Other animals have different nutrient requirements; for example, there are ten essential amino acids for fish that include arginine, histidine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. The biomasses produced using treated wastewater showed a promising amino acid profile for use in aquafeeds, since the concentration of these amino acids was exceptionally high. Protein is the most expensive ingredient in aquafeed, and its efficiency is determined by its content in each dietary digestible essential amino acid [23].

3.2. Fatty acids

Microalgae are also used at the industrial level as a source of lipids. Some algal lipids are accepted for use as human food; for example, *Schizochytrium* DHA-rich algal oils can be commercialised in the EU as human food [24]. Algal lipids are also relevant for the aquaculture industry; fish provide essential fatty acids for humans that are supplied mainly through the diet. Most of these are introduced into aquafeed as fish oil. However, the increasing cost of fish oil has led to an increased use of vegetable oils resulting in lower quality of fish muscle [25]. Microalgae could contribute to improving the lipid profile of farmed fish.

The lipid content of the biomass produced using freshwater, primary wastewater, secondary wastewater, and pig slurry diluted with freshwater was 4.0 ± 0.1, 8.6 ± 0.2, 7.2 ± 0.1, and 6.2 ± 0.3, respectively [10]. The fatty acid profile of the biomasses is shown in Table 2. In general, significant differences were observed in the fatty acid content depending on the type of water used for biomass production ($p < 0.05$). Saturated fatty acids (SFAs) was higher in biomasses produced using freshwater (13.9 mg·g^{-1}) and pig slurry (17.8 mg·g^{-1}). In these biomasses, *T. almeriensis* was the most abundant strain. In both cases, the most abundant fatty acid was palmitic acid (C16:0) followed by stearic acid (C18:0). In terms of monounsaturated fatty acids (MUFAs), the same trend was observed, with the highest concentration found in biomass produced using pig slurry (21.0 mg·g^{-1}) followed by freshwater (18.3 mg·g^{-1}). The most abundant fatty acid was oleic acid (C18: 1), which represented 88.7 and 82.1 % of the total content of MUFA, respectively. Oleic acid is the most abundant fatty acid in olive oil and its consumption is suggested as part of a healthy diet. The high content of oleic acid suggests a high oxidative and thermal stability of microalgal oil [26]. Oleic acid was also the most abundant MUFA in biomasses produced using wastewater, although its concentration was significantly lower ($p < 0.05$). Polyunsaturated fatty acids (PUFAs) are the most interesting fatty acids found in microalgae. They are essential for most animals and have several proven health effects, including the prevention of cardiovascular disease or inflammatory diseases [27]. The four evaluated samples had a high content of PUFAs, ranging from 15.2 mg·g^{-1} in the biomass produced using primary wastewater to 25.8 mg·g^{-1} in the biomass produced using secondary wastewater. When the biomass was produced using either freshwater and fertilisers or freshwater and pig slurry the content of PUFAs was 25.0 mg·g^{-1}. The most abundant fatty acid was α-linolenic acid (C18:3) followed by linoleic acid (C18:2). Their occurrence in microalgae is interesting because they are essential fatty acids for mammals and must be provided by food.

Table 2
Fatty acid profile of microalgal biomass produced using freshwater, wastewater, or diluted pig slurry. Values represent the means of three independent determinations ± SD. Different letters in the same row indicate statistical differences ($p < 0.05$).

Fatty acid	I- Freshwater (mg· g^{-1})*	II- Primary wastewater (mg· g^{-1}) *	III- Secondary wastewater (mg· g^{-1}) *	IV- Diluted pig slurry (mg· g^{-1}) *
Saturated fatty acids (SFA)				
C6:0	N.D.	0.017 ± 0.001 ^{A}	0.006 ± 0.000 ^{C}	0.009 ± 0.000 ^{B}
C8:0	0.010 ± 0.006 ^{B}	0.039 ± 0.001 ^{A}	0.007 ± 0.001 ^{B}	0.009 ± 0.000 ^{B}
C10:0	0.010 ± 0.000 ^{B}	0.027 ± 0.003 ^{A}	0.004 ± 0.001 ^{C}	0.004 ± 0.000 ^{C}
C11:0	N.D.	0.005 ± 0.000	N.D.	N.D.
C12:0	0.015 ± 0.000 ^{D}	0.126 ± 0.002 ^{A}	0.022 ± 0.001 ^{B}	0.019 ± 0.000 ^{C}
C14:0	0.182 ± 0.002 ^{D}	0.871 ± 0.012 ^{A}	0.321 ± 0.002 ^{B}	0.284 ± 0.005 ^{C}
C15:0	0.047 ± 0.003 ^{D}	0.170 ± 0.005 ^{A}	0.091 ± 0.002 ^{C}	0.143 ± 0.001 ^{B}
C16:0	11.362 ± 0.153 ^{B}	7.296 ± 0.001 ^{D}	10.015 ± 0.042 ^{C}	15.040 ± 0.028 ^{A}
C17:0	0.203 ± 0.003 ^{B}	0.166 ± 0.000 ^{D}	0.178 ± 0.002 ^{C}	0.359 ± 0.002 ^{A}
C18:0	1.537 ± 0.022 ^{A}	0.960 ± 0.001 ^{B}	0.417 ± 0.002 ^{C}	1.564 ± 0.004 ^{A}
C20:0	0.205 ± 0.004 ^{A}	0.102 ± 0.000 ^{B}	0.047 ± 0.000 ^{C}	0.087 ± 0.117 ^{BC}
C22:0	0.157 ± 0.000 ^{C}	0.140 ± 0.007 ^{C}	0.292 ± 0.006 ^{A}	0.210 ± 0.001 ^{B}
C24:0	0.249 ± 0.003 ^{A}	0.203 ± 0.010 ^{B}	0.126 ± 0.004 ^{C}	0.150 ± 0.003 ^{C}
Σ SFA	13.978 ± 0.191 ^{B}	10.123 ± 0.000 ^{D}	11.526 ± 0.045 ^{C}	17.877 ± 0.073 ^{A}
Monounsaturated fatty acids (MUFAs)				
C14:1	0.003 ± 0.004 ^{C}	0.040 ± 0.012 ^{A}	0.011 ± 0.001 ^{B}	0.019 ± 0.001 ^{B}
C15:1 cis10 (ω5)	0.005 ± 0.001 ^{A}	0.005 ± 0.001 ^{A}	0.007 ± 0.001 ^{A}	N.D.
C16:1 cis9 (ω7)	0.296 ± 0.004 ^{D}	1.774 ± 0.000 ^{A}	0.764 ± 0.004 ^{B}	0.482 ± 0.001 ^{C}
C17:1 cis10 (ω7)	0.006 ± 0.001 ^{C}	0.014 ± 0.000 ^{A}	0.011 ± 0.000 ^{B}	0.008 ± 0.001 ^{C}
C18:1 cis9 (ω9)	14.999 ± 0.212 ^{B}	4.618 ± 0.000 ^{D}	6.756 ± 0.020 ^{C}	18.672 ± 0.082 ^{A}
C18:1 (ω7)	2.912 ± 0.034 ^{B}	3.035 ± 0.005 ^{A}	2.085 ± 0.005 ^{C}	1.737 ± 0.011 ^{D}
C22:1 (ω9)	0.037 ± 0.001 ^{C}	0.088 ± 0.000 ^{B}	0.200 ± 0.016 ^{A}	0.075 ± 0.008 ^{B}
C24:1, cis15 (ω9)	0.020 ± 0.002 ^{B}	0.050 ± 0.005 ^{A}	0.052 ± 0.040 ^{A}	0.055 ± 0.001 ^{A}
Σ MUFA	18.277 ± 0.255 ^{B}	9.624 ± 0.011 ^{C}	9.886 ± 0.003 ^{C}	21.048 ± 0.063 ^{A}
Polyunsaturated fatty acids (PUFAs)				
C18:2 (ω6)	6.827 ± 0.104 ^{B}	5.050 ± 0.003 ^{D}	5.532 ± 0.017 ^{C}	9.769 ± 0.006 ^{A}
C20:2 cis11,14 (ω6)	0.797 ± 0.001 ^{C}	0.447 ± 0.003 ^{D}	1.692 ± 0.011 ^{B}	2.920 ± 0.007 ^{A}
C22:2 cis13,16 (ω6)	0.016 ± 0.016 ^{C}	0.217 ± 0.010 ^{A}	0.037 ± 0.002 ^{BC}	0.045 ± 0.017 ^{B}
C18:3, cis6,9,12 (ω6)	0.191 ± 0.005 ^{C}	0.247 ± 0.002 ^{B}	0.299 ± 0.001 ^{A}	0.304 ± 0.006 ^{A}
C18:3 (ω3)	17.064 ± 0.203 ^{B}	7.671 ± 0.000 ^{D}	18.323 ± 0.063 ^{A}	12.340 ± 0.035^{C}
C20:3, cis8, 11, 14 (ω6)	0.019 ± 0.019 ^{BC}	0.091 ± 0.020 ^{A}	0.013 ± 0.011 ^{C}	0.039 ± 0.009 ^{B}
C20:3, cis11, 14, 17 (ω3)	0.037 ± 0.020 ^{B}	0.076 ± 0.000 ^{A}	0.049 ± 0.000 ^{B}	0.049 ± 0.000 ^{B}
C20:4 (ω6)	0.032 ± 0.005 ^{C}	0.274 ± 0.002 ^{B}	0.150 ± 0.001 ^{A}	0.028 ± 0.002 ^{C}
C20:5, cis5,8,11,14,17 (ω3)	0.088 ± 0.001 ^{D}	1.044 ± 0.001 ^{A}	0.544 ± 0.005 ^{B}	0.156 ± 0.005 ^{C}
C22:5 cis7,10,13,16,19 (ω3)	N.D.	0.055 ± 0.001 ^{A}	0.025 ± 0.001 ^{B}	N.D.
C22:6, cis4,7,10,13,16,19 (ω3)	N.D.	0.109 ± 0.006 ^{A}	0.083 ± 0.014 ^{B}	0.017 ± 0.000^{C}
Σ PUFA	25.071 ± 0.262 ^{B}	15.281 ± 0.009 ^{C}	26.745 ± 0.101 ^{A}	25.039 ± 0.044 ^{B}

N.D. Not detected. * Data is expressed on a dry weight basis.

Their consumption ameliorates or prevents the morbidity and mortality of various degenerative diseases, especially those related to the brain and cardiovascular system [28]. Two fatty acids, namely docosapentaenoic acid (C22:5) and docosahexaenoic acid (C22:6), were not detected in the biomass produced using freshwater and were only present in the biomass produced using wastewater. This can be attributed to the different microbial populations present in both biomasses, probably some of the compounds present in wastewater ignited their synthesis. In general, the fatty acid profile of the biomasses was comparable to that of other *Tetradesmus* (*Scenedesmus*) [29]. Future works will assess the effect of biomass processing (e.g., drying) on the content and stability of these fatty acids, as previous works revealed that they can strongly affect their content [29].

3.3. Carotenoids

Carotenoids are pigments that are widely distributed in nature and include carotenes and xanthophylls. Animals cannot synthesise carotenoids de novo and therefore must obtain them from food [30]. For this reason, carotenoids have applications in different sectors including food

Table 3
Carotenoid profile of microalgal biomass produced using freshwater, wastewater, or diluted pig slurry. Values represent the means of three independent determinations ± SD. Different letters in the same row indicate statistical differences ($p < 0.05$).

Carotenoid	I- Freshwater (mg·g^{-1})*	II- Primary wastewater (mg·g^{-1})*	III- Secondary wastewater (mg·g^{-1})*	IV- Diluted pig slurry (mg·g^{-1})*
Lutein	1.154 ± 0.143 ^{A}	0.732 ± 0.190 ^{C}	0.943 ± 0.040 ^{B}	1.290 ± 0.190 ^{A}
Fucoxanthin	0.015 ± 0.009 ^{C}	0.073 ± 0.010 ^{A}	0.071 ± 0.029 ^{AB}	0.051 ± 0.010 ^{B}
Neoxanthin	0.109 ± 0.05 ^{A}	0.111 ± 0.001 ^{A}	0.125 ± 0.033 ^{A}	0.080 ± 0.011 ^{B}
Violaxanthin	0.224 ± 0.034 ^{A}	0.161 ± 0.008 ^{B}	0.167 ± 0.044 ^{B}	0.096 ± 0.005 ^{C}
Lycopene	N.D.	0.059 ± 0.002 ^{B}	0.191 ± 0.020 ^{A}	0.054 ± 0.019 ^{B}
β-carotene	0.324 ± 0.006 ^{A}	0.155 ± 0.026 ^{B}	0.341 ± 0.078 ^{A}	0.127 ± 0.017 ^{C}

N.D. Not detected.
* Data is expressed as mg of carotenoid per g of biomass. The total carotenoid content of the biomass produced using freshwater, primary urban wastewater, secondary urban wastewater, and diluted pig slurry was 3.3 ± 0.4, 2.6 ± 0.6, 3.6 ± 0.6, and 2.4 ± 0.1 mg·g^{-1}, respectively. The total carotenoid content was assessed spectrophotometrically.

Table 4
Relative abundance of the volatile organic compounds extracted from the microalga *Scenedesmus almeriensis* using a DVB/C-WR/PDMS or a DVB/PDMS fibre. Values represent the means of three independent determinations.

Fibre	DVB/C-WR/PDMS					DVB/PDMS				
Compound	LRI	I- Freshwater (RAAU)	II- Primary wastewater (RAAU)	III- Secondary wastewater (RAAU)	IV- Diluted pig slurry (RAAU)	LRI	I- Freshwater (RAAU)	II- Primary wastewater (RAAU)	III- Secondary wastewater (RAAU)	IV- Diluted pig slurry (RAAU)
1,3-Pentadiene	–	8.60E+00	1.28E+01	9.95E+01	6.86E+00	–	ND	ND	2.76E+01	ND
Heptane	704	7.20E+02	1.73E+03	4.63E+03	7.80E+02	–	1.19E+02	9.78E+01	9.70E+02	2.03E+02
Octane	799	9.64E+00	1.65E+02	4.83E+02	5.17E+01	801	2.35E+00	1.35E+02	1.44E+01	ND
Acetone	810	8.58E+00	ND	1.13E+02	5.61E+01	–	ND	ND	ND	ND
Cyclotrixiloxane, hexamethyl	849	2.28E+01	1.73E+01	2.55E+01	3.67E+00	851	6.11E+01	3.43E+01	6.69E+00	1.33E+01
Butanal	867	2.59E+01	9.51E+00	2.18E+01	ND	864	6.47E+01	ND	ND	ND
Cyclohexane, ethyl-	873	2.04E+00	ND	ND	ND	873	ND	8.88E+00	ND	ND
Ethyl acetate	879	1.11E+00	2.46E+01	ND	2.15E+00	–	ND	ND	ND	ND
2-Butanone	898	ND	8.54E+00	ND	ND	–	ND	ND	ND	ND
Butanal, 2-methyl-	909	ND	4.47E+00	ND	ND	–	ND	ND	ND	ND
Butanal, 3-methyl	913	ND	4.99E+00	ND	ND	–	ND	ND	ND	ND
Methylene chloride	919	8.20E+00	8.49E+00	ND	ND	917	1.13E+01	5.15E+00	ND	ND
Dimethyl ether	928	6.36E+00	4.08E+00	ND	5.94E+00	–	ND	ND	ND	ND
Furan, 2-ethyl-	946	ND	7.30E+00	1.16E+01	1.15E+00	942	2.09E+01	5.59E+00	1.06E+01	ND
Propanoic acid, ethyl ester	952	1.15E+00	4.51E+01	ND	ND	947	ND	3.17E+01	ND	ND
Propanoic acid, 2-methyl, ethyl ester	960	ND	1.88E+00	ND	ND	–	ND	ND	ND	ND
Propanoic acid, anhydride		ND	ND	ND	ND	1149	1.90E+01	4.25E+01	ND	4.91E+00
n-Propyl acetate	969	ND	1.04E+01	ND	ND	965	ND	4.52E+00	ND	ND
2-Pentanone	972	3.30E+00	4.85E+00	ND	ND	971	ND	ND	ND	3.02E+00
Pentanal	973	ND	1.11E+01	9.85E+00	1.91E+01	–	ND	ND	ND	ND
Nonane, 2,5-dimethyl-	1008	8.64E-01	ND	ND	ND	–	ND	ND	ND	ND
Trichloromethane	1012	5.06E+01	9.74E+01	3.05E+02	2.12E+02	1009	1.45E+01	2.55E+01	1.39E+02	7.87E+01
1-Penten-3-one	1016	ND	1.41E+01	ND	ND	–	ND	ND	ND	ND
Silanol, trimethyl-	1020	7.71E+00	ND	ND	ND	1017	1.42E+01	ND	ND	ND
3-Ethyl-1,5-octadiene	–	ND	ND	ND	ND	1025	ND	3.10E+00	ND	ND
3,5-Octadien-2-one	–	ND	ND	ND	ND	1546	ND	1.04E+01	ND	ND
Butanoic acid, ethyl ester	1032	ND	2.33E+01	ND	ND	1029	ND	9.78E+01	ND	ND
Toluene	1033	5.37E+02	2.10E+02	1.18E+03	5.35E+02	1031	3.56E+02	ND	3.19E+02	1.05E+02
Propanoic acid, propyl ester	1039	ND	7.37E+01	ND	ND	1038	ND	2.54E+01	ND	ND
Butanoic acid, 2-methyl-, ethyl ester	1048	ND	2.56E+01	ND	ND	1049	ND	3.22E+01	ND	ND
Disulfide, dimethyl	1066	ND	4.33E+00	ND	ND	1064	ND	6.42E+00	ND	ND
Acetic acid, butyl ester	1068	ND	3.16E+02	1.57E+02		1069	8.66E+00	6.40E+02	3.27E+02	ND
Propanoic acid, butyl ester	–	ND	ND	ND	ND	1136	ND	6.57E+02	ND	ND
Hexanal	1079	ND	3.54E+01	4.54E+01	2.35E+01	1080	6.65E+00	3.62E+01	2.13E+01	2.39E+01
Butanoic acid, propyl ester	–	ND	ND	ND	ND	1118	ND	2.06E+01	ND	ND
1-Propanol, 2-methyl-	1082	5.23E+00	5.25E+01	3.23E+01	4.27E+00	–	ND	ND	ND	ND
Ethylbenzene	1121	6.98E+00	7.80E+00	ND	2.34E+00	–	ND	ND	ND	ND
Pentanoic acid, ethyl ester	1133	ND	3.23E+00	ND	ND	1127	1.44E+01	6.22E+00	ND	ND
1-Butanol	1138	7.55E+02	1.09E+03	1.77E+03	5.65E+01	1134	1.47E+03	8.11E+02	1.94E+03	2.25E+01
1-Penten-3-ol	1151	3.28E+01	9.06E+01	9.20E+01	6.62E+00	1149	ND	ND	4.23E+01	ND
1-Butanol, 3-methyl-	1197	1.67E+01	4.52E+01	6.63E+00	4.10E+01	1194	2.19E+01	1.99E+01	ND	3.66E+01
Benzene, chloro	1207	9.96E+01	ND	ND	ND	1205	1.19E+02	ND	ND	ND
Butanoic acid, butyl ester	1223	ND	1.21E+01	ND	ND	1226	1.21E+03	7.77E+02	ND	6.48E+02
1-Pentanol	1242	ND	8.53E+00	ND	ND	–	ND	ND	ND	ND
Styrene	1258	1.40E+02	5.30E+01	ND	2.87E+01	1258	3.21E+02	1.16E+02	ND	ND
2-Butanone, 3-hydroxy	1282	ND	ND	ND	8.48E+01	1282	ND	ND	ND	9.30E+01
Pentanoic acid, butyl ester	1309	ND	1.77E+01	ND	ND	1341	ND	2.11E+01	ND	ND
1-Hexanol	1381	ND	3.35E+00	6.29E+00	ND	1360	3.67E+01	ND	ND	ND
Octanoic acid, ethyl ester	–	ND	ND	ND	ND	1459	1.94E+02	1.72E+01	6.64E+01	ND

(*continued on next page*)

Table 4 (*continued*)

Fibre	DVB/C-WR/PDMS					DVB/PDMS				
Compound	LRI	I- Freshwater (RAAU)	II- Primary wastewater (RAAU)	III- Secondary wastewater (RAAU)	IV- Diluted pig slurry (RAAU)	LRI	I- Freshwater (RAAU)	II- Primary wastewater (RAAU)	III- Secondary wastewater (RAAU)	IV- Diluted pig slurry (RAAU)
2-Hexene, 3,5,5-trimethyl-	–	ND	ND	ND	ND	1481	ND	5.53E+00	ND	ND
Cyclohexanol	1394	1.11E+02	2.93E+01	1.75E+01	6.18E+01	1392	4.07E+02	2.97E+01	2.53E+01	7.61E+01
Benzaldehyde	1559	2.29E+00	5.69E+00	ND	ND	1560	ND	9.25E+00	1.17E+01	6.49E+00
Hexadecane	1615	ND	9.54E+00	ND	ND	1589	3.33E+01	4.84E+02	2.12E+02	1.09E+01
Cyclohexanone, 2,6-dimethyl-	1631	ND	2.21E+01	ND	ND	1623	ND	6.78E+01	ND	ND
Cyclohexanone, 1,3,5-trimethyl-	–	ND	ND	ND	ND	1371	ND	ND	2.14E+01	ND
Pentadecane		ND	ND	ND	ND	1511	–	1.60E+01	ND	ND
β-Terpineol	1644	ND	3.69E+01	5.66E+00	ND	1633	–	1.91E+02	ND	ND
1-Tridecene	–	ND	ND	ND	ND	1637	1.21E+02	ND	ND	ND
β-Cyclocitral	–	ND	ND	ND	ND	1646	ND	2.23E+01	1.20E+01	ND
Heptadecane	–	ND	ND	ND	ND	1690	ND	7.18E+00	2.62E+01	3.07E+00
Butyl caprylate	1677	ND	6.65E+00	ND	ND		ND	ND	ND	ND
Propanoic acid	1802	ND	6.08E+01	ND	ND	–	ND	ND	ND	ND
Octadecane	1812	ND	ND	1.08E+01	6.95E+00	1771	6.06E+01	6.70E+01	4.37E+02	1.26E+02
8-Heptadecene	1821	ND	ND	1.82E+01	ND	1781	2.44E+02	1.66E+02	6.61E+02	1.47E+02
Dimethyl sulfone	–	ND	ND	ND	ND	1814	4.10E+01	ND	ND	ND
β-Ionone	–	ND	ND	ND	ND	1952	ND	1.71E+01	ND	ND
Butanoic acid	1904	ND	1.27E+02	ND	ND	–	ND	ND	ND	ND

Abbreviations: LRI, linear retention index; RAAU, relative abundance arbitrary units; ND, nor detected.

and feed production. Algal carotenoids are relevant for the industrial sector. In fact, they are one of the most popular microalgal compounds due to their high price and their interest in high-end industries such as the cosmetic, pharmaceutical, or functional food industries [31]. Astaxanthin from the microalga *Haematococcus pluvialis* and β-carotene from *Dunaliella salina* are already being commercialised [32]. Lutein derived from microalgae showed potential for use as a food additive in the past, but production costs cannot compete with lutein obtained from Marygold flowers [33].

Table 3 lists the carotenoid profile of the microalgal biomasses produced using the different types of water. Overall, the type of water used significantly affected the carotenoid profile of the biomass ($p < 0.05$), mainly due to the different abundance of microorganisms. Furthermore, the different composition of the culture media might also have contributed to the synthesis of carotenoids. Indeed, the production and accumulation of carotenoids is a common defence mechanism of extremophile strains against the harsh environments in which they grow [34]. In this work, the predominant carotenoid in all samples was lutein. Its concentration was higher in the biomasses produced in freshwater (both when produced using pig slurry or fertilisers as nutrient sources). This can be attributed to the higher proportion of *T. almeriensis* in these two samples, as this strain is especially rich in lutein [35]. The biomass produced using freshwater and commercial fertilisers had the highest carotenoid content, highlighting the concentration of β-carotene and violaxanthin, higher than in the other samples ($p < 0.05$). The carotenoid content of the biomasses was in line with that reported for other strains, including *Botryococcus braunii* (2.10 $mg{\cdot}g^{-1}$), *Chlorella vulgaris* (0.25–3.04 $mg{\cdot}g^{-1}$), *Haematococcus pluvialis* (1.89 $mg{\cdot}g^{-1}$), *or Nannochloropsis oculata* (1.65 $mg{\cdot}g^{-1}$) [36] . Carotenoids are valuable food ingredients used to increase the bioactive properties of foods [37]. Lycopene was detected mainly in wastewater-produced biomasses, probably because of the presence of lycopene in *Nitzschia* strains [38]. Carotenoids are also important compounds in animal feed and are responsible for the pigmentation of many fish and participate in different biological functions such as production against oxidation, immune response, and reproductive behaviour [39].

3.4. Volatile organic compounds

Finally, the content of volatile organic compounds was estimated for the different biomasses; the results about the identification and semi-quantitative information of the individual VOCs are shown in Table 4. In total, 67 VOCs were detected in the four samples studied. The results are in line with previous work that identified a similar amount of VOCs in microalgal biomass; for example, Van Durme et al. [40] detected 57 compounds in five microalgal strains. Other works identified a larger number of VOCs [41]. These differences might be caused by differences in the production and processing of the microalgae (e.g., freeze-drying vs. spray-drying). In addition, the variable number of VOCs detected in microalgal biomass can be attributed to the different methodologies used to extract and analyse the samples. Indeed, in the present study, two different fibres were studied (DVB/C-WR/PDMS and DVB/PDMS) and the profile of VOCs detected in the biomasses was significantly affected by the fibre used. When extracted using DVB/C-WR/PDMS, the maximum number of VOCs detected in a sample was 45 (primary wastewater). This number was reduced to 39 when using the same biomass and the DVB/PDMS fibre. The number of VOCs also varied significantly between samples. The biomass produced using pig slurry diluted in freshwater showed the lowest number of VOCs, 21 and 17 when extracted using DVB/C-WR/PDMS and DVB/PDMS, respectively. The results shown in Table 4 demonstrate the variability in individual volatile compounds and chemical families. Similar results were reported in a previous work when comparing different microalgal strains [17]. The different microbial compositions in the four biomasses [10] might partially explain these findings. Furthermore, the presence of VOCs in the culture medium, especially in the primary treated wastewater and the medium prepared with pig slurry, might have also affected the identification of VOCs. To the best of our knowledge, there are no studies evaluating the presence of VOCs in wastewater during its treatment using microalgae-based processes.

4. Conclusions

In this work, we identified how the biochemical composition of the microalgal biomass varies as a function of the type of water used to produce it. This has a huge impact on its potential uses. The protein content and abundance of essential amino acids were significantly affected; this work demonstrates the suitability of using microalgae produced in wastewater as animal feeds. The biomass produced using pig slurry had a chemical composition that revealed its potential use as

animal feed or as an agricultural biostimulant. In turn, the biomass produced using freshwater had a high concentration of essential amino acids and polyunsaturated fatty acids, demonstrating its suitability for use as human food. Overall, the biomass showed potential for being used in different industrial applications, especially as a source of ingredients for animal feed and as a source of high-value agricultural products such as plant biostimulants. Future works should evaluate their potential bioactive properties, which together with their biochemical composition will identify the best-suited application for the biomasses produced using different types of water.

CRediT authorship contribution statement

Silvia Villaró: Investigation, Formal analysis, Writing – original draft. **María Cuaresma Franco:** Investigation, Formal analysis, Resources. **Marco García-Vaquero:** Investigation, Formal analysis, Resources. **Lara Morán:** Investigation, Formal analysis, Resources. **Francisco Javier Alarcón:** Investigation, Formal analysis, Resources. **Tomás Lafarga:** Formal analysis, Supervision, Funding acquisition, 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

Data related with the biomass production is accessible at the SABANA Data Center (http://www2.ual.es/sabana/data-center-2/). The rest of the data will be made available on request.

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