Bioaccessibility Characterization of Organic Matter, Nitrogen, and Phosphorus from Microalgae-Bacteria Aggregates

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ORIGINAL PAPER

Bioaccessibility Characterization of Organic Matter, Nitrogen, and Phosphorus from Microalgae-Bacteria Aggregates

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Abstract

The quality of microalgae-bacteria biomass as an organic soil fertilizer may depend on the biomass's microbial composition, morphology, and growth history. This study aims to characterize the molecular complexity and bioaccessibility of organic matter, nitrogen, and phosphorus from microalgae-bacteria aggregates with different morphologies (flocs and granules) grown under nutrient-abundant and starvation conditions. A biochemical fractionation method was used based on sequential chemical extraction and fluorescence spectroscopy. Microalgae-bacteria aggregates were cultured and collected from photobioreactors using contrasting growth conditions to generate (i) loose flocs, (ii) consolidated flocs, (iii) smooth granules, and (iv) filamentous granules. The organic matter, nitrogen, and phosphorus from consolidated flocs were mostly extractable, accounting for up to 94% of their total content. In contrast, the organic matter from loose flocs was up to 50% non-extractable. The extractability of loose flocs was improved under starvation conditions. All microalgae-bacteria aggregates showed a low structural complexity, corresponding to an abundance of simple microbial-related constituents like tyrosine and tryptophane. Differences between the gradients of bioaccessibility for each microalgae-bacteria structure were related to the abundance of microorganisms and their metabolic products. The findings of this study have implications for the development of sustainable and environment-friendly organic fertilizers.

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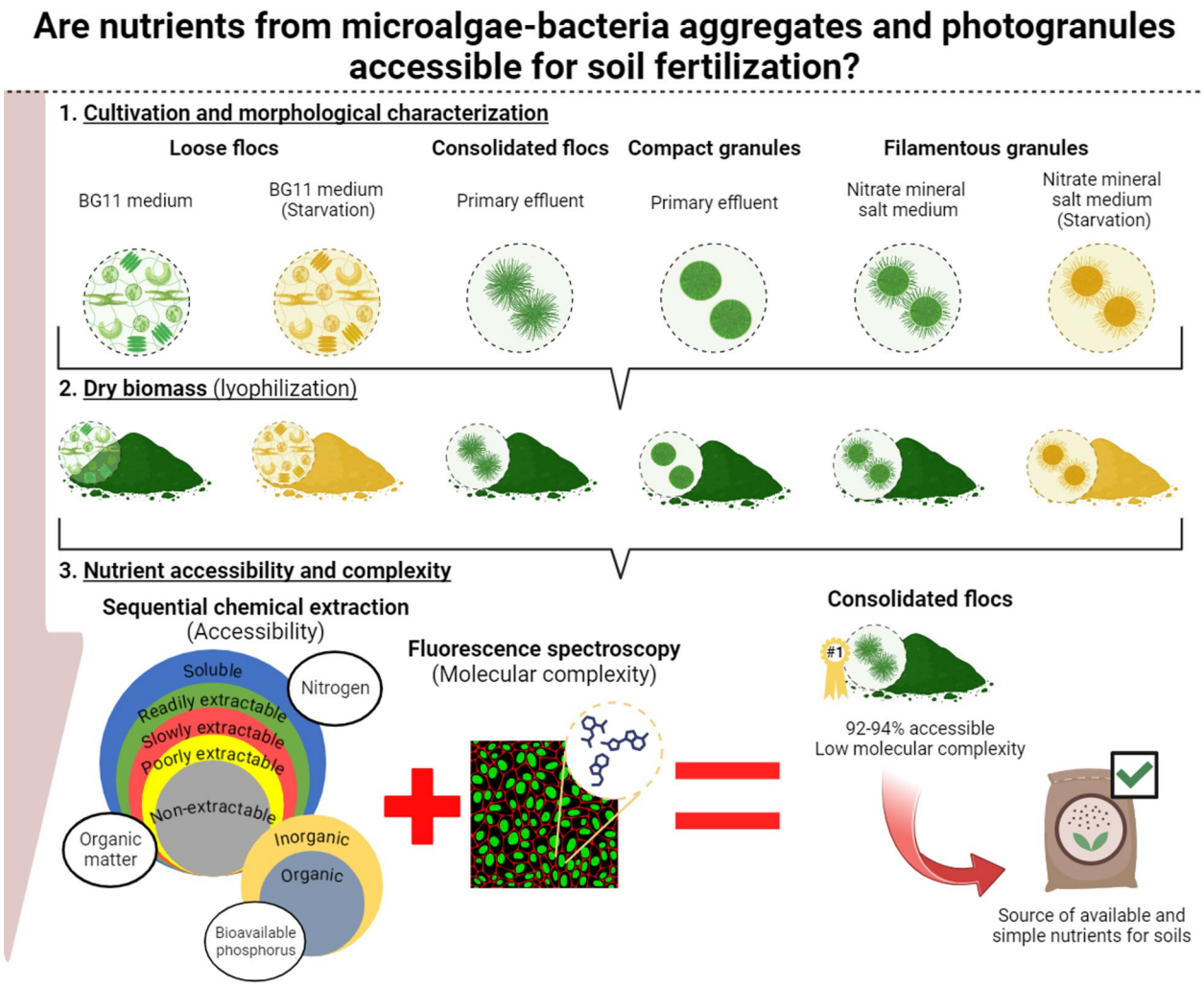
Graphical Abstract



Keywords Biochemical fractioning · Biomass valorization · Fluorescence spectroscopy · Nutrients bioaccessibility · Oxygenic photogranule · Soil fertilization

Hihglights

- Organic matter and nutrient bioaccessibility were assessed in microalga-bacteria structures.
- The morphology of floccular or granular structures influenced the bioaccessibility.
- Maximal bioaccessibility of organic matter and nutrients was observed in flocs.
- Starvation improves the bioaccessibility of organic matter in loose flocs.
- Simple molecular-like groups prevail in microalgae-bacteria aggregates.

Introduction

Modern agriculture is driven by the energy-intensive synthesis of fertilizers (*i.e.*, the Haber–Bosch process) and the extraction of mineral ore deposits (*e.g.*, phosphate rock). With a projected global human population growth from eight to nine billion by 2050, a similar increase in food demand and utilization of these fertilizers can be expected. However, synthetic fertilizers negatively affect the environment and soil quality over the long term. Current manufacturing practices of the fertilizing industry are energy-intensive and responsible for 2.5% or 1203 Tg of global carbon dioxide emissions [1] and the continuous depletion of mineral deposits worldwide [2]. The limited retention

capacity of nutrients in soils has resulted in their excessive application, which has been linked to the eutrophication of ground and surface bodies and the limited growth of endemic soil microorganisms [3]. A need for more sustainable and innovative organic fertilizers that minimize energy consumption and dependency on fossil resources while maintaining crop growth and yield is evident.

Microalgae, including eukaryotic microalgae, diatoms, and cyanobacteria, may be used as organic fertilizers because they convert nutrients into biomass rich in essential elements for soil fertilization, namely nitrogen and phosphorus [4]. Furthermore, microalgae biomass has notably contributed to plant growth through several mechanisms, including phytohormone production, root association formation, and protection against phytopathogens and pests [5]. These promising attributes have resulted in improvements in crop performance that are equal to or even superior to those achieved using synthetic fertilizers. Álvarez-González et al. [6] observed a significant increase in shoot and leaf weight, as well as the number of leaves of basil (*Ocimum basilicum* L.) by harnessing the high nitrogen content of the eukaryotic microalga *Scenedesmus* sp. biomass cultivated in municipal wastewater. Similar enhancements in potato plants' growth, development, and nitrogen assimilation (*Solanum tuberosum*) were reported using spray-dried eukaryotic microalga *Asterarcys quadricellulare* biomass [7]. Height, leaves, and other essential aspects of onion plants (*Allium cepa* L.) were also improved by mixing soil with cow manure and the cyanobacterium *Spirulina platensis*, compared to cow manure alone [8].

Growing heterotrophic and phototrophic microorganisms syntrophically was recognized as a promising strategy to boost bioproduct yields [9]. The key to syntrophic interactions is the production of oxygen by microalgae through photosynthesis. In return, heterotrophic bacteria oxidize organic matter and produce carbon dioxide to sustain microalgae growth. Compared to pure microalgae monocultures, co-culturing microalgae, and heterotrophic bacteria has improved nutrient assimilation and biomass yield, tolerance against pollutants, and stability against competing microorganisms [10]. Moreover, combining these microorganisms has also been observed to promote biomass aggregation, allowing efficient and inexpensive harvesting by gravity settling [11]. Studies describing the mechanisms of microalgae-bacteria aggregation have been reported. Aggregates can be in the form of flocs, compact granules [12], and filamentous granules [13], dominated by different eukaryotic microalgae or cyanobacteria genera.

The feasibility of using microalgae-bacteria aggregates as feedstock for organic fertilizer production has previously been discussed [14]. However, their fertilizing value has yet to be systematically determined. Previous studies have established that organic substrates can be categorized into different fractions, each defined by i) their complexity (molecular structure) and ii) their bioaccessibility (availability as a source of organic matter, nitrogen, and phosphorus to soil microorganisms) [15]. This categorization has proven to be a valuable approach for a better diagnosis of the fertilizing value of organic materials, as it delivers a more comprehensive prediction of the fate of nitrogen, phosphorus, and organic matter after soil amendment [16]. Assessing the complexity and bioaccessibility of microalgae-bacteria aggregates offers valuable insights into their composition and serves as a foundation for the potential optimization of fertilizer production.

This study comprehensively characterizes morphologically distinct microalgae-bacteria aggregates in terms of molecular complexity and organic matter, nitrogen, and phosphorus bioaccessibility as potential organic soil fertilizers. Biochemical fractionation methods were employed using sequential chemical extraction and fluorescence spectroscopy [17]. This investigation also compared the molecular complexity and organic matter, nitrogen, and phosphorus bioaccessibility between selected microalgae-bacteria aggregates grown under nutrient-abundant and nutrient-reduced conditions to analyze changes in their composition during starvation. The underlying hypothesis is that the morphology of microalgae-bacteria aggregates influences the molecular complexity and the bioaccessibility of organic matter, nitrogen, and phosphorus. Microalgae-bacteria flocs, being more fragile structures, may present more bioaccessible nutrients, whereas, in the more compact microalgae-bacteria granules, the nutrients may be less bioaccessible.

Materials and Methods

Origin, Morphology, and Preparation of Microalgae-Bacteria Aggregates

Four morphologically distinct samples of microalgae-bacteria aggregates used for wastewater treatment or biomass production were collected from three photobioreactors (PBRs) operated under different conditions. Sample A consisted of microalgae-bacteria aggregates and was collected from flat-panel PBRs with a working volume of 13 L (total volume of 15 L). The culture was grown in fresh BG-11 medium [19] in which the phosphorus concentration was increased from 0.23 mM (39 mg $K_2HPO_4 \cdot L^{-1}$) to 1.1 mM (192 mg $K_2HPO_4 \cdot L^{-1}$). Phosphorus was increased to obtain a nitrogen-to-phosphorus molar ratio of 38:1. This ratio is within the optimum range (16:1–49:1) for sustaining the growth of eukaryotic microalgae and cyanobacteria [21]. Cultures were grown at 25 ± 5 °C and pH between 7.0 and 8.0. Illumination was induced using cool-white LED lights at photosynthetically active radiation (PAR) of 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$. An airflow

of 0.15 $L_{air} \cdot L_{culture}^{-1} \cdot min^{-1}$ was always kept, maintaining a continuous mixture of the cultures.

Sample B and Sample C consisted of two morphologically distinct microalgae-bacteria aggregates found in a high-rate algae pond (HRAP) used to treat wastewater. The HRAP has a working volume of 50 L (total volume 80 L), 0.26 m^2 of surface area, and a consistent liquid velocity of 0.2 $m \cdot s^{-1}$. This pond was continuously fed with primary effluent from a municipal wastewater treatment plant in Santa Rosa Jauregui, Queretaro, Mexico. The composition of the primary effluent was as follows: 517 ± 133 mg $COD \cdot L^{-1}$, 86 ± 15 mg $N\text{-}NH_4^+ \cdot L^{-1}$, 43 ± 4 mg $N\text{-}NO_3^- \cdot L^{-1}$, 25 ± 10 mg $N\text{-}NO_2^-$, and 43 ± 4 mg $P\text{-}PO_4^{3-} \cdot L^{-1}$. More on the design and operating conditions of the HRAP can be found in [22].

Sample D was obtained from sequencing batch PBRs with a working volume of 4 L and fed with nitrate mineral salt (NMS) growth medium containing 246 mg $CH_3COONa \cdot L^{-1}$, 5.4 mg $KH_2PO4 \cdot L^{-1}$, 11 mg $Na_2HPO_4.12H_2O \cdot L^{-1}$, 100 mg $(NH_4)_2SO_4 \cdot L^{-1}$, 2.5 mg $Na_2EDTA.2H_2O \cdot L^{-1}$, 0.001 mg $Na_2MoO_4.2H_2O \cdot L^{-1}$, 0.01 mg $FeSO_4.7H_2O \cdot L^{-1}$, 0.02 mg $H_3BO_3 \cdot L^{-1}$, 0.004 mg $ZnSO_4.7H_2O \cdot L^{-1}$, 0.001 mg $MnCl_2 \cdot L^{-1}$, 0.01 mg $CoCl_2.6H_2O \cdot L^{-1}$, 0.014 mg $CuSO_4.5H_2O \cdot L^{-1}$, 0.2 mg $FeCl_3 \cdot L^{-1}$ and 0.001 mg $NiCl_2.6H_2O \cdot L^{-1}$. Cultures were grown at an average temperature of 25 °C, pH of 8.5 ± 0.5, and hydraulic retention time (HRT) of 8 h. Illumination was constantly provided using cool-white LED lights at a PAR of 62 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Overhead stirring at 100 rpm was used to mix the biomass continuously.

The settling velocity and size distribution of the four different aggregate types were determined using the APHA 2710 E method [18] and stereomicroscopy [19], respectively. Genus identification of eukaryotic microalgae and cyanobacteria was carried out according to the guidelines by Wehr and Sheath [20] using an ECLIPSE 90i microscope (Nikon, Japan) equipped with a NIS-Elements V.4.30 image acquisition system (Nikon, Japan). Collected microalgae-bacteria aggregates were harvested through centrifugation (3000 g, 20 min, 5 ºC), frozen at −20 °C for at least 3 h, lyophilized overnight (−80 to −85 °C, 0.05 hPa), and ground using 4.8- mm stainless steel beads in a bead mill. Dry matter was determined gravimetrically using the APHA 2540 B method [18].

Assessment of Total Organic Matter, Total Nitrogen, and Total Bioavailable Phosphorus Content

Total organic matter (TOM), total nitrogen (TN), and total bioavailable phosphorus (TBP) content for each freeze-dried and ground type of microalgae-bacteria aggregate were measured. For TOM, 250 mg of freeze-dried, ground microalgae-bacteria aggregates were placed in a propylene vessel containing 10 mL of 96% $w \cdot w^{-1}$ H_2SO_4. After 24 h, the mixture was diluted with distilled water until a final weight of 250 g was reached, and TOM was measured in the liquid extract using Aqualytic Vario COD tubes for a range of 0–1500 mg $COD \cdot L^{-1}$ (Tintometer GmbH, Germany). For TN, 2–3 mg of freeze-dried, ground microalgae-bacteria aggregates were analyzed using a CHNS/O FlashSmart Elemental Analyzer (ThermoFisher, USA). TBP was determined by placing 300 mg of freeze-dried, ground microalgae-bacteria aggregates in 30 mL of distilled water. Samples were shaken for 24 h in an orbital shaker at 280 rpm and then centrifuged (18,600 g, 20 min, 4 °C). The liquid extract was recovered, filtered (0.45 µm), and stored for analysis. The same procedure was repeated using residual pellets from the previous step but in a 0.5 M $NaHCO_3$ (pH 8.5) solution. Phosphorus concentration in each liquid extract was determined using HACH LCK 348 (HACH GmbH, Germany) kits, totaled, and considered as the TBP content as described in [16].

Sequential Chemical Extraction for Organic Matter, Nitrogen, and Phosphorus Fractions Characterization

A biochemical fractionation method based on sequential chemical extraction of organic matter and nitrogen bioaccessibility was carried out following the ISBAMO fractionation protocol described by Fernández-Domínguez et al. [17]. For each type of freeze-dried, ground microalgae-bacteria aggregates, 1.0 g was weighed and subjected to four different extraction solvents: (1) 10 mM $CaCl_2$, (2) 10 mM NaCl + 10 mM NaOH, (3) 100 mM NaOH, and (4) 72% $w \cdot w^{-1}$ H_2SO_4) using an Accelerated Solvent Extractor DIONEX ASE-350 (Thermo Fisher, USA). Each solvent, sequentially more aggressive to organic substrates [17], allowed the recovery of the resulting four liquid ISBAMO fractions. These fractions contain the organic matter and nitrogen within microalgae-bacteria aggregates according to their degree of bioaccessibility. The four ISBAMO fractions were defined as (1) soluble (extracted with 10 mM $CaCl_2$), (2) readily extractable (extracted with 10 mM NaCl + 10 mM NaOH), (3) slowly extractable (extracted with 100 mM NaOH), and (4) poorly extractable (extracted with 72% $w \cdot w^{-1}$ H_2SO_4). The organic matter and nitrogen concentrations of each ISBAMO fraction were determined using Aqualytic Vario COD tubes for a range of 0–1500 mg $COD \cdot L^{-1}$ (Tintometer GmbH, Germany) and HACH LCK 338 kit (HACH GmbH, Germany), respectively. Finally, a fifth non-extractable fraction was calculated by subtracting the organic matter or nitrogen content of each ISBAMO fraction from the TOM or TN values determined in Sect. "Effect of nutrient starvation".

Inorganic and organic phosphorus fractions of the TBP in microalgae-bacteria aggregates were determined following a modified Olsen-phosphorus methodology [16]. Liquid extracts from distilled water and 0.5 M $NaHCO_3$ extractions were obtained for TBP estimation from Sect. "Total organic matter, total nitrogen, and total bioavailable phosphorus content in microalgae-bacteria aggregates". were analyzed for inorganic phosphorus using a HACH LCK 348 kit (HACH GmbH, Germany). In this study, the inorganic phosphorus determined for liquid extracts from distilled water and 0.5 M $NaHCO_3$ extractions were summed and considered as inorganic phosphorus in the TBP content. Organic phosphorus was determined by subtracting inorganic phosphorus from TBP content with TBP values obtained in Sect. "Total organic matter, total nitrogen, and total bioavailable phosphorus content in microalgae-bacteria aggregates".

Fluorescence Spectroscopy Analysis for Molecular Complexity Characterization

Following the methodology described by Fernández-Domínguez et al. [17], the fluorescence spectra of the ISBAMO fractions of the microalgae-bacteria aggregates were acquired using an LS55 luminescence spectrometer (Perkin Elmer, USA). For the analysis, each fraction was filtered using a 1.6 µm pore-size glass fiber filter, mounted on a 1 cm path-length quartz cuvette, and exposed to excitation wavelengths between 200 and 600 nm at fixed increments of 10 nm. The scanning monochromator speed was set at 1200 $nm{\cdot}s^{-1}$, and the fluorescence emission values were recorded each 0.5 nm using the FL Winlab V.4.00.03 software (Perkin Elmer, USA). These records aimed to obtain a comprehensive fluorescence excitation-emission matrix per 10 nm increment.

The matrices were then integrated to determine the structural complexity of microalgae-bacteria aggregates [23]. The integration algorithm interpolates the measured data with known fluorescence values of molecular intermediates (*e.g.*, proteins, cellulose, etc.) and converts the fluorescence matrices to a grey-level bitmap. These images were then split into seven fluorescence zones, each corresponding to a group of molecules with similar structural complexity [24]. Zones I-III correspond to simple proteins, amino acid-like molecules, or soluble sugars. In contrast, more complex, polymer-like molecules (*e.g.*, humic acid-like substances) give a strong signal in zones IV-VII. The zone fluorescence volume (Eq. 1) the fluorescence proportion (Eq. 2), and the fluorescence complexity index (Eq. 3) were calculated per liquid fraction [24].

$$V_f(x) = \frac{V_{f\,raw}(x)}{OM_S} \times \frac{\sum_1^7 S(x)}{S(x)} \quad (1)$$

$$P_f(x) = \frac{V_f(x)}{\sum_1^7 V_f(x)} \times 100 \quad (2)$$

$$FluorescenceComplexityIndex = \frac{\sum_4^7 V_f(x)}{\sum_1^3 V_f(x)} \quad (3)$$

where $V_f(x)$ is the normalized fluorescence volume of zone x, $V_{f\,raw}(x)$ is the raw volume of zone x, OM_S is the organic matter (mg $COD{\cdot}L^{-1}$) concentration of the ISBAMO fraction, $S(x)$ is the area of zone x, and $P_f(x)$ is the proportion of fluorescence in zone x (%).

Statistical Analyses

Experiments were conducted in duplicate. All statistical analyses were performed using R version 4.2.2 (R Core Team, USA). Data presented in tables and figures were calculated as mean values with corresponding standard deviations using the get_summary_stats function of the rstatix [25] package. Statistically significant differences ($p \leq 0.05$) were determined through a one-way analysis of variance followed by Tukey's honestly significant difference post-hoc test, using the functions aov and HSD.test of the rstatix [25] and agricolae [26] packages, respectively.

Results and Discussion

Morphology of Microalgae-Bacteria Aggregates Samples

The collected microalgae-bacteria aggregates samples were characterized by settling velocity, diameter, and genus-level identification (Table 1). Samples were considered as floccular biomass in the form of loose flocs (Sample A) and consolidated flocs (Sample B) and granular biomass in the form of smooth granules (Sample C) and filamentous granules (Sample D), considering an increasing degree of granulation. An increased settling velocity reflects a higher degree of granulation. Fundamentally, the aggregates consistently contained unicellular eukaryotic microalgae, like *Scenedesmus*, and filamentous cyanobacteria, like *Oscillatoria*, commonly found in freshwater environments [27]. Notably, microalgae-bacteria aggregates also included various other non-phototrophic microorganisms. Representative images of the different types of microalgae-bacteria aggregates used in this study are presented in Fig. 1.

Table 1 Physical and morphological characteristics of microalgae-bacteria aggregates. See Fig. 1 for representative images of the different sample types

	Sample type	Settling velocity ($m\ h^{-1}$)	Diameter (mm)	Identified microalgae and cyanobacteria
Floccular biomass	A) Loose flocs	≤ 1.0	≤ 0.1	Chlorella Scenedesmus Nitzschia Oscillatoria Phormidium
	B) Consolidated flocs	4.7 ± 0.1	2.2 ± 0.9	Chlorella Stigeoclonium
Granular biomass	C) Smooth granules	6.4 ± 0.4	4.0 ± 1.4	Chlorella Stigeoclonium Nitzschia
	D) Filamentous granules	≥ 13.0	2.0 ± 1.4	Oscillatoria

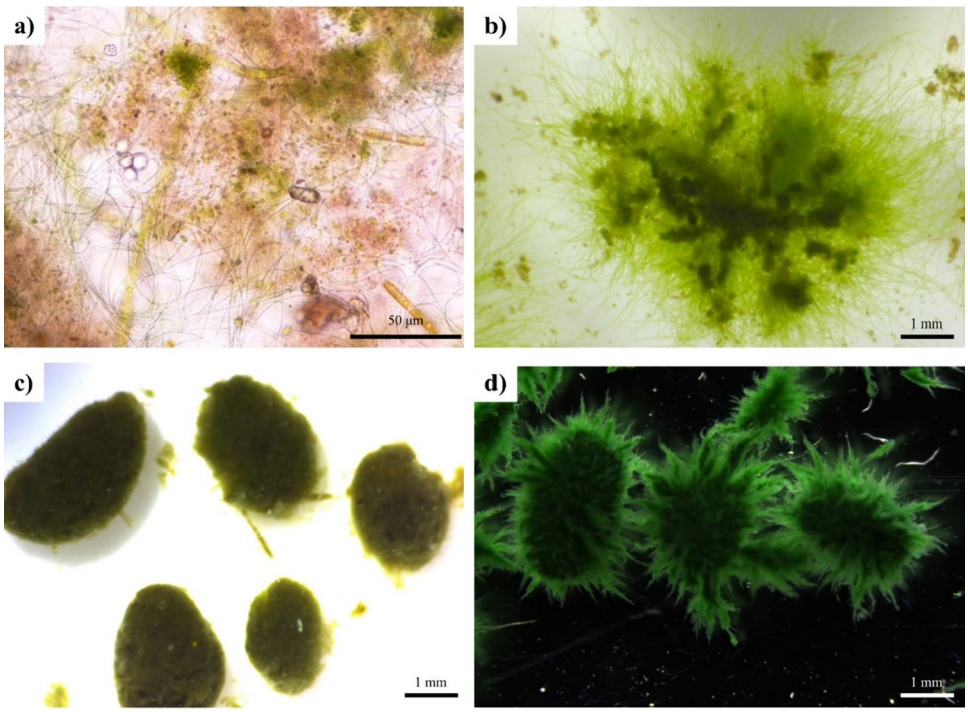


Fig. 1 Representative images of **a** loose flocs, **b** consolidated flocs, **c** smooth granules, and **d** filamentous granules

Total Organic Matter, Total Nitrogen, and Total Bioavailable Phosphorus Content in Microalgae-Bacteria Aggregates

The initial hypothesis proposed that the morphology of microalgae-bacteria aggregates could influence the bioaccessibility of organic matter, nitrogen, phosphorus content, and molecular complexity. TOM (expressed as COD), TN, and TBP content of freeze-dried, ground microalgae-bacteria aggregates were determined. The results of this characterization are presented in Table 2. Statistically significant differences ($p \leq 0.05$) were observed when comparing TOM, TN, and TBP values between analyzed samples. The results between the microalgae-bacteria aggregate samples with the highest TOM, TN, and TBP values led to the consideration that factors other than morphology, *i.e.*, microbial diversity distribution and extracellular polymeric substances (EPS) secretion, could have influenced these values. The following paragraphs address the individual explanations for each parameter to understand these outcomes further.

Granular biomass (smooth granules and filamentous granules) had a higher TOM content than floccular biomass (loose flocs and consolidated flocs). TOM across all microalgae-bacteria aggregates samples ranged between the theoretical COD values for microalgae biomass and bacteria biomass, calculated as 952 mg COD g dry $matter^{-1}$ and 1420 mg COD·g dry $matter^{-1}$, respectively [28]. It has been suggested that differences between theoretical COD values could be attributed to the inherent carbon, oxygen, hydrogen, and nitrogen ratio, *i.e.*, the elemental ratio, of each

Table 2 Total organic matter (TOM), total nitrogen (TN), and total bioavailable phosphorus (TBP) in different microalgae-bacteria aggregates.

	Sample type	TOM (mg COD·g dry matter^{-1})	TN (mg TN·g dry matter^{-1})	TBP (mg TBP·g dry matter^{-1})
Floccular biomass	A) Loose flocs	1004 ± 2^{c}	93 ± 3^{a}	14 ± 1^{b}
	B) Consolidated flocs	1097 ± 17^{b}	88 ± 4^{a}	17 ± 1^{a}
Granular biomass	C) Smooth granules	1208 ± 71^{a}	59 ± 3^{c}	17 ± 1^{a}
	D) Filamentous granules	1223 ± 1^{a}	73 ± 2^{b}	13 ± 1^{b}

Lowercase letters indicate significant differences ($p \leq 0.05$) between samples for each parameter

type of biomass [28]. For microalgae, the common biomass elemental ratio is $CH_{2.48}O_{1.04}N_{0.15}P_{0.01}$ [29]. In contrast, the elemental ratio of bacteria is $CH_{1.40}O_{0.40}N_{0.20}P_{0.02}$ [30]. The significantly higher TOM content observed in granular biomass could be attributed to a higher bacteria proportion over the microalgae biomass when compared to floccular biomass. These findings agree with those of previous studies, where the overall bacteria biomass accounted for approximately 30% of the total biomass concentration in floccular microalgae-bacteria aggregates [31]. In contrast, non-phototrophic and phototrophic bacteria gene sequence types in photogranules were found at maximum abundances of 17 and 67%, respectively [32]. Considering these proportions and assuming that the prevailing population in floccular microalgae-bacteria aggregates and photogranules were made up of microalgae with their respective elemental ratio, the theoretical COD values could be calculated at 1086 mg COD·g dry matter^{-1} and 1340 mg COD·g dry matter^{-1}, which are similar to the data reported in Table 2.

Granular biomass had a significantly lower TN content than that of loose flocs. The TN content in all microalgae-bacteria aggregates was within the theoretical TN range for microalgae biomass and bacteria biomass. Based on the biomass mentioned above elemental ratios of microalgae and bacteria, the theoretical TN content was calculated to be 63 mg TN·g dry matter^{-1} and 124 mg TN·g dry matter^{-1}, respectively. Differences in the theoretical TN content could be attributed to the lower proportion of nitrogen per microalgae biomass compared to bacteria biomass. Granular biomass had a lower TN content than floccular biomass, even lower than the theoretical TN value calculated to be 114 mg TN·g dry matter^{-1} based on the microalgae and bacteria proportions mentioned in [32]. These observations indicate that factors other than microalgae and bacteria could have contributed to the low TN content of granular biomass. The overall formation of microalgae-bacteria aggregates depends on the association between these microorganisms and the quality of EPS. EPS are mainly composed of proteins and polysaccharides. The proteins-to-polysaccharides ratio seems linked to the central aggregation mechanism in microalgae-bacteria aggregates [11]. In this context, proteins are the primary reservoir of nitrogen, whereas polysaccharides determine the carbon content [33]. According to Arcila and Buitrón [34], high protein and, thus, nitrogen content in the EPS could be found in microalgae-bacteria granules when the carbon-to-nitrogen ratio in growth media was below 5. In the present study, gas exchange and pH control (8.0 to 9.0) favored the transformation of atmospheric CO_2 into HCO_3^- in the growth medium, influencing the carbon-to-nitrogen ratio during granular biomass development, as previously observed [34]. This condition likely resulted in a carbon-to-nitrogen ratio ≥ 5, which negatively affected the protein and, thus, nitrogen content in the EPS of granular biomass.

TBP content in consolidated flocs and smooth granules was significantly higher than in loose flocs and filamentous granules. TBP content across all microalgae-bacteria aggregates was also higher than that of microalgae biomass but lower than that of bacterial biomass. The theoretical phosphorus content for microalgae and bacteria was calculated to be 9 mg TBP g dry matter^{-1} and 20 mg TBP·g dry matter^{-1}, using their respective elemental ratios introduced above. Differences in these theoretical values could be attributed to the lower share of phosphorus per microalgae biomass compared to bacteria biomass. The significantly higher TBP in consolidated flocs and smooth granules could also be attributed to other microorganisms contributing to the phosphorus content of the biomass. Considering that these aggregates were cultivated in wastewater, polyphosphate-accumulating organisms (PAOs) growth could have influenced the TBP content of the consolidated flocs and smooth granules. That is supported by Wang et al. [35], who obtained PAOs from secondary effluent sludge and co-cultured them with the eukaryotic microalga *Chlorella pyrenoidosa*.

Moreover, PAOs, known to assimilate substantial amounts of inorganic phosphorus as polyphosphates (up to 38 mg TBP g dry matter^{-1}), were successfully integrated into photogranules [36]. The physicochemical gradients in the granules provided a niche for PAOs. These PAO-enriched photogranules showed a notably higher phosphorus content than non-enriched granules.

Extractable Fractions of Organic Matter, Nitrogen, and Phosphorus in Microalgae-Bacteria Aggregates

Biomass fractions along a gradient of bioaccessibility of organic matter, nitrogen, and phosphorus in different microalgae-bacteria aggregates were analyzed. The results obtained for organic matter, nitrogen, and phosphorus bioaccessibility are shown in Fig. 2 and Tables S1, S2, and S3 in the supplementary data. Consolidated flocs, followed by filamentous granules, had the highest amount of extractable organic matter and nitrogen, given by the soluble, readily extractable, slowly extractable, and poorly extractable fractions. Consolidated flocs contained more readily extractable (*i.e.*, EPS extractions as proteins and lipids) and slowly extractable (*i.e.*, protein-like compounds). In contrast, filamentous granules are rich in poorly extractable fractions, which are associated with carbohydrates-like (*e.g.*, cellulose and hemicellulose) compounds and poorly accessible proteins, given the nitrogen concentration of this fraction. Consolidated flocs and filamentous granules also had the highest proportion of inorganic phosphorus in the TBP.

Microalgae-bacteria flocs were initially hypothesized to be fragile, more bioaccessible structures than the compact microalgae-bacteria granules. However, the results show that the morphology of microalgae-bacteria aggregates, whether flocs or granules, did not influence the bioaccessibility of organic matter, nitrogen, and phosphorus. Additional events during the formation of the different microalgae-bacteria aggregates, *e.g.*, microbial diversity distribution and EPS secretion, could have played a pivotal role in shaping the observed bioaccessibility profiles.

The higher extractability and, thus, higher organic matter and nitrogen bioaccessibility in consolidated flocs could be attributed to an abundance of macromolecules like polysaccharides, proteins, and lipids. These macromolecules are typically the main components of the soluble, readily extractable, and slowly extractable ISBAMO fractions [23]. The contrasting growth and operating conditions for developing granular and floccular biomass in this study could have impacted their macromolecular content. Previous studies reported that the occurrence of macromolecules in microalgae-bacteria aggregates was associated with different abiotic factors, including nutrient availability, light intensity, temperature, shear stress, and pH [37]. These environmental perturbations directly affect the cellular integrity and change the intracellular distribution of carbon flux, determining whether one or another macromolecule is produced. For instance, lipids and carbohydrates in the eukaryotic microalga *Scenedemus acuminatus* increased due to limited nitrogen availability [38]. That occurs as these macromolecules are the primary carbon and energy reserves in microalgae that allow them to cope with environmental stress.

Similarly, the protein content in microalgae-bacteria aggregates decreased from 69 dried weight to 58% dried weight when HRT was increased from 6 to 10 d [11]. Such a decrease was linked to the depletion of nitrogen caused by the growth of microalgae and bacteria. It is possible that operating and process conditions used for growing consolidated flocs (*i.e.*, wastewater growth medium, 6 d HRT, 12 h photoperiods, 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$, pH 8.4 ± 0.4, 22 °C temperature) combined with their fragile structure contributed to their higher nutrient bioaccessibility.

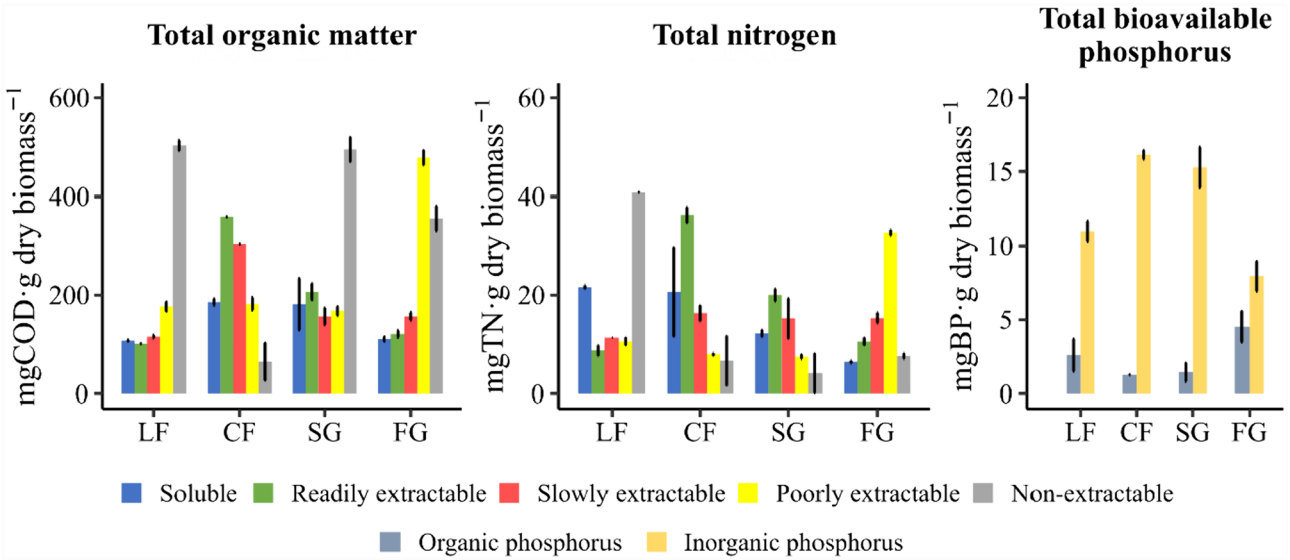


Fig. 2 Contribution of each extracted biochemical fraction to the total organic matter (TOM), total nitrogen (TN), and total bioavailable phosphorus (TBP) in loose flocs (LF), consolidated flocs (CF), smooth granules (SG), and filamentous granules (FG)

The lower share of extractable fractions and, thus, lower bioaccessibility of organic matter and nitrogen in loose flocs, smooth granules, and filamentous granules could be attributed to non-extractable humic-like substances. The literature supports this, as the non-extractable ISBAMO fraction contains water-insoluble, non-extractable humic-like substances [23]. EPS secretion may explain the abundance of these compounds in loose flocs, smooth granules, and filamentous granules. Arcila and Buitron [11] observed that the formation of microalgae-bacteria aggregates in the form of granules was related to EPS secretion as the biomass age, or solids retention time, increased. Humic substances are integral components of the EPS matrix [39]. These authors reported that humic substances, including fulvic acids, humic acids, and water-insoluble humin, could be taken up by the EPS matrix from their surrounding medium, *e.g.*, wastewater effluents or dead cells. Further studies confirmed that syntrophic interactions between eukaryotic microalga *Chlorella pyrenoidosa* and bacteria could decompose humic-like substances in stabilized landfill leachates, thus resulting in water-insoluble humin [40]. Given that only smooth granules were grown in wastewater, the origin of water-insoluble, non-extractable humic-like substances in loose flocs and filamentous granules could be related to other factors, including partially/fully decomposed microbial constituents from dead cells, including partially/fully decomposed dead microbial cells [41]. These could have come from decaying, slow-growing microorganisms, *e.g.*, diatoms, whose debris has been observed in the core of filamentous granules with large diameters (≥ 3.0 mm) [42].

The prevalence of inorganic phosphorus in the TBP content of consolidated flocs and filamentous granules is unexpected. In other phototrophic systems, the organic fraction of phosphorus dominated: In the cyanobacterium *Synechocystis* sp. PCC 6803, organic phosphorus accounted for nearly 77% of the total phosphorus content [43]. In the eukaryotic microalga *Scenedesmus* sp., 71% of the total phosphorus was stored in an organic form when grown in a synthetic medium [44]. The high inorganic phosphorus fraction in the bioavailable phosphorus in this study may result from the activity of PAOs. PAO-enriched aerobic granules exhibited an inorganic phosphorus fraction as high as 77% of the total phosphorus content [45], similar to the one observed for the different microalgae-bacteria aggregates used in this study.

Effect of Nutrient Starvation

Nutrient availability in the growth medium affects the development and biochemical composition of microalgae-bacteria aggregates [11]. Therefore, organic matter, nitrogen, and phosphorus bioaccessibility in loose flocs and filamentous granules were determined before (nutrient abundant) and after a starvation period. Two contrasted biomass types, loose flocs, and filamentous granules, were grown under nutrient starvation for over 8 and 3 d, respectively, using the growth media described in the supplementary material (Table S4 for loose flocs and Table S5 for filamentous granules). The other operating conditions were kept as described in Sect. "Morphology of microalgae-bacteria aggregates samples". As shown in Fig. 3, the biomass with contrasted spatial structures, loose flocs versus filamentous granules, differed in their shift in biochemical compositions when starved. The amount of poorly extractable organic matter in loose flocs significantly increased from 177 ± 10 mg TN·g dry matter^{-1} in nutrient-abundant conditions to 442 ± 19 mg TN·g dry matter^{-1} after starvation. The non-extractable TOM for this biomass significantly decreased from 502 ± 11 mg COD g dry matter^{-1} in nutrient-abundant conditions to 55 ± 10 mg COD g dry matter^{-1} after starvation. Similarly, the poorly extractable fraction of nitrogen in loose flocs also increased from 11 ± 1 mg TN g dry matter^{-1} to 25 ± 2 mg TN g dry matter^{-1} before and after starvation conditions, respectively.

The notable increase in the poorly extractable fraction of starved loose flocs correlates with an abundance of cellulose-like and hemicellulose-like key compounds targeted by this fraction [23]. Under nutrient-abundant conditions, microalgae store polysaccharides in many ways, including polymeric cellulose-like and hemicellulose-like substances in the inner layer of their cellular wall. These cellular constituents not only provide rigidity and protection against environmental interferences but also represent up to 7.1% (cellulose) and 16.3% (hemicellulose) of their dry matter content in microalgae-bacteria aggregates [46]. However, previous studies have reported that cellulose and hemicellulose content in microalgae could also be positively influenced by starvation. The eukaryotic microalga *Nannochloropsis salina* grown under nitrogen deficiency experienced a twofold increase in cellulose content compared to growth under nutrient abundance [47]. The authors proposed that as an alternative sink, microalgae channel excess energy and carbon intermediates from photosynthesis toward synthesizing storage molecules, including cellulose-like compounds. This redirection was seen as a response to inhibiting anabolic pathways requiring nitrogen, *i.e.*, amino acid and nucleotide synthesis, which usually works as microalgae's primary energy and carbon sink.

The critical components of the non-extractable ISBAMO fraction are water-insoluble, non-extractable humic-like substances that come from EPS or partially/fully degraded microbial constituents. Therefore, decreased non-extractable fractions after starvation could be associated with limited degradation of microbial constituents because of decayed microbial activity. Starvation leads the cell into a dormant-like state with no metabolic activity to synthesize microbial constituents and potentially transform them

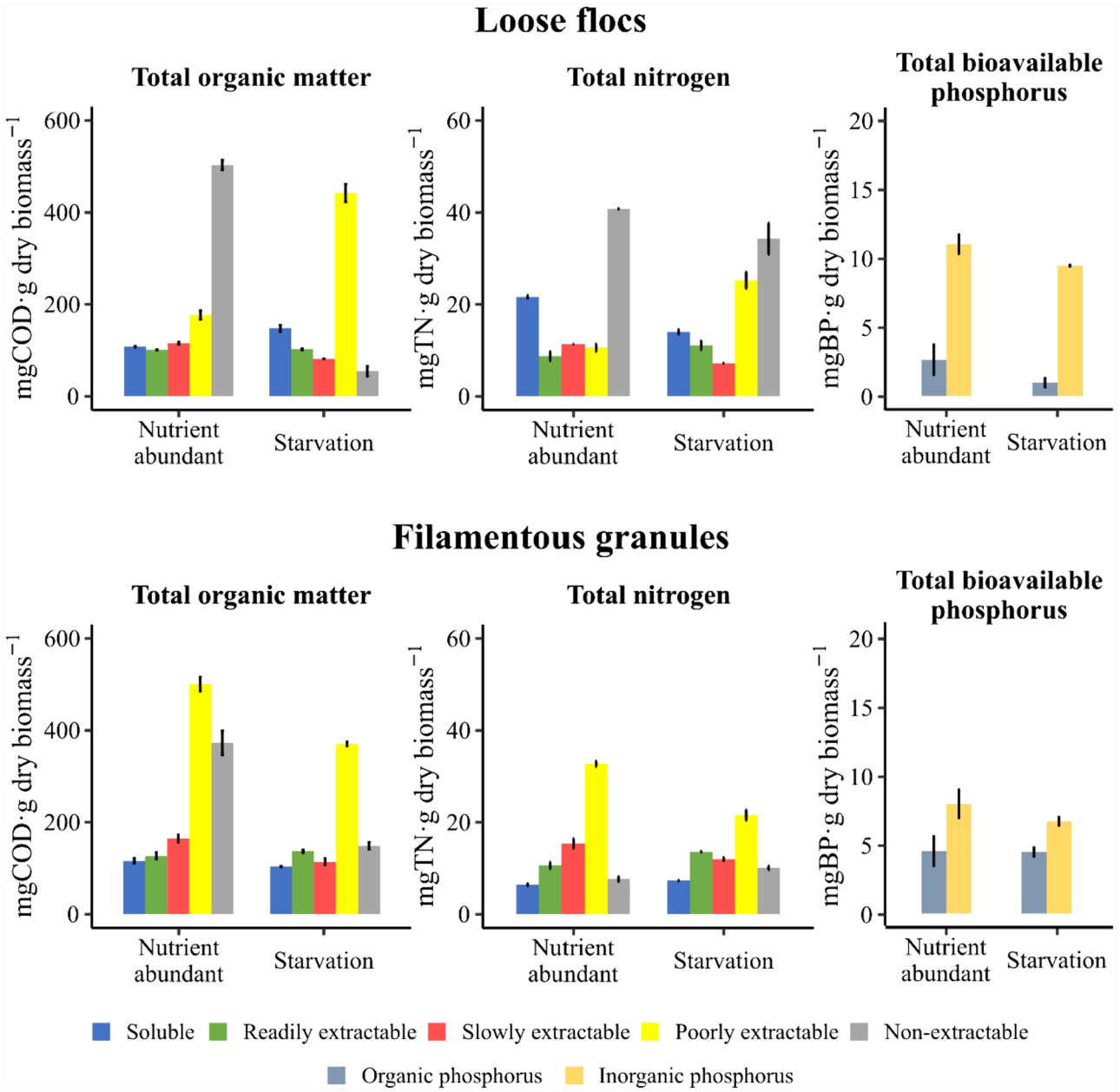


Fig. 3 Contribution of each extracted biochemical fraction to the total organic matter (TOM), total nitrogen (TN), and total bioavailable phosphorus (TBP) observed for loose flocs and filamentous granules before and after starvation conditions

into water-insoluble, non-extractable humic-like substances [48]. Previous studies confirmed these observations, where the lack of one or more nutrients during the formation of microalgae-bacteria aggregates decreased the abundance of humic-like substances in anaerobic digestate effluent [49].

Complexity of Microalgae-Bacteria Aggregates

The structural complexity of the ISBAMO fractions was determined to complement the bioaccessibility characterization of the different types of microalgae-bacteria aggregates. This complexity assessment allowed (i) the identification of groups of molecules based on their fluorescence excitation and emission wavelengths and (ii) the estimation of a complexity index based on all fluorescence information [15, 23]. This complexity index is defined by dividing the sum of fluorescence volumes of complex, humic-like substances by the sum of fluorescence volumes of simpler compounds (*e.g.*, tyrosine, tryptophane, and microbial-related constituents). A fluorescence complexity index (FCI) greater than 1.0 indicates a dominance of complex, humic-like substances, whereas

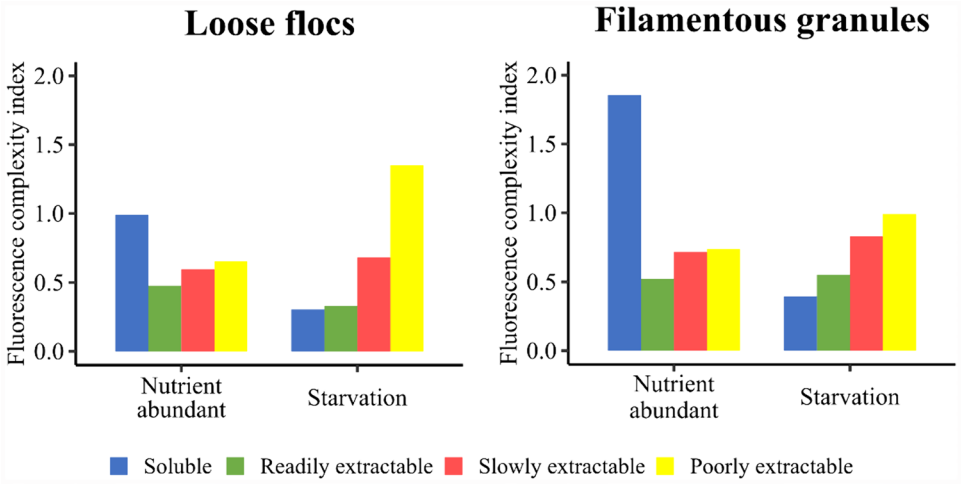


Fig. 4 Fluorescence complexity index (FCI) for the different biochemical fractions from loose flocs and filamentous granules before and after starvation conditions

an index smaller than 1.0 indicates a dominance of simpler compounds [24].

The fluorescence complexity indices from ISBAMO fractions for the samples from the starvation experiments, *i.e.*, loose flocs and filamentous granules, are presented in Fig. 4. Most aggregate types had an FCI lower or equal to 1.0, indicating that most bioaccessible fractions are primarily simple constituents, *e.g.*, tyrosine, tryptophane, and microbial-related constituents. These results follow earlier studies in the eukaryotic microalga *Scenedesmus acuminatus* grown under nutrient abundance and starvation, where up to 32% of its dry matter is in the form of amino acids, including tyrosine [38]. Similarly, the *Chlorella* genus commonly contains about 17 and 10% of its total dried biomass in soluble polysaccharides [50] and triacylglycerides [51], respectively.

Whatever the spatial structuration of the biomass (loose flocs or filamentous granules), two fractions had an FCI greater or equal to 1.0, indicating an abundance of complex, humic-like substances. These specific fractions were the soluble fraction from filamentous granules grown under nutrient-abundant conditions and the poorly extractable fraction from loose flocs grown under starvation. Even though the discussed results did not distinguish between the contribution of external (*e.g.*, EPS) and internal (e.g., microbial-related constituents) compounds to the complexity of microalgae-bacteria aggregates, the overall complexification of these fractions could be related to the adsorption of humic-like substances by EPS during microalgae-bacteria aggregation [40] or to the partial decay of cells [49]. These sources of biomass complexity should be considered in future studies.

Conclusions

Biochemical fractionation methods were applied to morphologically distinct microalgae-bacteria aggregates to characterize their molecular complexity and organic matter, nitrogen, and phosphorus bioaccessibility. This study shows that morphologically distinct microalgae-bacteria aggregates have different total organic matter, total nitrogen, and total bioavailable phosphorus content. Floccular biomass in consolidated flocs contains more bioaccessible fractions of organic matter, nitrogen, and phosphorus than other analyzed structures. This biomass also presents the highest proportions of soluble, readily extractable, and slowly extractable organic matter and nitrogen, as well as bioavailable phosphorus in the form of inorganic phosphorus. After starvation, changes in the poorly extractable and non-extractable fractions of organic matter in floccular biomass were observed, suggesting that humic-like substances mostly accumulate under these conditions. All microalgae-bacteria aggregates presented a low molecular complexity, highlighting their benefits as soil fertilizer. The results indicated that microalgae-bacteria aggregates are a promising source of highly bioaccessible organic matter, nitrogen, and phosphorus.

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Author Contributions ER-F: conceptualization, validation, formal analysis, investigation, data curation, writing—original draft, writing—review and editing, visualization. SG-O: writing—original draft, writing—review and editing, formal analysis, visualization. KGC-A: analysis, investigation, data curation, writing—review and editing. JJ:

conceptualization, methodology, writing—review and editing, supervision. GB, KM, JH: conceptualization, resources, writing—review and editing, supervision, funding acquisition.

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Data Availability The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should raw data files be needed in another format, they are available from the corresponding author upon reasonable request.

Declarations

Conflict of Interests The authors have no relevant financial or non-financial interests to disclose.

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