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PhD Thesis

Integrated algae bio refinery: nutrient and carbon recycling from waste

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Preface

This PhD thesis comprises the research carried out at the Department of Agricultural and Environmental Sciences - Production, Land, Agroenergy - University of Milan. Professor Fabrizio Adani was the supervisor of the whole work. This thesis is about integrated algae bio refinery: nutrient and carbon recycling from waste and it is organized in fiver chapters.

Chapter I explains the context of the work, namely the aim of circular economy, introducing the possibility and importance to utilize waste streams treatment with microalgae cultivation.

Chapter II is represented by a submitted work about the structure and diversity of microalgae-microbial consortia isolated from various local organic wastes. This chapter demonstrated the potential for isolating and cultivating microalgae from different organic wastes, along with their Eukaryotic and Prokaryotic compositions.

Chapter III is represented by a submitted work about the growth performances, biochemical compositions and nutrients recovery ability of twelves microalgae consortia isolated from various wastes and grown on nano-filtered pig slurry.

Chapter IV is represented by a publish work about the utilization with ultrafiltered digestate and glycerol to support mixotrophic growth of *Phaeodactylum tricornutum*. In this chapter, the mixotrophic biomass productivity, biomass composition, N efficiency use, and total energy balance applied by waste stream and glycerol were recorded and compared to autotrophic condition.

Chapter V is represented by the conclusions of this work.

Contents

Chapter I

Introduction

1. Introduction

In recent years, many countries have experienced challenges in terms of resource depletion and environmental remediation, accompanied by the increasing of waste generation, which can arise social and economic impacts (Amulya et al., 2016). Driven by these sustained growth of resource consumption and waste generation, the circular economy has received much attention as a solution to surmount the consumption and production demand of goods (Ubando et al., 2020).

Microalgae are a key component of circular economy where they can be used as a source of biomass, products and energy (Stiles et al., 2018). To illustrate, microalgae are photosynthetic microorganisms that are capable to efficiently utilize solar energy to amass biomass for numerous microalgal-based products production, e.g. biofuels, pharmaceuticals and nutraceuticals (Chew et al., 2017; Gabriel Acien Fernandez et al., 2012). Moreover, microalgal biomass has been considered as advanced (third generation) biomass feedstocks, providing benefits such as (i) high growth rate (over 1 d^{-1}) accompanying by high biomass productivity and yield (Acién Fernández et al., 2018); (ii) essential biological compounds in biomass i.e., lipids, carbohydrates, and proteins along with many therapeutically active enzymes, pigments, sterols, and vitamins. (Leu and Boussiba, 2014; Venkata Mohan et al., 2015); (iii) being capable to grow in widely different environments, not requiring fertile land or usable water even on wastewaters (Chisti., 2013).

However, microalgae production involves huge consumptions of water and fertilizers (Sandefur et al., 2016), representing more than 20% of the total production costs (Lam and Lee, 2012). For instance, to produce 100 tons of microalgae biomass up to 200 tons of CO_2, 10 tons of N, and 1 tons of P are consumed (Acién Fernández et al., 2018). In order to reduce this production cost, the utilization of wastewaters as nutrients source is recommended. Organic wastes are rich in a number of macro nutrients (e.g. N, P, K, S, Mg, Ca, Fe, and Na) and may contain a number of trace elements (e.g. Co, Fe, Se, Mo and Ni). Instead of disposing them by incineration and/or landfilled which could consume

large amount of land resources and cause irreversible damage to the environment, they can be used for microalgae cultivation (Stiles et al., 2018).

Various kinds of wastes, from domestic to industrial, with different consistency (solid, liquid or gaseous form) has been exploited for algal biomass production then used for different applications as well as for nutrient removal (Mohan et al., 2016; Kumar and Bharadvaja, 2021). Some studies (Franchino et al., 2013; Ledda et al., 2016) have already shown the high efficiency of algae in removing nutrients from wastes. Subsequently, microalgae can uptake nutrients from waste turning as valuable biomass, up to 1 kg of dry microalgae biomass can be produced per m^3 of sewage, whereas more than 10 kg of dry microalgae biomass can be produced per m^3 of manure (Acién Fernández et al., 2018). It was reported that the utilization of microalgae-based processes reduced to half the energy consumption of conventional wastewater treatment and allows recovering up to 90% of the nutrients contained into wastewater (Acién Fernández et al., 2018). Hence, microalgae production coupled to the waste treatment presents an opportunity to establish a circular economy solution for reducing overall residual waste component, algae production cost, as well as recovering the nutrients contained in waste to achieve and favor sustainable economics (Acién et al., 2012; Kumar and Bharadvaja, 2021; Olguín, 2012).

Under those circumstances, this Ph.D thesis aimed to provide the effectiveness of algae-waste based system. Particularly, this work mainly focused on integrating microalgae with various organic waste streams treatment by achieving nutrient recovery of the wastes as well as producing algal biomasses. Instead of applying the microalgae pure species, the innovation of this work (Chapter II) explored and demonstrated the potential for isolating and cultivating microalgae-microbial consortia (AC) from different organic wastes as original growing substrates, providing the knowledge of the structure, diversity and population of Eukaryotic and Prokaryotic compositions of these microbial-microalgae consortia communities. The survived and well adapted ACs were analyzed by next generation sequencing technology. Then in Chapter III, these ACs have been applied on nutrient rich waste

stream (nanofiltrated pig slurry) in autotrophic condition to be evaluated their growth ability for producing biomass and nutrients recovery ability for treating wastes. Furthermore, biochemical compositions have been tested along with the fatty acids and amino acids profiles for further application potential.

Microalgae can not only grow under autotrophic condition, but they can also grow under mixotrophic nutrition by combining both the autotrophic and heterotrophic mechanisms by assimilating available organic compounds as well as atmospheric CO_2 as carbon source (Venkata Mohan et al., 2015). A difference approach in Chapter IV using as substrate a similar waste stream studied in Chapter 2 added of C source to support microalgae growing in mixotrophic, i.e. glycerol, mode was conducted. Phaeodactylum *tricornutum* was selected since this strain can grow in mixotrophic condition in the presence of diverse organic compounds and it has been included among potential candidates for biodiesel production due to its high lipid content (Silaban et al., 2014). Thus, this chapter represents an attempt to assess the ability of the algae *P. tricornutum* to grow mixotrophically and providing comparison with autotrophic condition.

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Chapter II

The structure and diversity of microalgae-microbial consortia isolated from various local organic wastes

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The structure and diversity of microalgae-microbial consortia isolated from various local organic wastes.

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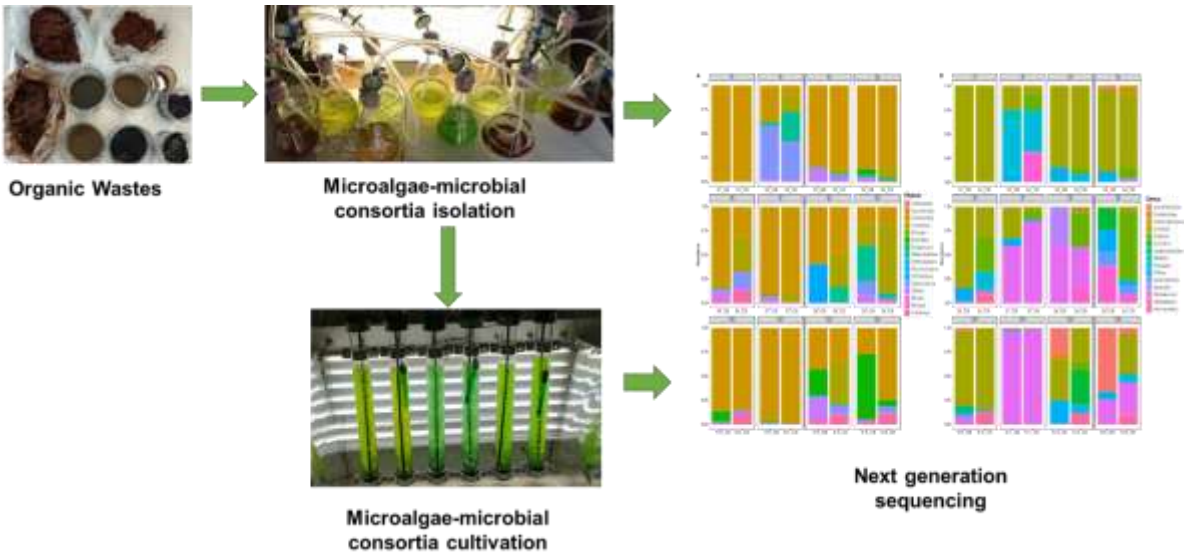
Abstract

This study demonstrated the potential for isolating and cultivating microalgae from different organic wastes sampled locally. To do so, sixteen organic wastes, having different origins (cow slurry, urban municipal wastewater and sewage sludge) and consistency (solid/slurry/liquid fractions), were sampled from plants/farms located in the Lombardy Region of northern Italy and completely characterized. Twelve microbial consortia were isolated from wastes and their Eukaryotic and Prokaryotic compositions were analyzed by next generation sequencing of 18S and 16S rRNA genes.

Most of the eukaryotic communities were dominated by the phylum Chlorophyta led by the *Chlorella* genus, which demonstrated an ability to survive in different waste conditions regardless of chemical-biological factors. *Tetradesmus*, the second most represented genus grew preferentially in substrates characterized by less stressing chemical-physical parameters. Cyanobacteria, Proteobacteria and Planctomycetota were the most abundant bacterial phyla. Cyanobacteria co-existed well with Proteobacteria and Planctomycetota, along with *Chlorella*, *Tetradesmus* and *Scenedesmus*.

Key words: Microalgae; Next generation sequencing (NGS); Eukaryotic; Prokaryotic Organic wastes.



Graphic Abstract

1. Introduction

Due to the growing world population, global industrialization, economic and industrial development, the amounts of industrial and municipal waste along with agricultural residues, have been increasing rapidly over recent decades. Demichelis et al. (2019) reported that 4.06 Mt of wastewater and sewage sludge, 1.7 Mt of the organic fraction of municipal solid waste and 5.7 Mt of agricultural livestock waste were produced during 2016 to 2017.

Organic wastes need to be correctly disposed of, avoiding environmental impacts and health issues (Oliveira et al., 2017). Wastewater treatment, composting and anaerobic digestion have been proposed to treat organic waste streams (Uddin et al., 2021; Yong et al., 2021). These biological processes produce secondary by-products, i.e., sewage sludge, compost and digestate which could be sources of organic matter and nutrients which might be usefully recovered for subsequent productive processes in a circular way, i.e. within the Circular Economy (Abad et al., 2019; Lupu et al., 2021).

Compost, sewage sludge and more recently digestate have been proposed as organic amendments and fertilizers, aiming to replace the use of mineral fertilizers and improve soil organic matter and thus contributing to a more sustainable agriculture (Riva et al., 2016).

Organic wastes were reported, also, to be an excellent source of energy-rich organic C-molecules and

macro- and micro-nutrients for microalgae cultivation (Wallace et al., 2015; Shahid et al., 2020). However, microalgae cultivation has been successful when wastewater was used to supply mineral nutrients and organic carbon for their growth (Aketo et al., 2020; Pena et al., 2020; Khalid et al., 2019; Nayak and Ghosh, 2019; Li et al., 2021; Park et al., 2010).

Previous studies have proven that waste could be a suitable growth medium for the cultivation of certain pure microalgae species, i.e., *Chlorella* (Ji et al., 2013; Kim et al., 2017), *Tetradesmus* (Rugnini et al., 2019) and *Scenedesmus* (Ferro et al., 2020; Kim et al., 2017). Nevertheless, in practice, only a relatively small number of microalgae species have been developed and used extensively because of the necessity to control the stability of the microalgae populations (Izadpanah et al., 2018), and the risks of culture contamination (Moreno-García et al., 2021). Bacteria, fungi, cyanobacteria and other microalgae establish mutualistic or competitive relationships with the inoculated microalgae strain, depending on the different species and environmental conditions (González-González and De-Bashan, 2021). Therefore, rather than attempting to combat the contamination of monocultures by unwanted and detrimental species, a different approach can be taken by cultivating native microalgae consortia isolated directly from local wastes, without a strict control of the microbial population stability. The ability of microalgae to grow in a substrate depends on both the chemical-biological parameters characterizing the substrate and the organisms' ability to colonize the substrate (Agrawal, 2009). Research showed that microalgae are capable of producing highly resistant spores which enable them to survive in adverse conditions (Agrawal, 2009). Different algae have a relatively large tolerance range for changes in environmental conditions. For example, *Nostoc punctiforme* and *Anabaena circinalis* can germinate in the dark in the presence of organic carbon acting as a suitable source of energy (Agrawal, 2009). The brown alga *Macrocystis integrifolia* sporophyte can grow even at low temperature, i.e. 8 °C (Buschmann et al., 2004). Palmer (1974) surveyed and found several microalgal genera surviving in a wide range of waste stabilization ponds, i.e., *Chlorella*, *Ankistrodesmus*, *Scenedesmus*, *Euglena*, *Chlamydomonas*, *Oscillatoria*, *Micractinium* and *Golenkinia*, in order of abundance and frequency of occurrence. Furthermore, Palmer (1969)

listed the algae in the order of their tolerance to organic pollutants as reported by 165 authors, which demonstrated that algae could both survive and adapt well in organic wastes. Specifically, algae have been used to treat human sewage (Abdel-Raouf et al., 2012), livestock wastes (Lincoln and Hill, 1980), agro-industrial wastes (Syafiqah Hazman et al., 2018), industrial wastes (Stirk and Van Staden, 2000) and piggery effluent (De Pauw et al., 1980). Algae-based systems for the removal of toxic minerals such as Pb, Cd, Hg, Sc, Sn, As and Br are also being developed (Aydinoglu et al., 2013; Soeder et al., 1978.). It is evident that microalgae have several interesting advantages including their relatively easy to provide growth conditions, and the ability to colonize both diverse and extreme conditions (Jacob et al., 2021).

Therefore, microalgae growing on particular wastes are well adapted to nutrient-rich substrates (Elsrer, 1999), which will lead to further scientific-technological developments such as in nutrient recovery strategy and in mixotrophic culture (Do et al., 2020; Mulbry et al., 2008).

This paper aimed to investigate the presence of useful algae-microbial consortia in sixteen different selected organic wastes rich in mineral nutrients and organic carbon, as the first step for a subsequent use of these consortia to produce algae biomasses.

Molecular metabarcoding characterization was applied to identify the algae and bacteria taxa and their relative abundance and to provide information on the composition of microalgae-microbial communities obtained from the different wastes.

2. Materials and Methods

2.1 Waste sampling

Sixteen organic wastes samples with different origins (cow slurry, urban municipal wastewater, sewage sludge) and types (solid/slurry/liquid fractions) were sampled from plants/farms located in the Lombardy Region of northern Italy (Table 1). The waste samples were marked as S1 to S16. All collected samples were immediately brought to the laboratory and stored at 4˚C before further analysis.

Table 1 Raw wastes sampling and origin details

	Sample Name	Origin	Stage	Location	Plant/farm Scale	Plant Volume m^3
S1	Cow slurry	Cattle Stables	Input	Mantova	150 animals	-
S2	Liquid digestate of cow slurry	Biogas Plant	Output	Suzzara	1 MW, 5 digesters	12000
S3	Solid digestate of cow slurry	Biogas plant	Output	Suzzara	1 MW, 5 digesters	12000
S4	Liquid digestate of cow slurry	Biogas plant	Output	Pegognaga	1 MW, 4 digesters	10000
S5	Cow slurry	Cattle Stables	Input	Pegognaga	100 animals	-
S6	Solid digestate of cow slurry	Biogas plant	Output	Pegognaga	1 MW, 4 digesters	10000
S7	Cow slurry	Cattle stables	Input	Lodi	400 animals	-
S8	Digestate of cow slurry	Cattle stables	Output	Bologna	-	-
S9	Wastewater	Urban wastewater treatment plant	Output	Peschiera Borromeo	$5.2x10^5$ equivalent inhabitants	154095 m^2
S10	Digestate of OFMSW[a]	Biogas plant fed by OFMSW	Output	Lodi	1 MW, 4 digesters	8000
S11	Liquid fraction of OFMSW	Biogas plant fed by OFMSW	Input	Lodi	1 MW, 4 digesters	8000
S12	OFMSW	Biogas plant fed by OFMSW	Input	Lodi	1 MW, 4 digesters	8000
S13	Sewage Sludge	Biogas Plant	Input	Vellezzo Bellini (PV)	1.6 MW, 3 digesters	13500
S14	Digestate of sewage sludge	Biogas Plant	Output	Vellezzo Bellini (PV)	1.6 MW, 3 digesters	13500
S15	Cow slurry	Cattle stables	Input	Bologna	-	-
S16	Cattle manure	Cattle stables	Input	Lodi	-	-

[a]OFMSW: Organic fraction of municipal solid wastes

2.2 Waste chemical and biological characterization

The waste samples were dried for 24 h at 105˚ C (APHA 2005), shredded in a blender and passed through a 2-mm mesh. Total solids (TS), volatile solid (VS) and total organic carbon (TOC) were determined according to standard procedures (APHA 2005). The pH and electrical conductivity (EC) were measured potentiometrically using an Orion-520A pH-meter and a WTW-LF537 (GE) conductivity electrode, respectively. Total Nitrogen (TKN), Ammonia nitrogen (NH_4^+-N), pH, volatile fatty acids (VFA) and alkalinity (ALK) were determined on fresh materials by using the analytical method for wastewater sludge (IRSA CNR, 1994). Optical density was measured as absorbance at 750 nm by using a Jeneway 7350 UV-visible spectrophotometer. Macro and microelement concentrations including Na, Mg, K, Ca, P, Mn, Fe, Cu, Zn, Cr, Co, Ni, As, Se, Mo, Cd, Pb were determined by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, Aurora M90 BRUKER), preceded by microwave assisted (Multiwave ECO, Anton Paar GmbH) nitric acid digestion (EPA, 2007) of fresh samples. All chemical analyses were performed in triplicate. The

biological property of samples, i.e. biological stability, was determined by the anaerobic biogas production (ABP) (Schievano et al., 2008). All biological tests were performed in duplicate.

2.3 Original biomasses cultivation

2.3.1 Experimental design

Waste samples (inocula) were diluted by sterilized BG-11 Medium (BG-11, a growth mineral solution widely used and suitable for most kinds of freshwater algae growth, according to Andersen et al., 2005) to get final optical densities of 0.1, 0.3 and 0.5. Diluted samples were maintained in 500 mL Erlenmeyer flasks under constant aeration and mixed by using filtered air (filter of 0.2 µm) with a continuous illumination of 50 µE m^{-2} s^{-1}, provided by fluorescent white tubes, at a controlled temperature of 22 ± 1°C. BG-11 nutrient solution at 0.3 optical density for at least 8 weeks resulted in the isolation of the most microalgae consortia (original biomass – OB). Then the microalgae consortia isolated from wastes were cultivated in Photo Bio Reactors (PBRs) of 0.5 L working volume. pH was set at up at 8 and it was maintained by using pure CO_2 injection adopting an "on-demand" modality. Room temperature (25°C) and constant air flux (10 L min^{-1}) were provided as well as light that was provided by cold fluorescent lamps at irradiance of 312 µE m^{-2} s^{-1} at PBR surface, adopting a 12h:12h photoperiod regime. The OB (original biomass) was dosed at 10% v/v, i.e., 0.2-0.3 g L^{-1} was placed into the reactors and culture medium (BG-11) was added to start the trials to obtain cultivated biomass (CB). Two replicates/microalgae consortium were carried out.

To monitor the culture growth, biomasses' dry weights (TS) were determined by sampling 10 mL of biomass suspension from each PBR every 2 days. The samples were centrifuged at 4,000 rpm for 10 min and then washed with an equivalent volume of distilled water to remove salts. Biomass samples were then filtered by 1.2-µm filter (GF/C, Whatman Ltd., Maidstone, UK), dried overnight at 80 °C and weighted.

2.4 Microalgae consortia molecular characterization

In order to investigate the diversity and population of microbial consortia, DNA extraction was carried out on both the original biomass (OB) and cultivated biomass (CB) during the exponential growth phase. The consortia biomasses were collected by centrifugation at 4,000 rpm for 10 min and 8,000 rpm for another 10 mins and then stored at -80° C until further analysis. Then the lyophilized biomass samples were collected for DNA extraction by DNeasy plant mini kit Qiagen, following the procedure described by the manufacturer. The extracted DNA samples were stored at -20˚ C for further use. DNA concentration and purity were determined by a nanodrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., USA).

For next generation sequencing (NGS), a library for 16S and 18S marker genes was prepared following Illumina Protocol. NGS can be used to sequence entire genomes and to generate extensive data from diverse microbial communities in a timely manner (Kim et al., 2014). For the 16S, the hypervariable V3-V4 region was amplified using the 341F and 805R primers (Herlemann et al., 2011) while for 18S, the V9 region was amplified using the 1389F and 1510R primers (Piredda et al., 2017) both modified with the required Illumina sequencing adaptors. 16S and 18S PCR amplification was performed on a total volume of 25 µl: 12.5 µl of appTaq RedMix (Appleton Wood Ltd., UK), 1 µl of forward and 1 µl of reversed primers modified with Illumina over-hanger (10 uM) (IDT, Belgium), 2.5 µl of extracted DNA and 8 µl of PCR grade water (Merck, Germany). Thermal protocol for 16S gene was 95° C for 3 mins followed by 30 cycles at 95° C for 15s, 57° C for 15s and 72° C for 30s, with a final extension step at 72° C for 7 mins. For 18S marker gene the thermal protocol was 98° C for 3 mins, followed by 30 cycles at 98° C for 10s, 56° C for 30s and 72° C for 15s with a final extension step at 72° C for 7 mins. PCR products were cleaned using Agencourt AMPure XP PCR Purification beads (Beckman Coulter), following the manufacturer's instructions. 2.5 µl of purified PCR product was used in a short secondary PCR, to attach Nextera XT indices, in the presence of 2.5 µl of Nextera i5 and i7 index, 12.5 µl Appletonwood Taq and 5 µl of PCR water. Thermal cycling conditions consisted of an initial denaturation step of 3 min at 95° C followed by 8 cycles each of 30

s at 95° C, 30s at 55° C and 30 s at 72° C followed by a final extension step of 5 min at 72° C. PCR products were purified using Agencourt AMPure XP PCR Purification beads as described previously. PCR products were quantified using PicoGreen® dsDNA quantification assays (Thermo Fisher Scientific), on a POLAR star Omega (BMG Labtech) plate reader. Nextera XT amplicons were then pooled in equimolar concentration. The length of amplicons was verified with Agilent bioanalyzer DNA kit (Agilent, USA). Final quantification of the pooled amplicon library was determined with the NEBNext® Library Quant Kit for Illumina® (New England BioLabs) prior to sequencing on the Illumina MiSeq (2 X 300 bp) at the University of Essex (UK).

2.5 Data analysis

2.5.1 Multivariate analysis

Multivariate analyses were used to identify environmental parameters that were most strongly associated with each other, and to define environmental factors to eukaryotes and prokaryotes species associations. Principal component analysis (PCA) (Ter Braak, 1986) was used to identify trends between highly correlating chemical-biological parameters. This analysis provides information on the most meaningful parameters, which describe the whole dataset with minimum loss of original information (Singh et al., 2005). Analyses were performed by XLSTAT version 2016.02.28451.

2.5.2 Metabarcoding statistical analysis.

Reads were processed as described in Dumbrell et al. (2016). Briefly, quality filtering was carried out in Sickle, followed by error correction in SPAdes. Reads were paired ended using PEAR inside Pandaseq. Chimera check and clustering at 97% of similarity was performed using the vsearch algorithm. Repsets were imported in Qiime2 and taxonomy was assigned using sklearn classifier using SILVA database for 16S while for 18S taxonomy has been assigned using blastn algorithm.

Statistical analyses were performed in R studio. Figures were generated using gglpot2 library. Richness data were log transformed to meet the normality criteria and the equality of the variance (car package). A pairwise t-test was used to test if the richness in batch/original was significant.

Phyloseq package was used to generate relative abundance and for the NMDS plot. Sequence data from this study were deposited in the SRA archive under the project accession numbers: PRJNA752495 for eukaryotic communities (18S) and PRJNA752492 for bacterial sequences (16S).

3. Results and Discussion

3.1 Organic wastes characterization

Many factors such as light, pH, and nutrients influence microalgae survival and growth (Izadpanah et al., 2018); thus it is critical to understand the original substrates' features. To do so, the selected organic wastes were characterized for chemical-physical and biological parameters and results are reported in Table 2, reporting data referred to the fresh materials to better describe the real growth conditions.

Results show that wastes differed greatly from each other (Table 2a and Table 2b), thus providing a wide range of substrates. TS varied from 15 ± 1 g kg^{-1} (S4) to 257 ± 10 (S6) g kg^{-1} while VS was from 8 ± 0.2 g kg^{-1} (S2) to 221 ± 0.6 g kg^{-1}(S3). TS and VS reflected indirectly the waste turbidity, which would directly affect light availability for microalgae growth. pH varied from 6.4 ± 0.3 (S12) up to 9.1 ± 0.2 (0.1) (S3 and S6). Regarding pH, maintaining a suitable pH condition is critical for algae, as the tolerated pH range for most algal species has been reported to be between 7 and 9, with the optimum range being 8.2-8.7, though there are species that dwell in more acid/basic environments (Barsanti and Gualtieri, 2006). Total alkalinity (TA), a parameter close to pH, varied widely between 2.1 ± 0.1 g $CaCO_3$ kg^{-1} (S9) and 18.7 ± 0.5 g $CaCO_3$ kg^{-1} (S10).

Furthermore, three macronutrients, i.e., carbon, (TOC) (important for heterotrophic and mixotrophic algae growth), nitrogen (N) and phosphorus (P) also showed wide differences. In particular (Table 2), we found the following range of variation for C, N, N-NH_4 and P: 5 ± 0 g kg^{-1} (S4) to 110 ± 0.2 g kg^{-1} (S6), 1.5 ± 0 g kg^{-1} (S4) to 8.8 ± 0.11 g kg^{-1} (S13), 0.3 g kg^{-1} (S9) to 3.5 g kg^{-1} (S10) and 5 ± 0 g kg^{-1} (S2) to 299 ± 4 g kg^{-1} (S13), respectively. Moreover, apart from C, N and P, also the elements S, K,

Na, Fe, Mg, Ca and trace elements such as B, Cu, Mn, Zn, Mo, Co, V and Se, that are essential nutrient for microalgae, showed a wide variability depending on feedstock composition (Table 2) (Stiles et al., 2018).

Table 2a Chemical Characterization of raw wastes

Waste sample	TS g kg^{-1} FM	VS g kg^{-1} FM	pH	TN g kg^{-1} FM	N-NH_4 g kg^{-1} FM	TVFAs g kg^{-1} FM	TA g $CaCO_3$ kg^{-1} FM	TOC g kg^{-1} FM	TP g kg^{-1} FM	EC ds m^{-1}	ABP ml g^{-1} FM
S-1	78 ± 1	57 ± 0.6	8.4 ± 0.2	4.1 ± 0.05	1.6 ± 0.1	276 ± 30	17.7 ± 0.2	34 ± 1	63 ± 2	29.7 ± 0.9 [c]	20.5 ± 1.6
S-2	19 ± 3	8 ± 0.2	8.6 ± 0.1	2.5 ± 0.01	1.3 ± 0	1749 ± 94	8.7 ± 2.4	6 ± 0.1	5 ± 0	26 ± 1.3 [c]	2.8 ± 0.1
S-3	242 ± 2	221 ± 0.6	9.1 ± 0.2 [b]	4.2 ± 0.09	0.9 ± 0	154 ± 12	7.1 ± 0	95 ± 0.4	159 ± 10	21.3 ± 1.2 [d]	52.6 ± 2.8
S-4	15 ± 1	9 ± 0.1	7.5 ± 0.1	1.5 ± 0	0.9 ± 0	1334 ± 64	6.9 ± 0.1	5 ± 0	9 ± 1	20 ± 1.6 [c]	3.9 ± 0.2
S-5	46 ± 1	29 ± 0.1	7.9 ± 0	3.7 ± 0.11	1.5 ± 0.1	718 ± 10	12. 9 ± 0.6	17 ± 0.8	60 ± 1	29.9 ± 4 [c]	7.4 ± 1.1
S-6	257 ± 10	218 ± 1.3	9.1 ± 0.1 [b]	5.4 ± 0.26	0.9 ± 0	623 ± 20	6.7 ± 0	110 ± 0.2	151 ± 14	23 ± 1.9 [e]	68 ± 3.6
S-7	65 ± 1	58 ± 0.4	7.2 ± 0	3.1 ± 0.14	1.0 ± 0	7469 ± 183	13.3 ± 0.5	27 ± 0	47 ± 4	23.8 ± 1.2 [c]	30.2 ± 2.6
S-8	66 ± 0	37 ± 0.1	7.8 ± 0	5.9 ± 0.06	2.6 ± 0.1	408 ± 7	11.8 ± 0.1	21 ± 1	156 ± 8	27.6 ± 1.7 [c]	14.9 ± 1.1
S-9	16 ± 0	13 ± 0.1	6.7 ± 0.2	1.2 ± 0.05	0.3 ± 0	598 ± 40	2.1 ± 0.1	6 ± 0.3	54 ± 2	2.8 ± 0.6 [c]	4.6 ± 0.4
S-10	20 ± 3	12 ± 0.1	8.1 ± 0.1	5.6 ± 0.43	3.5 ± 0.4	64 ± 0	18.7 ± 0.5	7.8 ± 0.4	18 ± 1	43.1 ± 1.7 [c]	5.7 ± 0.2
S-11	17 ± 1	10 ± 0.1	8.1 ± 0.3	5.5 ± 0.07	3.3 ± 0.1	513 ± 92	16.6 ± 0	4 ± 0.2	12 ± 0	44 ± 0.1 [c]	5.3 ± 0.2
S-12	87 ± 11	66 ± 0.1	6.4 ± 0.3	7.7 ± 0.01	3.4 ± 0	17565 ± 208	16.1 ± 0.1	35 ± 0.3	71 ± 6	30.4 ± 2.3 [c]	42.7 ± 2.3
S-13	119 ± 0	85 ± 0.4	7.4 ± 0.1	8.8 ± 0.11	2.4 ± 0.1	10631 ± 190	5.7 ± 0.1	41 ± 0.3	299 ± 4	23.9 ± 1.1 [c]	33.6 ± 3.4
S-14	97 ± 1	58 ± 0.6	8.0 ± 0.2	7.7 ± 0.07	2.9 ± 0.1	3309 ± 411	7.0 ± 0.2	23 ± 1	289 ± 0	24.7 ± 2.6 [c]	19.2 ± 1.1
S-15	40 ± 7	29 ± 0.2	7.6 ± 0.2	2.8 ± 0.02	0.9 ± 0.2	277 ± 9	7.4 ± 0.3	14 ± 1	46 ± 0	19.5 ± 0.6 [c]	9.4 ± 0
S-16	162 ± 5	136 ± 2	8.7 ± 0 [b]	5.8 ± 0.06	1.4 ± 0.1	766 ± 171	12.1 ± 0.1	7 ± 0.5	109 ± 4	32.3 ± 0.8 [d]	52.5 ± 1.6

[a] FM: fresh materials
[b] pH dilution rate sample: water =1:10
[c] EC dilution rate sample: water =1:5
[d] EC dilution rate sample: water =1:10
[e] EC dilution rate sample: water =1:25

Table 2b Element composition of raw wastes

	Na	Mg	K	Ca	Fe	Mn	Cr	Cu	Zn	Co	Ni	As	Se	Mo	Cd	Pb
	mg kg^{-1} FM[a]															
S-1	104 ± 11	70 ± 2	421 ± 12	2156 ± 71	27 ± 1	1.6 ± 0.1	0.1 ± 0	0.6 ± 0.1	2.7 ± 0.2	u.d.l	0.031 ± 0	0.018 ± 0	0.025 ± 0.011	0.045 ± 0.007	u.d.l	0.02 ± 0.007
S-2	64 ± 2	11 ± 0	368 ± 31	792 ± 12	4 ± 0	0.2 ± 0	u.d.l[b]	0.1 ± 0	0.3 ± 0	u.d.l	0.005 ± 0.001	0.005 ± 0	0.009 ± 0	0.021 ± 0	0.001 ± 0	0.003 ± 0
S-3	84 ± 3	146 ± 10	421 ± 69	1059 ± 128	20 ± 1	2.1 ± 0.1	0.3 ± 0	0.5 ± 0.1	5.4 ± 0.3	u.d.l	u.d.l	0.065 ± 0.024	0.103 ± 0.006	0.141 ± 0.014	u.d.l	0.027 ± 0.007
S-4	54 ± 4	16 ± 2	185 ± 14	2155 ± 34	1 ± 0	0.1 ± 0	u.d.l	0.1 ± 0	0.2 ± 0	u.d.l	0.004 ± 0	0.005 ± 0.001	0.007 ± 0	0.007 ± 0	0.001 ± 0	0.002 ± 0
S-5	85 ± 0	63 ± 0	375 ± 5	2681 ± 47	11 ± 0	0.8 ± 0.7	0.1 ± 0	0.4 ± 0	1.7 ± 0	u.d.l	0.07 ± 0	0.013 ± 0	0.019 ± 0.002	0.024 ± 0.001	0.002 ± 0	0.013 ± 0
S-6	90 ± 7	138 ± 12	378 ± 16	1031 ± 227	24 ± 2	2.2 ± 0.2	0.2 ± 0	1.0 ± 0.4	3.5 ± 0.2	u.d.l	0.017 ± 0	0.035 ± 0.002	0.075 ± 0.033	0.122 ± 0.067	0.004 ± 0	0.017 ± 0.01
S-7	63 ± 1	51 ± 2	223 ± 5	1495 ± 35	8 ± 0	0.9 ± 0.1	0.1 ± 0	0.3 ± 0	1.5 ± 0	u.d.l	0.018 ± 0.001	0.008 ± 0	0.014 ± 0.007	0.032 ± 0.005	u.d.l	0.01 ± 0.003
S-8	205 ±7	43 ± 1	119 ± 0	2785 ± 110	80 ± 3	35 ± 0.1	0.3 ± 0	7.5 ± 0.1	45.7 ± 0	0.2 ± 0	0.182 ± 0.004	0.024 ± 0	0.148 ± 0	0.018 ± 0.014	0.005 ± 0	0.053 ± 0.002
S-9	12 ± 1	14 ± 0	14 ± 0	4334 ± 22	13 ± 1	0.2 ± 0	0.1 ± 0	0.4 ± 0	1.1 ± 0	u.d.l	0.068 ± 0	0.01 ± 0	0.008 ± 0	0.007 ± 0.003	0.002 ± 0	0.078 ± 0
S-10	160 ± 1	2 ± 0	194 ± 3	894 ± 14	6 ± 0	0.1 ± 0	u.d.l	0.1 ± 0	0.5 ± 0	u.d.l	0.046 ± 0.002	0.009 ± 0	0.008 ± 0	0.017 ± 0	0.002 ± 0	0.011 ± 0.003
S-11	130 ± 1	2 ± 0	166 ± 1	513 ± 2	3 ± 0	0.1 ± 0	u.d.l	0.1 ± 0	0.3 ± 0	u.d.l	0.035 ± 0.002	0.006 ± 0.001	0.007 ± 0	0.003 ± 0	u.d.l	0.006 ± 0.001
S-12	247 ± 28	31 ± 0	276 ± 8	2701 ± 83	27 ± 1	1.6 ± 0	0.1 ± 0	0.5 ± 0	1.6 ± 0	u.d.l	0.061 ± 0	0.02 ± 0.005	0.029 ± 0.017	0.046 ± 0.006	u.d.l	0.07 ± 0.02
S-13	36 ± 1	65 ± 2	58 ± 3	2701 ± 77	141 ± 2	3.1 ± 0.1	0.9 ± 0	3.7 ± 0.4	10.2 ± 0.4	0.009 ± 0	0.552 ± 0.026	0.102 ± 0.001	0.047 ± 0.007	0.086 ± 0.010	0.016 ± 0	0.612 ± 0.021
S-14	30 ± 1	66 ± 1	48 ± 1	3446 ± 28	227 ± 2	4.2 ± 0	1.1 ± 0.1	3.6 ± 0.2	10.5 ± 0.5	0.033 ± 0	0.65 ± 0.05	0.127 ± 0.004	0.052 ± 0.002	0.088 ± 0.044	0.021 ± 0	0.645 ± 0.022
S-15	38 ± 1	38 ± 0	225 ± 8	1884 ± 2	26 ± 1	1 ± 0	0.1 ± 0	0.3 ± 0	1.3 ± 0.2	u.d.l	0.037 ± 0.01	0.004 ± 0	0.012 ± 0.001	0.022 ± 0.008	0.001 ± 0	0.007 ± 0
S-16	147 ± 24	146 ± 5	585 ± 3	2525 ± 246	34 ± 1	3.6 ± 0.2	0.3 ± 0	0.8 ± 0.1	4.2 ± 0.3	u.d.l	0.1 ± 0.02	0.014 ± 0.001	0.039 ± 0.005	0.059 ± 0.025	u.d.l	0.023 ± 0.002

[a]FM: fresh materials

[b]u.d.l: under detection level

3.2 Taxonomic profiling of original biomasses

3.2.1 Eukaryotic Communities

For Eukaryotic communities a total of 3,565 OTUs were obtained but only 2,183 OTUs could be assigned to the Eukaryotic domain (100% Eukaryotic). At phylum level (Fig. 2a), the original biomasses were dominated by Chlorophyta (from 60% in S8_OB to 99% in OB-S1) except for samples S2_OB, S9_OB, S13_OB and S16_OB which were characterized by the abundant presence of the phyla Opisthokonta (S2_OB, 55% ± 3) and Discosea (S16_OB 68% ± 1). Contrarily, there was no one dominant phylum in Samples S9_OB and S13_OB which were characterized by a mix of phyla i.e., S9_OB: Heterolobosea 37% ± 2, Ciliophora 23% ± 2, Chorophyta 18% ± 1 and Opisthokonta 14% ± 1, and S13_OB: Chlorophyta 42% ± 3, Discosea 27% ± 0. At genus level, *Chlorella* was the most common microalgae found in both original and cultivated biomasses, followed by *Tetradesmus* (S7_OB, S8_OB and S11_OB), both belonging to Chlorophyta. Other important genera found were the microalgae predators *Colpoda* and *Vahlkampfia* (Hailei et al., 2017; Wahi et al., 2018). Original biomasses obtained could be divided into three groups (Groups 1-3) for genus composition (Fig. 2A Originals): Group 1 (S1_OB, S3_OB, S4_OB, S6_OB and S10_OB) mainly dominated by the presence of *Chlorella*; and Group 2 (S7_OB, S8_OB and S11_OB) dominated by *Tetradesmus*. Group 3 (S2_OB, S9_OB, S13_OB and S16_OB) did not show any dominating microalgae genus, but these samples were characterized by mixtures of different protozoa and by only a small number of different algae.

The driving forces structuring microbial communities are concurrently regulated by both external factors such as chemical-biological parameters of the growth media (Nyhom and Kalqvist, 1989) and internal factors such as the interaction between eukaryotes and prokaryotes (Zachar and Boza, 2020). In this study, 27 chemical-biological parameters characterizing the original organic wastes (Table 1) were determined (Table 2) to understand how waste properties affected microbial and algae population.

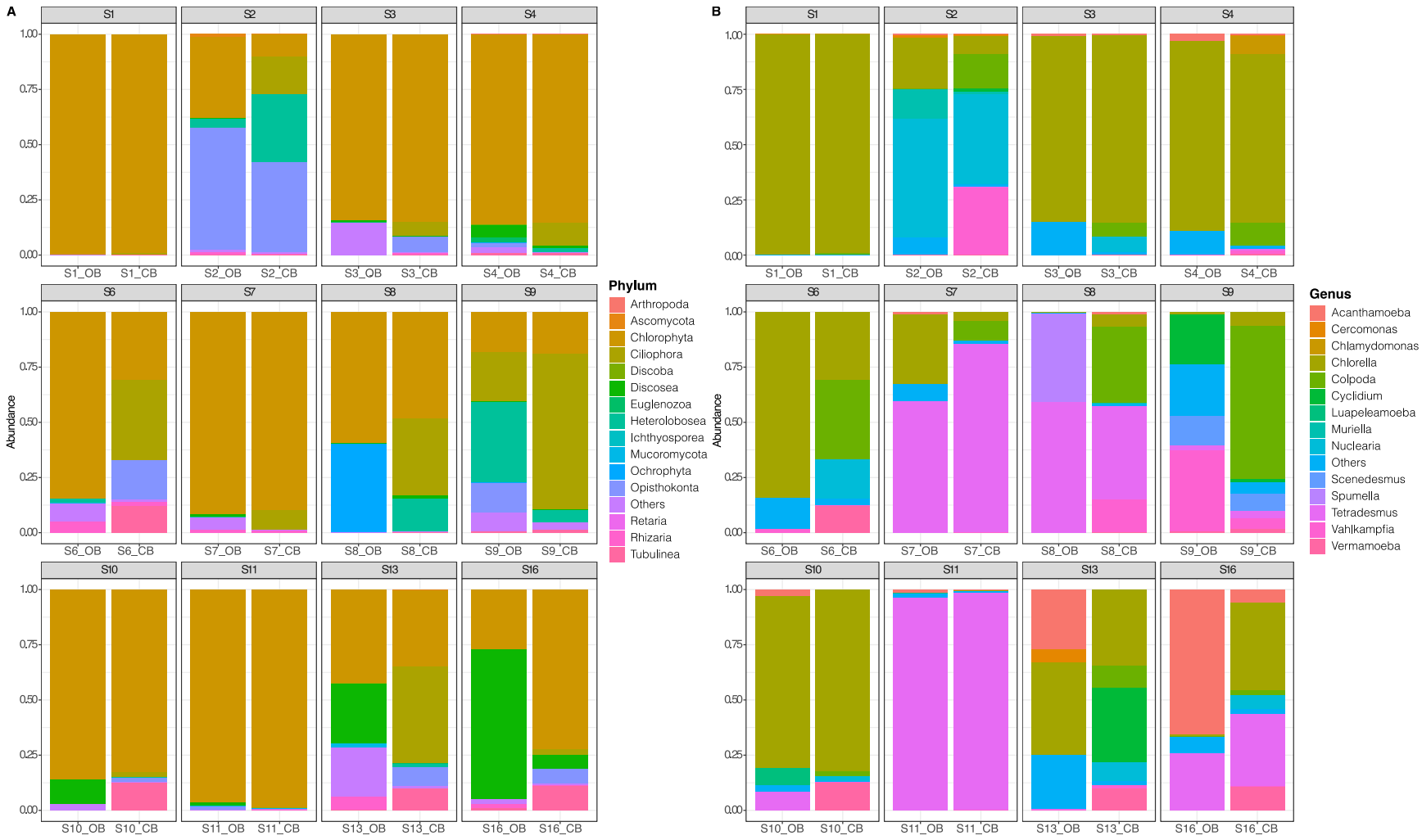
To do so, PCA was performed to describe chemical-biological parameters vs. dominating microalgae (Fig. 2a) and vs. dominating prokaryotes (Fig. 2b).

Two main factors (PC) were found to cover 61.8% of the total variance. PC1 correlated to heavy metals (Fe, Cu, Cr, Ni, As, Cd and Pb), TKN and P contents, while PC2 correlated to TS, VS, TOC, ABP, Mg pH and Mo. In particular, PC1 (in forward direction) carried inorganic nutrients and PC2 (forward direction) carried organic matter contents (TS, VS and TOC) and its quality, i.e. biological stability (ABP), and pH. VFA, EC and Na did not play important roles as their positions were near to the centre. *Chlorella* survived in almost all conditions regardless of the wide range of TS, pH, nitrogen, macro and microelements concentrations of original organic wastes, which accounted for its high abundance, i.e., 78-99.9 % of Eukaryotes (Figure 1a). The exception was for S7_OB, that was characterized by the strong presence of *Tetradesmus* (60 % of Eukaryotes); S2_OB in which the presence of *Muriella* (13 % of Eukaryotes) was also reported; and S13_OB that was characterized by an organic substrate rich in heavy metals (HMs), which probably limited the growth of algae.

Chlorella is a small spherical (coccoid) green unicellular simple alga, that replicates exclusively by asexual reproduction, i.e., autospore formation; in addition it is easy to cultivate and it grows rapidly (Kumar et al., 2015). *Chlorella* is widely diffused and it has been reported to occur on damp soils, walls, bark of trees, freshwater pools, sewage and sewage treatment plants (Palmer, 1969).

Chlorella was reported as having high tolerance to non-ideal growing environments (Gacheva and Pilarski, 2008). According to Agrawal and Singh (2000), the vegetative cell of *Chlorella* has high tolerance to dryness because of its small cell size and/or to the presence of sporopollenin protecting the cell wall. This fact can explain the presence of *Chlorella* in OB-samples S3_OB and S6_OB that, although characterized for organic substrate having the highest TS (242 and 257 g kg^{-1} FM), showed 84% of *Chlorella*. Again, previous findings reported that *Chlorella* is capable of surviving in different conditions, for example, in a wide pH range from pH 3 to pH 10.5 (Mayo, 1997; Khalil et al., 2010).

a


A
B
S1
S2
S3
S4
S6
S7
S8
S9
S10
S11
S13
S16
Abundance
1.00
0.75
0.50
0.25
0.00
S1_OB S1_CB
S2_OB S2_CB
S3_OB S3_CB
S4_OB S4_CB
S6_OB S6_CB
S7_OB S7_CB
S8_OB S8_CB
S9_OB S9_CB
S10_OB S10_CB
S11_OB S11_CB
S13_OB S13_CB
S16_OB S16_CB
Phylum
Arthropoda
Ascomycota
Chlorophyta
Ciliophora
Discoba
Discosea
Euglenozoa
Heterolobosea
Ichthyosporea
Mucoromycota
Ochrophyta
Opisthokonta
Others
Retaria
Rhizaria
Tubulinea
Genus
Acanthamoeba
Cercomonas
Chlamydomonas
Chlorella
Colpoda
Cyclidium
Luapeleamoeba
Muriella
Nuclearia
Others
Scenedesmus
Spumella
Tetradesmus
Vahlkampfia
Vermamoeba

b

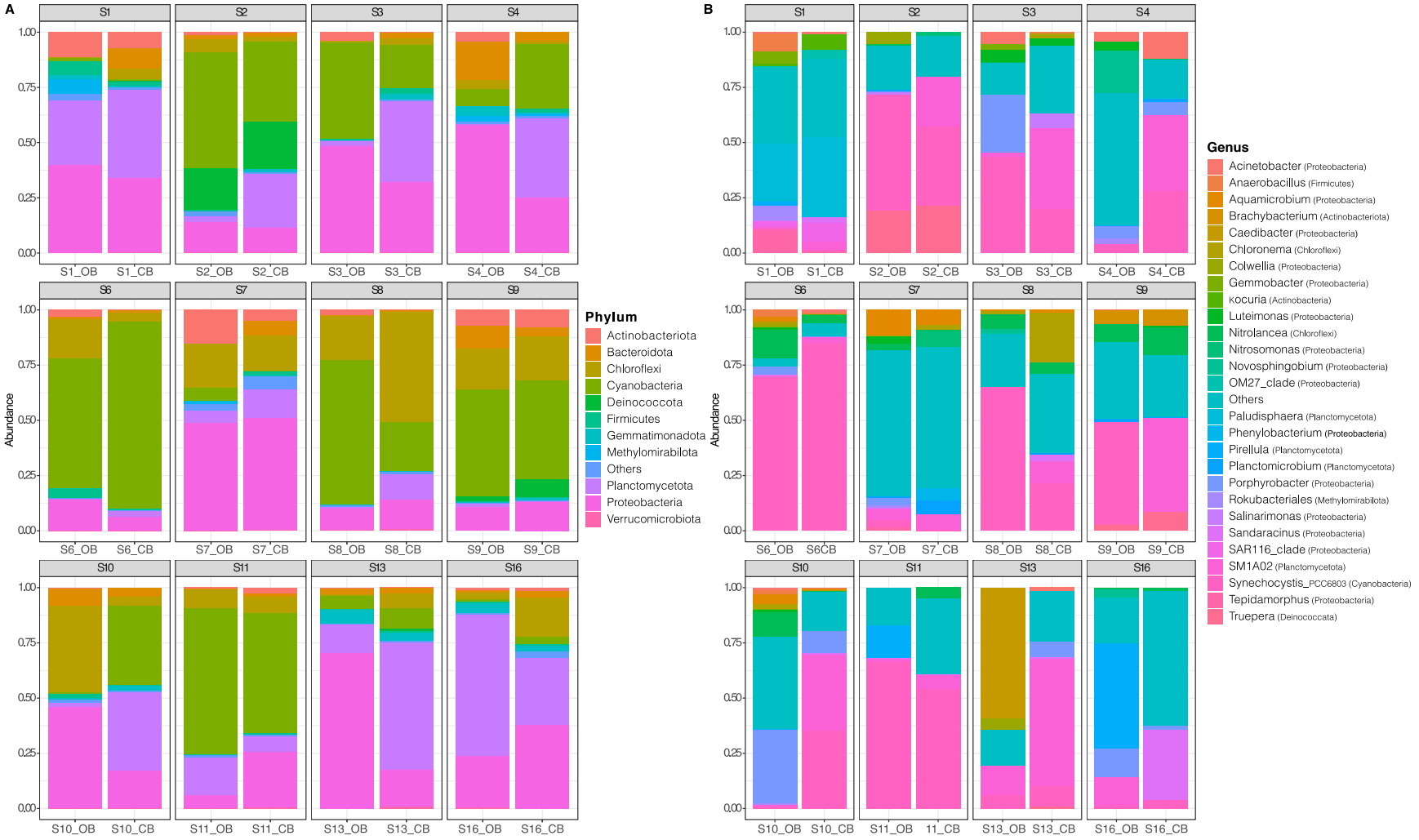


Figure 1. Eukaryotic (a) and prokaryotic (b) composition associated to each treatment. For the original samples (OB) each bar plot is the average of 3 replicates while for the cultivated samples (CB) is the average of 4 samples (2 biological replicates with 2 technical replicates for each biological sample). Only biomasses derived from S2 and S7 had two replicates (see Material and Methods for a better description). A) Phylum composition and B) Genus composition, for clarity only genera above 5% are shown. S-1 to S-16 represent the microalgae and bacteria consortia.

Some HMs are essential for microalgae, but exposing microalgae to HMs beyond their tolerance range very likely affects their growth and metabolism (Danouche et al., 2020; Expósito et al., 2021). *Chlorella* sp. was reported to be sensitive to As and Zn (Expósito et al., 2021), Cu (Xia and Tian, 2009; Ouyang et al., 2012), Cr (Lu et al., 2021) and Pb (Nanda et al., 2021). *Chlorella* can develop specific adaptive mechanisms to HMs (e.g. for Cu and Zn) (El-Naggar and Sheikh, 2014), thanks to the presence of anti-stress molecules such as brassinolide, an important hormone able to activate enzymatic and non-enzymatic systems responding to HM stress (Bajguz, 2010). The high HM contents (Table 2) reported for S13 and S14 may be the responsible for the low or null algae growth in S13 and S14 substrates (Figure 1a).

Some OB-samples were characterized by the presence of *Tetradesmus*, i.e. 96% in S11_OB, 60% in S7_OB and 59% in S8_OB, which was the second most abundant microalgae genus. *Tetradesmus* has been reported to be a robust species able to grow under unfavorable conditions (Nordin et al., 2019; Oliveira et al., 2021); the finding of this algae in some of the organic wastes studied seemed to confirm this fact.

Dahiya et al. (2021) found that *Tetradesmus wisconsinensis* was one of the dominant species in a wastewater plant in India and *Tetradesmus obliquus* has been reported to grow in nitrogen and phosphorus-rich wastewater both heterotrophically and mixotrophically (Ferreira et al., 2019; Martínez et al., 1999).

The multivariate analysis (PCA) performed for organic waste chemical-biological parameters (Fig 2a) indicated that *Tetradesmus* was mainly distributed in the lower part of the PCA axes (except for S16_OB, characterized by a much lower algae content, i.e. 26%) indicating that *Tetradesmus* preferred lower pH and TS content, and lower nutrient concentration than *Chlorella* (Fig. 2a). Hodaifa et al. (2009) found the highest specific growth rate of *Tetradesmus obliquus* when the medium was maintained at a constant pH value of 7. Nevertheless, the S11 substrate that was characterized by a pH of 8 showed a eukaryotic OB composed 96% by *Tetradesmus*, but S11 also showed a very low TS

content (TS of 1.7 ± 0.1). S16 substrate, that contained both high TS and pH, and high volatile fatty acids (VFAs), showed a low presence of *Tetradesmus* (26%), probably because this environment (VFAs) limited the growth of algae (Figure 1b).

Thus, the results described above seem to suggest that *Tetradesmus* survived in organic wastes that were not characterized for extreme chemical-biological parameters, unlike *Chlorella*, which seemed to be much less affected by chemical-biological parameters of the organic wastes. On the other hand, the presence of *Tetradesmus* excluded (or strongly reduced) the presence of *Chlorella*, suggesting that in the absence of extreme growing conditions the former, when present, grew better than the latter (Bani et al., 2020). This fact suggested that extreme chemical-biological parameters selected *Chlorella* (Low-De´carie et al., 2016).

Substrate S12, that was characterized for both low TS and pH, did not show any algae growth, probably because of very high VFA (17,565±208 g kg^{-1}FM) content that inhibited algae growth (Lacroux et al., 2020). Nevertheless, sometimes, substrate features were not able to explain algae growth. For example, S2 and S7 substrates, for which algae growth was detected, showed similar characteristics (Table 2) to S5 and S15 that did not show any algae growth. However, other internal factors, such as interactions with bacteria, should be considered in discussing microalgae presence.

3.2.2 Bacterial Communities

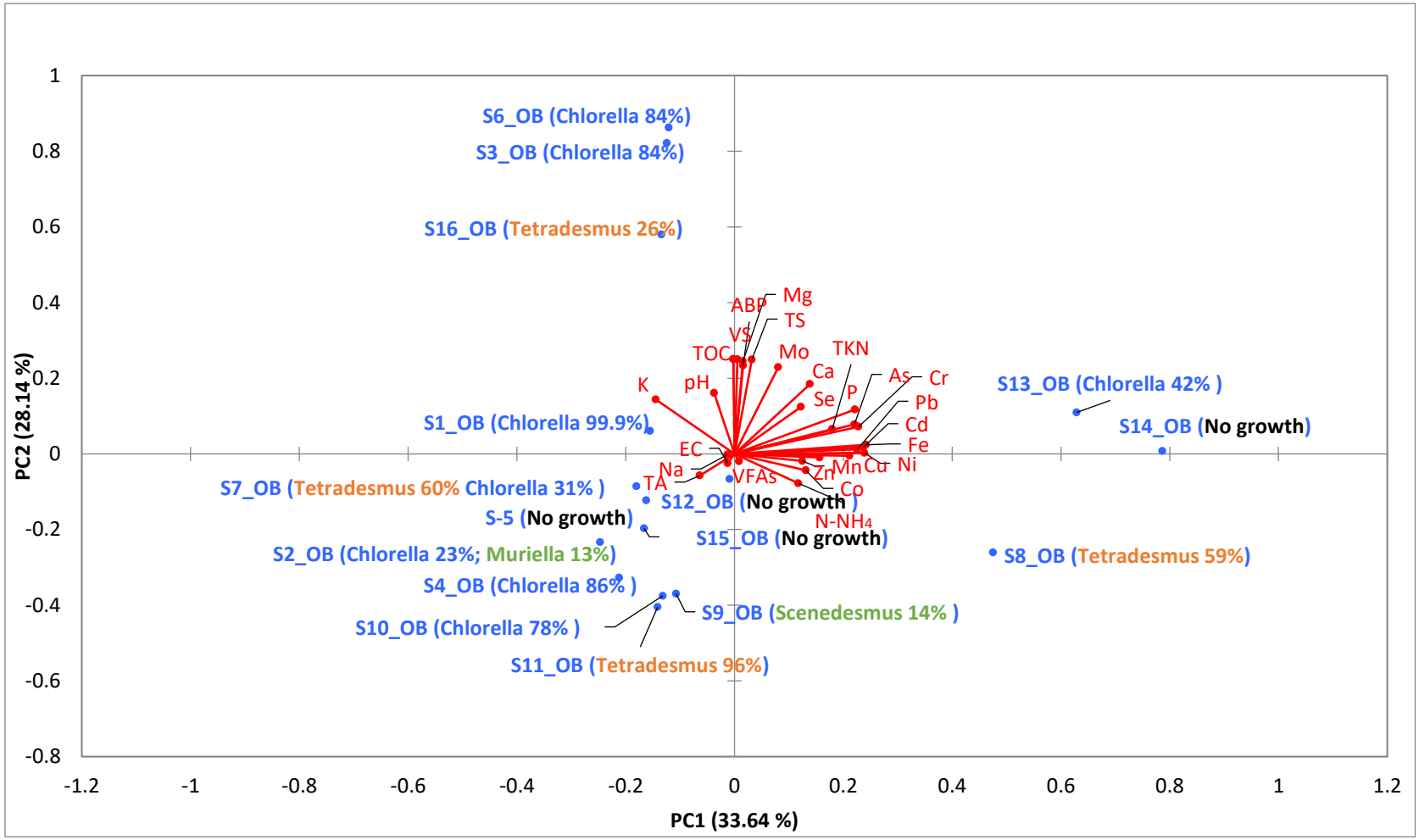
641 OTUs were assigned to bacteria after removing OTUs shared with the controls and the ones that were assigned to Chloroplast and Mitochondria (862 OTUs were produced before any cleaning). The bacterial community (100% OTU) was dominated by Cyanobacteria, Proteobacteria and Planctomycetota which are commonly found in both wastewater and digestate media (Caprio et al., 2018). A first group of OB samples was dominated by Cyanobacteria, which almost reached 50% of prokaryotic content, i.e. S2_OB (53% ± 4), S6_OB (59% ± 13), S8_OB (65% ± 7), S11_OB (66% ± 2), S3_OB (44% ± 5) and S9_OB (47% ± 4). The most abundant genus of this phylum was

Synechocystis that accounted for 47% ± 4 in S9_OB becoming the dominant genus in S6_OB in which it accounted for 70% ± 7 of relative abundance (Fig. 2b). Cyanobacteria, also known as photosynthetic bacteria, are prokaryotes able to survive in waste due to their capability to tolerate high levels of pollutants, to degrade highly persistent organic contaminants and to remove heavy metals such as Cr, Co, Cu and Zn (Trentin et al., 2019). They are the only planktonic group capable of utilizing atmospheric nitrogen via biological N_2 fixation, and as such, can circumvent N-limited conditions. They are also capable of using alternate pathways for the acquisition of carbon and nitrogen to counteract the reduction of photosynthesis efficiency in an unfavorable environment (Yu et al., 2013). Additionally, Cyanobacteria make a good combination with green algae because of their ability to produce some growth promoting substances that result in a symbiotic relationship (Gutierrez-Wing et al., 2012). As can be seen in Fig. 1a and Fig. 1b, S2_OB (*Chlorella* 23%, *Muriella* 13% of Eukaryotes, *Synechocystis* 53% of Prokaryotes), S3_OB (*Chlorella* 84% and *Synechocystis* 44%), S6_OB (*Chlorella* 84% and *Synechocystis* 70%), S8_OB (*Tetradesmus* 59% and *Synechocystis* 65%), S9_OB (*Scenedesmus* 14% and *Synechocystis* 47%) and S11_OB (*Tetradesmus* 96% and *Synechocystis* 66%) were good examples. *Synechocystis 6803*, one strain of Cyanobacteria, demonstrates adaptable growth ability under photoautotrophic, mixotrophic and heterotrophic conditions (Vermaas, 1996). They have developed sophisticated regulatory systems to adapt cellular processes and maintain metabolic homeostasis in response to many environmental fluctuations, such as nutrient availability and the ambient chemical-biological properties (Spät et al., 2015). As can be seen from Fig. 2B, *Synechocystis* tended to appear both at the top and at the bottom of PC2, showing its ability to co-exist with *Chlorella* (S2_OB, S3_OB and S6_OB), as well as *Tetradesmus* (S8_OB and S11_OB) and *Scenedesmus* (S9_OB).

Proteobacteria and Planctomycetota co-dominated the remaining communities (Fig. 2b) with the exceptions of samples S4_OB, S7_OB and S10_OB, in which Proteobacteria represented the main phylum. No clear dominance could be found for all these samples at genus level; for example S13_OB

was dominated by *Caedibacter* (59% ± 6) which included an endosymbiont of *Paramecium* which is commonly present in sewage sludge (Beier et al., 2002). Other common genera found were: *Pirellula* (S16_OB 48% ± 4) and *Paludisphaera* (S16_OB 36% ± 10), which are commonly found in wastewater samples due to their high metabolic flexibility in using multiple compounds (Chouari et al., 2003). Furthermore, small amounts of *Sandaracinus* and *Luteimonas* (data not shown) were found in OB cultures, above all in digestate-derived substrates, since they are able to degrade both simple (Sharma et al., 2016) or recalcitrant organic molecules (Akyol et al., 2019). Moreover, due to the fact that the media (wastes) were enriched in N compounds, many N oxidizing bacteria were present in the initial community, e.g., *Nitrolancea* that is a nitrite oxidizing bacterium present in different OBs (S6_OB 13% ± 2 vs 4% ± 1 in batch). These bacteria were then lost in the CBs because, probably, of the effect of both temperature batch growth temperature (25°C), which was lower than their optimum (37°C), and low ability to compete with other bacteria in the consortia (Spieck et al., 2020).

a


S6_OB (Chlorella 84%)
S3_OB (Chlorella 84%)
S16_OB (Tetradesmus 26%)
S1_OB (Chlorella 99.9%)
S7_OB (Tetradesmus 60% Chlorella 31%)
S-5 (No growth)
S2_OB (Chlorella 23%; Muriella 13%)
S4_OB (Chlorella 86%)
S10_OB (Chlorella 78%)
S11_OB (Tetradesmus 96%)
S9_OB (Scenedesmus 14%)
S15_OB (No growth)
S12_OB (No growth)
TA
VFAs
EC
Na
K
pH
ABP
VS
TOC
TS
Mg
Mo
TKN
Ca
As
Cr
P
Se
Pb
Cd
Fe
Mn
Cu
Ni
Zn
Co
N-NH4
S13_OB (Chlorella 42%)
S14_OB (No growth)
S8_OB (Tetradesmus 59%)
PC2 (28.14 %)
PC1 (33.64 %)
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1.2
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1
1.2

b

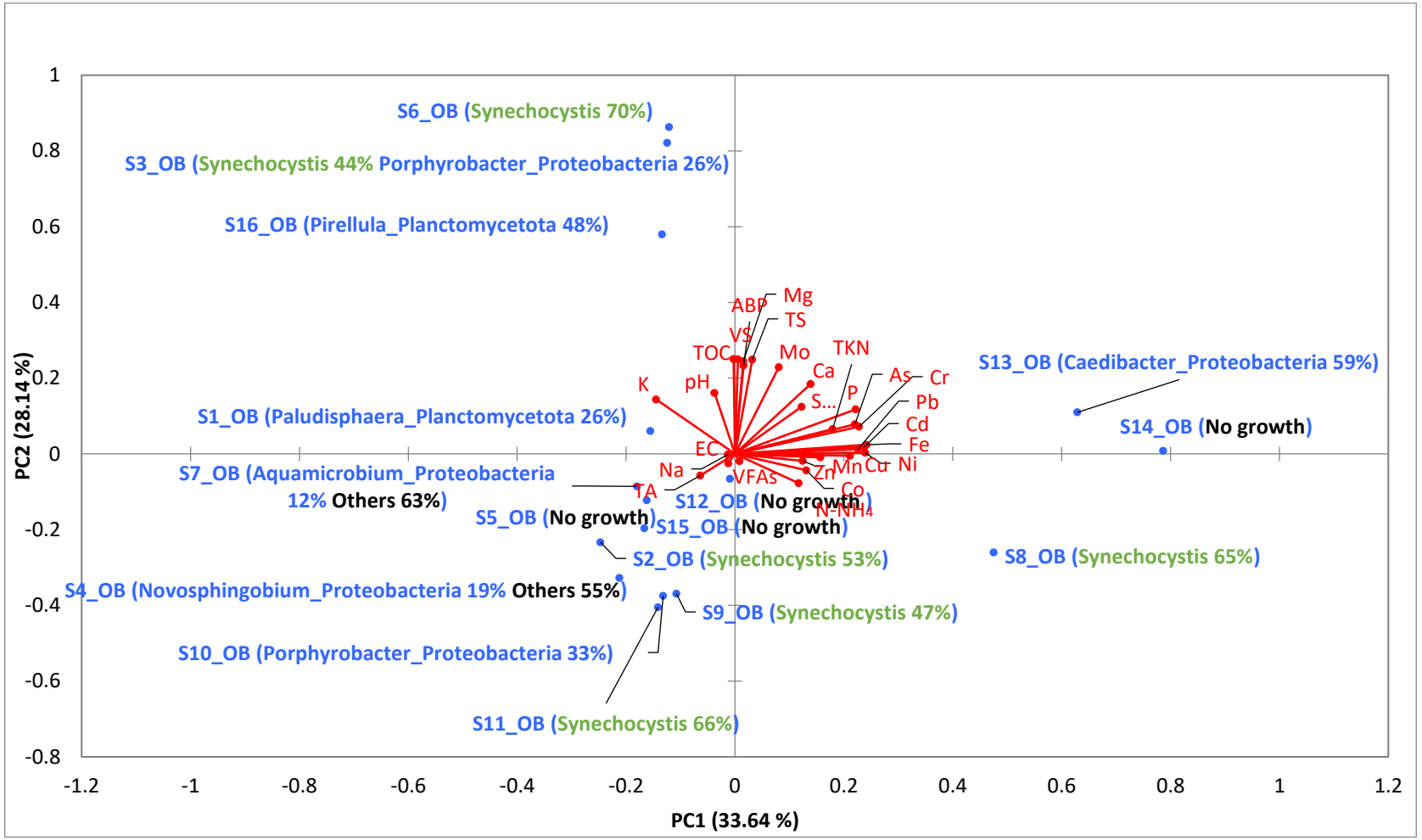


Figure 2. Principal component plot of waste chemical-biological parameters vs. eukaryotic microalgae (a), and prokaryotic bacteria and cyanobacteria abundance (b). S1_OB to S16_OB represent the microalgae and bacteria consortia obtained from the corresponding organic waste. "No growth" means that there was not microalgae consortia biomass growth for metabarcoding analysis.

3.2.3 Algae-bacteria consortia interactions

Besides chemical-biological parameters, bacteria communities are another major factor affecting algal communities, as previously reported (Choi et al., 2010; Nie et al., 2020). Algae and bacteria synergistically affect each other's physiology and metabolism (Ramanan et al., 2016). Interrelations between bacteria and microalgae are multifaceted and complicated; for example, bacteria naturally can rely on photosynthetic phytoplankton to obtain the organic carbon needed to maintain their growth; in turn, phytoplankton can depend on bacteria to mineralize organic matter into inorganic substitutes, ultimately supporting the growth of algae (Yang et al., 2020). Figure 3 shows prokaryote distribution in the different samples represented by two PCs derived from PCA analysis and the relationships with eukaryote communities (algae). PC1 and PC2 that covered 26.6 % and 15.2% of the total variability, were able to separate OB-samples. Samples S1_OB, S3_OB, S4_OB, S6_OB and S10_OB, that showed the highest abundance of *Chlorella*, were preferentially distributed in the left part of PC1 and up part of PC2 (Figure 3). *Paludisphaera* (Planctomycetota) that accounted for 26.5% of the prokaryotic community of S1_OB, is a chemo-organotrophic aerobe capable of growth under micro-oxic conditions (Kulichevskaya et al., 2016), that makes it a good combination with *Chlorella* to get mutual benefits from each other. Samples S3_OB, S4_OB and S10_OB were characterized for the presence of Proteobacteria, such as *Roseomonas, Acinetobacter*, *Luteimonas* and *Porphyrobacter*, while S6_OB showed the presence of *Nitrolancea* (13%) in phylum Chloroflexi.

Porphyrobacter was present above all in S3_OB and S10_OB, i.e. 26% and 33%, respectively, while for S4_OB about 55% of undetectable genera made it impossible to define the most influential genus, although *Novosphingobium* (Proteobacteria) contributed 19%. *Porphyrobacter* is an aerobic and chemohetero-trophic bacterium (Fuerst et al., 1993) with potential applications of hydrocarbon degradation, algalytic activity and bioleaching (Xu et al., 2018). *Porphyrobacter* (Xu et al., 2018) and *Novosphingobium* (Thn, 2018) have both been commonly found in diverse and contaminated environments. *Novosphingobium* species can rearrange their genomes and functional profiles to adapt

to local environments. As for S6_OB, Chloroflexi, also known as green filamentous bacteria, can grow photosynthetically under anaerobic conditions or in the dark by respiration under aerobic conditions (Jagannathan and Golbeck, 2009). Luis et al. (2017) demonstrated that Chloroflexi were dominant in the bacterial community of a biogas reactor fed by sludge and *Chlorella* biomass. Thus, *Chlorella*-dominated communities can be stable with various Proteobacteria and/or Planctomycetota, and also make a good combination with Cyanobacteria.

Tetradesmus, *Scenedesmus* and *Muriella* tended to group in the left part of axis PC1 (Figure 3). S11_OB (96% ± 2) and S16_OB (26% ± 1) were affected above all by the presence of *Pirellula* (Planctomycetota), accounting for 14% and 48% respectively.

Pirellula, the bacteria that are responsible for nitrogen transformations, can utilize NO_2^--N to oxidize NH_4^+-N and generate N_2 under hypoxic or anaerobic environments. *Pirellula* removing ammonia would be inhibitory to algal growth (Choi et al., 2010), that can explain the fact that in the presence of more *Pirellula*, a lower *Tetradesmus* abundance was found. As for S13_OB, a significant abundance of *Caedibacter* (59%) appeared in the culture while *Chlorella* (42%) were found to be less dense. It is important to highlight that *Caedibacter* was probably not the reason for the scarcity of *Chlorella*, as it is reported to be capable to increasing its host's (in this work it refers to algae) fitness via manipulation of metabolic pathways and cell cycle control (Grosser et al., 2018) rather than negatively affecting the growth of its host (Dziallas et al., 2012).

The results seem to indicate that Planctomycetota presence was close to that of both *Tetradesmus* and *Chlorella*, unlike that of the Proteobacteria, of which the presence was generally found for OB characterized by *Chlorella* alone. The mixed populations can perform functions which are difficult or even impossible for individual strains or species (Brenner et al., 2008).

Compared to the unialgal culture, co-culture provides robustness to environmental fluctuations, culture stability, mutual benefits of nutrients distributions and resistance to invasion by other species

(Subashchandrabose et al., 2011). However, it is worth stating that how positive or negative interactions modulate the dynamics of bacterial-eukaryotic communities is still far from being fully understood.

3.3 Original biomasses selected vs. cultivated biomasses

This paper aimed to investigate the presence of useful algae-microbial consortia able to growth on substrate rich in nutrients (N and P) and C, for subsequent processing production purposes.

To do so, OBs isolated were successively cultivated under standardized batch conditions for algae growth (see section 2.4) and the cultivated microbial populations (CB) obtained were investigated.

Results indicate that S9_CB, S11_CB and S1_CB did not show any difference with respect to the original biomasses (OB) isolated from organic wastes (Figure 4a). For the other CBs, eukaryotes and bacterial communities were strongly influenced, as expected, by the original biomass (OB), as shown by the NMDS results (Fig. 4a and Fig. 5a) and supported by the PERMANOVA analyses (origin R^2= 0.61 and p-value < 0.05, condition R^2=0.07 and p-value < 0.05, origin*condition R^2= 0.19 and p-value < 0.05).

Cultivated biomass richness (Figure 5a) did not seem to be affected by the batch growth, with the exception only of S10_CB (pairwise t-test, p-value < 0.05). In batch cultivated biomass samples, a *Chlorella* reads count reduction was usually associated with an increase of *Colpoda* reads count (see for examples S6_CB, S2_CB and S4_CB (Figure 2a)). However, recent studies had found that *Colpoda* sp. are also able to prevent the collapse of *Chlorella* sp. in open ponds, as it eliminated bacterial cells that could damage the microalgae (Haberkorn et al., 2020; Hailei et al., 2017). Thus, the interaction between algae and bacteria is beneficial or harmful to each other also depending on the cultivation conditions.

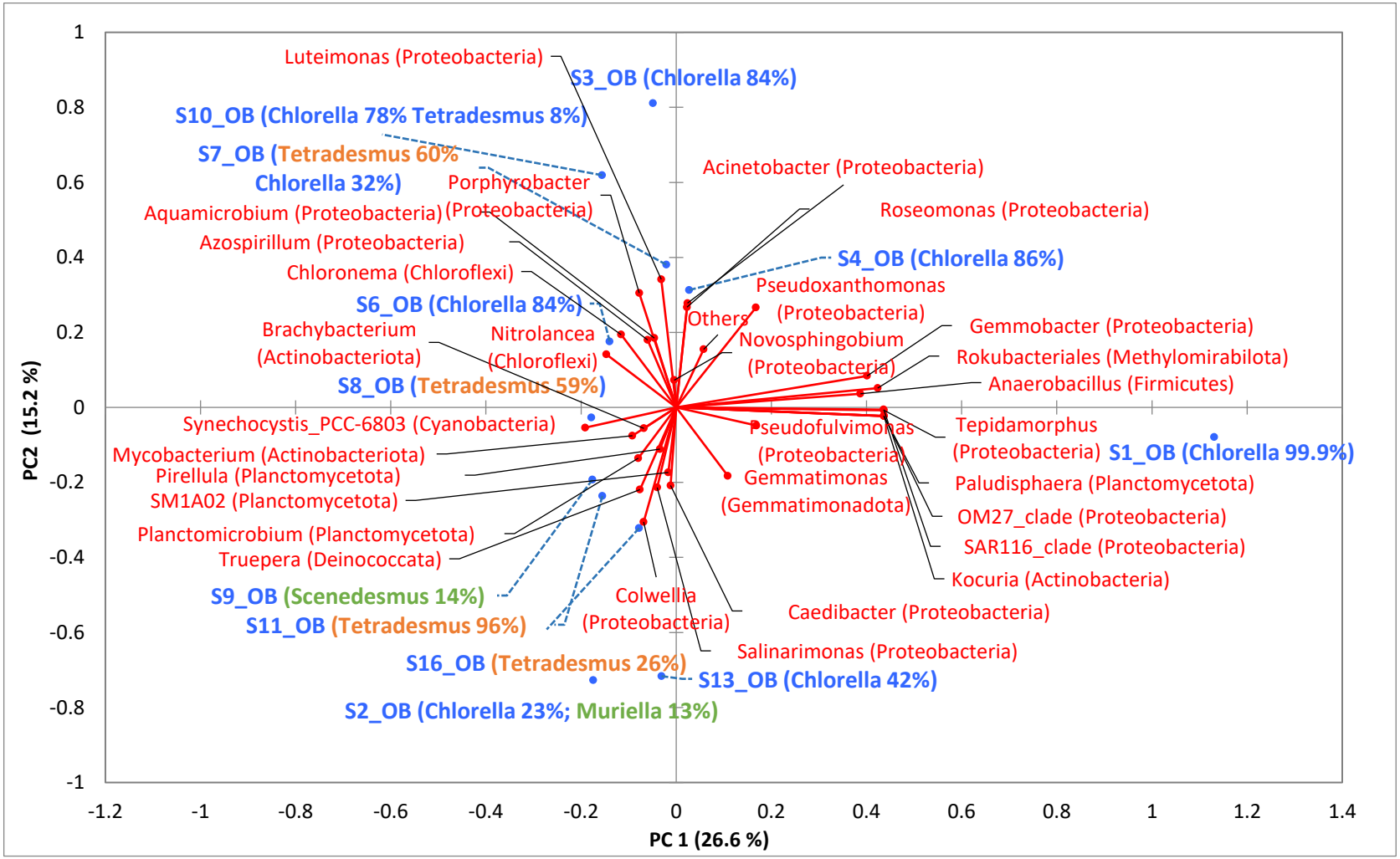


Figure 3. Principal component plot of prokaryotic bacteria and eukaryotes abundance. S1_OB to S16_OB represent the microalgae and bacteria consortia obtained from the corresponding organic waste.

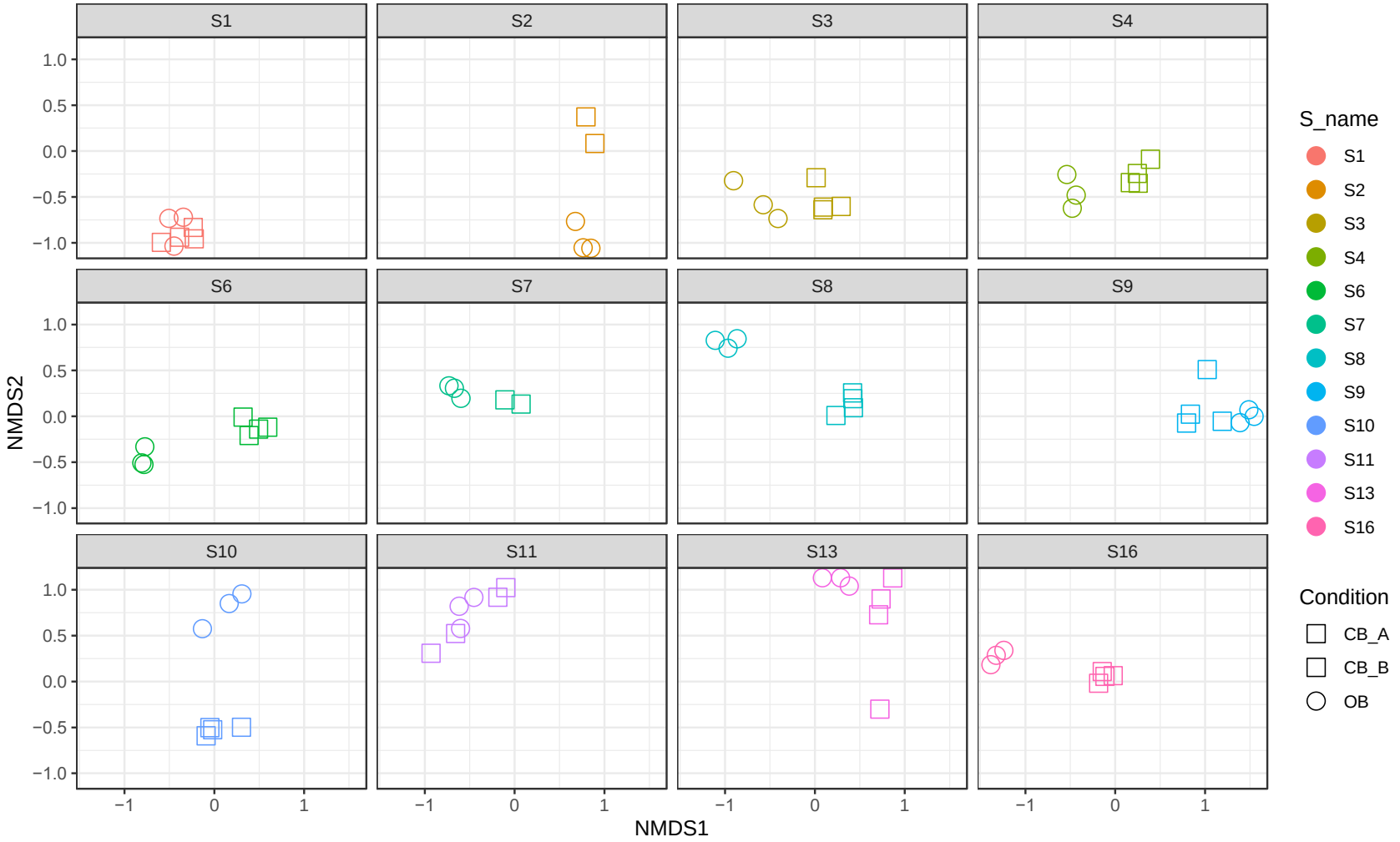
Again, *Tetradesmus* decreasing, i.e., in S8_CB, from 59% to 42% and *Scenedesmus* decreasing, i.e. in S9_CB from 14% to 8%, were both accompanied by *Colpoda* sp. increase, i.e. from 10% in OBs to 35% and 69% in CBs, respectively. These results supported once again the idea that understanding the interaction between the different organisms is essential to tailor effective strategies for successful microalgae cultivation.

Bacterial communities, even if they maintain the phyla composition, as shown in Section 3.2.2, have different community structures, as shown by Figure 4b, with the only exceptions of S9_OB and S11_OB that were similar to S9_CB and S11_CB. S9_OB communities were originally from wastewater while all the others were sampled from digestate or slurry.

The bacterial community was influenced by both the origin of the samples (OB or CB) and the type of inoculum (digestate, wastewater, manure etc.), as supported by the PERMANOVA results (origin R^2= 0.44 and p-value < 0.05, condition R^2=0.07 and p-value < 0.05, origin*condition R^2= 0.25 and p-value < 0.05).

Alpha diversity for bacteria did not show variation between the OBs and the respective CBs, with the exception of only two communities (Fig. 5b). S-6 is one of them (pairwise t-test, p-value< 0.005), however the difference can be easily explained as a drop in richness as *Synechocystis*, which was the main genus accounting for almost the totality of the community (see previous paragraph and Fig. 2b).

a


S1
S2
S3
S4
S6
S7
S8
S9
S10
S11
S13
S16
NMDS1
NMDS2
S_name
S1
S2
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S7
S8
S9
S10
S11
S13
S16
Condition
CB_A
CB_B
OB

b

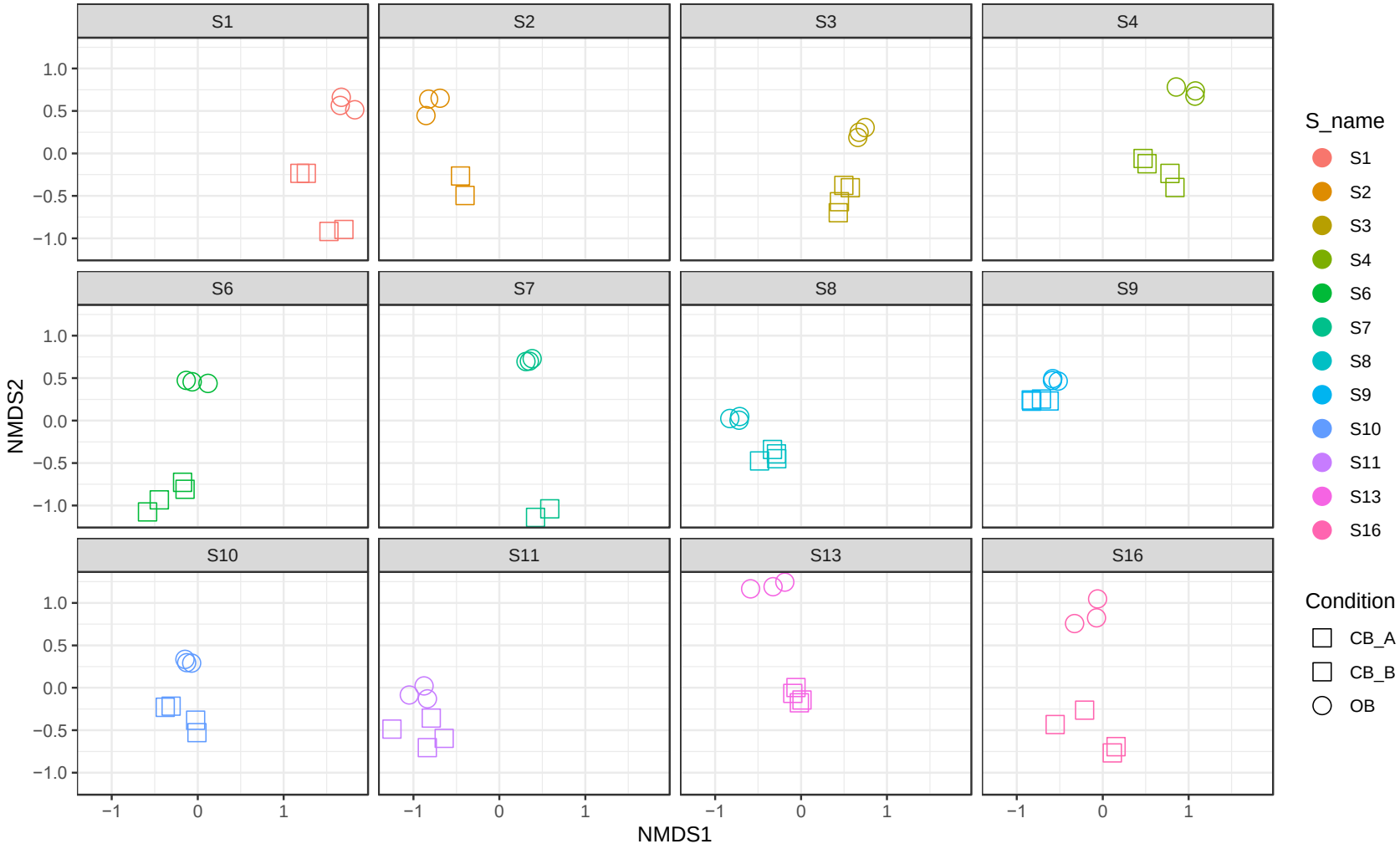
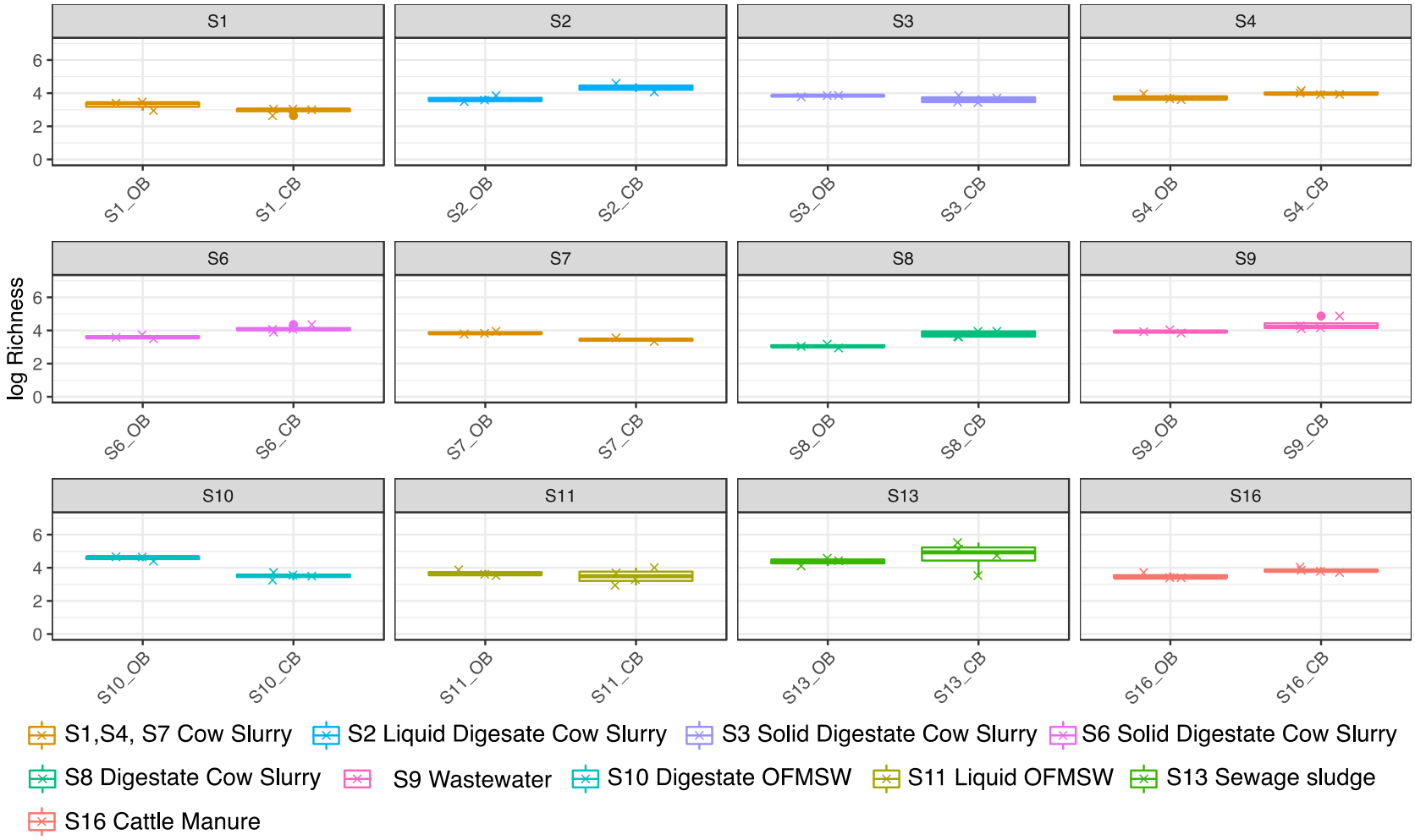


Figure 4. NMDS for eukaryotic community (a) and prokaryotic (b) community. Each panel represents organic wastes (different colors) from which microbial consortia have been obtained. Different shapes represent the different growth condition: (circle) original eukaryotic/prokaryotic community obtained from organic wastes (OB) and (square) eukaryotic/prokaryotic community cultivated starting from original community (CB from trial A and B). For each original biomass (OB) there are 3 replicates and 2 replicates for each cultivated community (CB), so that in total 4 replicates for the CBs. Only S-2 and S-7 had 2 replicates (see Material and Methods for a better description).

a


S1
S2
S3
S4
S6
S7
S8
S9
S10
S11
S13
S16
log Richness
0
2
4
6
S1_OB
S1_CB
S2_OB
S2_CB
S3_OB
S3_CB
S4_OB
S4_CB
S6_OB
S6_CB
S7_OB
S7_CB
S8_OB
S8_CB
S9_OB
S9_CB
S10_OB
S10_CB
S11_OB
S11_CB
S13_OB
S13_CB
S16_OB
S16_CB
S1,S4, S7 Cow Slurry
S2 Liquid Digesate Cow Slurry
S3 Solid Digestate Cow Slurry
S6 Solid Digestate Cow Slurry
S8 Digestate Cow Slurry
S9 Wastewater
S10 Digestate OFMSW
S11 Liquid OFMSW
S13 Sewage sludge
S16 Cattle Manure

b

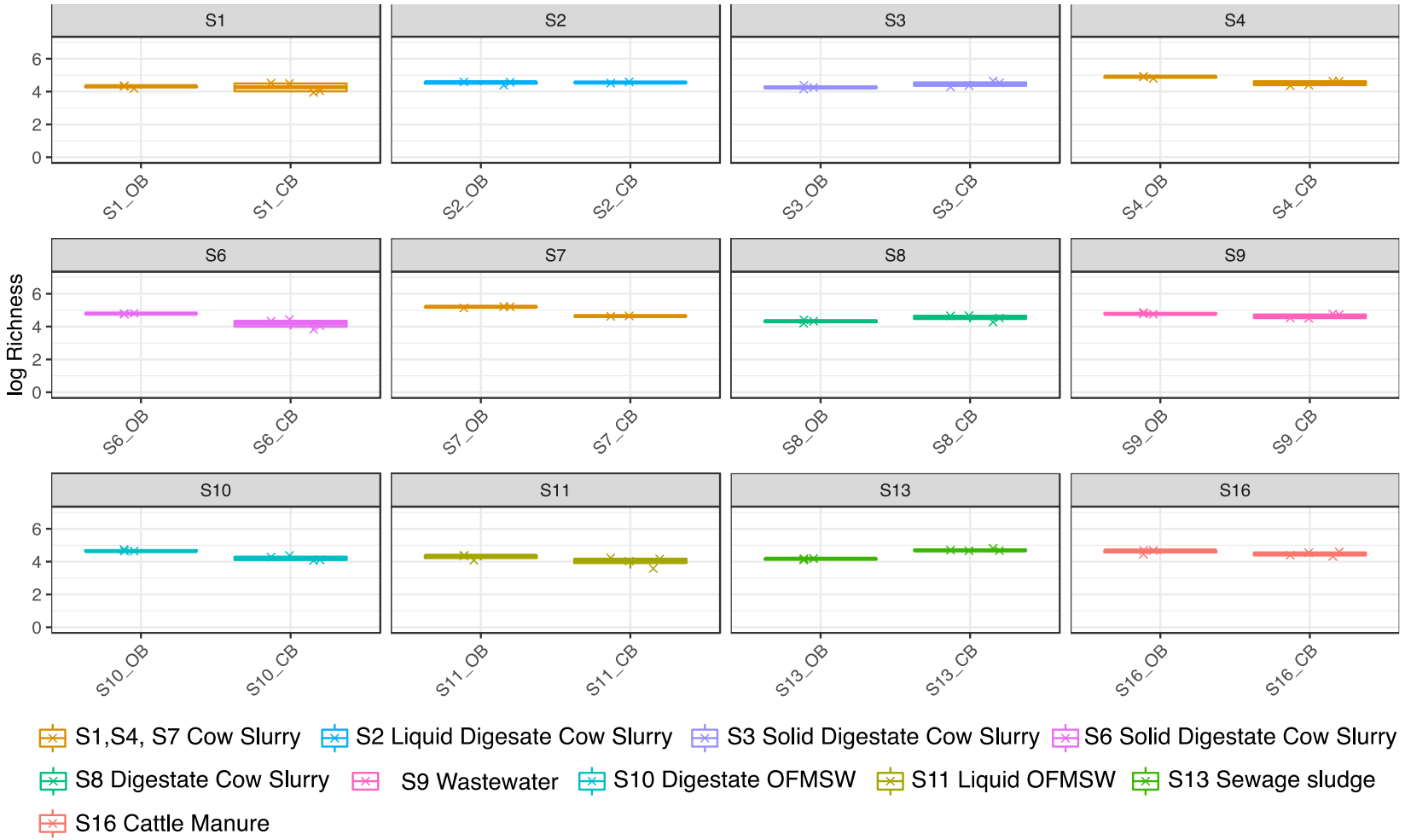


Figure 5. Eukaryotic (a) and prokaryotic (b) community richness. Each panel represents a different original (S-_OB) and cultivated (S-_CB) biomasses. Cultivated biomass box plots include 4 replicates while original boxplots show the results of 3 replicates. Only S-2 and S-7 had 2 replicates for batch condition.

4. Conclusion

Microalgae are useful in treating organic wastes producing useful biomasses to be used for different purposes. Nevertheless, organic substrates are characterized by high concentrations of C and nutrients (N above all) that make microalgae cultivation very difficult.

The isolation of microalgae directly from organic waste can be a winning approach in obtaining useful microalgae-microbial consortia, self-adapted to grown on wastes for commercial purposes. Working on sixteen different organic wastes, twelve consortia were isolated and completely characterized. *Chlorella* and *Tetradesmus* were the most represented microalgae in combination with prokaryotic communities, i.e. Cyanobacteria, Proteobacteria and Planctomycetota.

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Chapter III

Growth performance, biochemical composition and nutrients recovery ability of twelve microalgae consortia isolated from various local organic wastes grown on nano-filtered pig slurry

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Growth performance, biochemical composition and nutrients recovery ability of twelve microalgae consortia isolated from various local organic wastes grown on nano-filtered pig slurry.

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Abstract

This paper demonstrated the growth ability of twelve algae-microbial consortia (AC) isolated from organic wastes when a pig slurry-derived wastewater (NFP) was used as growth substrate in autotrophic cultivation. Nutrients recovery ability, and biochemical composition, along with fatty acids and amino acids profiles of algae consortia, were evaluated and compared.

Three algae-microbial consortia, i.e. a *Chlorella*-dominated consortium (AC_1), a *Tetradesmus* & *Synechocystis* co-dominated consortium (AC_10) and a *Chlorella* & *Tetradesmus* co-dominated consortium (AC_12) were found to have the best growth rates (μ of 0.55 ± 0.04, 0.52 ± 0.06 and 0.58 ± 0.03 d^{-1}, respectively), which made them good candidates for further applications. Nevertheless, biochemical composition suggested that ACs showed high carbohydrates and lipid contents but both low protein and essential amino acid contents, probably because of the low N concentration of NFP. AC_1 and AC_12 showed an optimal ω6: ω3 ration of 3.1, and 3.6 which make them interesting from a nutritional point of view.

Key words: Amino acid; Biochemical compositions; Fatty acid; Microalgae-microbial Consortia; Nutrient recovery; Organic waste.

1. Introduction

Microalgae cultivation is considered as an efficient tool in the framework of a circular economy, combining wastewater treatment and the production of valuable biomass for different purposes (Stiles et al., 2018). Microalgae cultivation implies high consumption of water, inorganic nutrients, and CO_2 (Acién Fernández et al., 2018). The utilization of chemical fertilizers as nutrient sources is costly and reduces the environmental sustainability of microalgae-based processes (Olguín, 2012). The chemical composition of several kinds of wastewaters is quite similar to the culture media usually adopted for microalgae growth, hence the possibility to recover inorganic nutrients, water and CO_2 from wastewaters and organic wastes provides an environmentally friendly and cheap method for algae production (Acién Fernández et al., 2018; Ledda et al., 2015). In particular, inorganic nitrogen (N) and phosphorus (P) uptake by microalgae can be used as an efficient bioremediation tool for wastewater treatment, transforming these nutrients into energy-rich biomass which can be further processed to make biofuels or other valuable products such as biofertilizers, bioplastics and so forth (Batista et al., 2013).

One of the main bottlenecks hampering the full exploitation of this tool is that among the wide variety of microalgal species present in nature, only a few species are currently known to survive and grow in wastewater or high carbon-rich wastes (Stiles et al., 2018). It has been reported that compared to algae monospecies cultures, algal-bacteria consortia may be more suitable for cultivation on wastewater, by acting symbiotically, especially in terms of preventing contamination and enabling long-term cultivation in open systems (Acién Fernández et al., 2018; González-Fernández et al., 2011). In this mutualistic equilibrium, the O_2 released by algal photosynthesis is utilized by aerobic-heterotrophic bacteria to mineralize organic compounds, and bacterial respiration provides CO_2 as a carbon (C) source to the algae. Additionally, it has been demonstrated that algae-bacteria consortia systems take up nutrients from digestate more efficiently than monoculture systems (Kerckhof et al., 2014; Vulsteke et al., 2017).

Several types of organic wastes and wastewaters are characterised by the presence of ammonium (NH_4^+/NH_3) as a proportion of the total N content or as the unique N form. Ammonium is the preferred N source for microalgae, since, unlike nitrate, it does not need the energy-consuming reduction steps once it is taken up by the cells (Kumar and Bera, 2020). However, the ammoniacal N form can also be toxic for microalgae, its toxicity depending on the equilibrium between the ionized (NH_4^+) and the unionized, volatile (NH_3) form, which is in turn determined mainly by pH and secondarily by temperature (Collos and Harrison, 2014). Although both N forms are toxic to some extent, NH_3 is the more toxic, since it is uncharged and so able to cross cell membranes in an uncontrolled way, impairing the pH gradient across thylakoid membranes (uncoupling photophosphorylation) and interfering with PSII, affecting photosynthesis (Kumar and Bera, 2020; Li et al., 2019). On the other hand, NH_3 is very volatile, and can be lost to the atmosphere, especially in aerated culture systems (Collos and Harrison, 2014). Therefore, the feasibility to use NH_4-containing wastewaters as the nutrient source for microalgae culture depends mainly on the possibility of matching the wastewater total NH_4-N concentration and the tolerance of the cultured microalgae. Collos and Harrison (2014) detected a wide variability among microalgae phyla in the optimal, tolerated, and toxic concentrations of ammonium, providing evidence for a potential biodiversity that can be exploited to optimize the use of microalgae for N recovery from wastewater.

Thus, the main objective of this study was to examine and compare the potential of twelve microalgae consortia, originally selected from different waste streams rich in diverse N forms, to be cultivated using a nanofiltered permeate from pig slurry as the growth medium, rich in NH_4-N. We evaluated the growth performance, nutrient removal efficiency as well as the biomass quality in terms of the biochemical composition along with fatty acids and amino acids profiles, to give an insight into the most robust algae consortia, especially as regards their adaptability to NH_4-N sources.

2. Materials and Methods

2.1 Nanofiltered Permeate sampling and characterization

The culture medium used during batch trials was the nano-filtered permeate (NFP) sampled at a full-scale pig slurry treatment plant located in Brescia province in Northern Italy. Briefly, the raw slurry was separated after screw pressing into liquid (L1) and solid fraction (S1); then the liquid fraction went through a vibrating screen (0.1 - 0.3 mm), to give a second liquid (L2) and solid fraction (S2). Lastly, the L2 passed through 0.01µm nano-filtration step, giving NFP. NFP was immediately stored at 4˚C and characterized upon arrival after sampling. Total nitrogen (TN), ammonia nitrogen (NH_4^+-N); pH and chemical oxygen demand (COD) were determined on fresh materials according to the analytical method for wastewater sludge (IRSA CNR, 1994). Macro and microelement contents including Na, Mg, K, Ca, P, Mn, Fe, Cu, Zn, Cr, Co, Ni, As, Se, Mo, Cd, Pb were determined by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, Aurora M90 BRUKER, Germany), preceded by microwave assisted (Multiwave ECO, Anton Paar GmbH, Germany) nitric acid digestion (EPA, 2007) of fresh samples. All analyses were performed in triplicate.

2.2 Microalgae consortia and preparation of inoculum

The algae consortia used in these trials were obtained previously by direct isolation from organic wastes sampled directly at full scale plants located in Northern Italy, aiming to select algae-microbial consortia well adapted to substrates rich in C and high in nutrient contents (Su et al., 2021). Algae consortia (AC) were maintained in 500 mL Erlenmeyer flasks in BG-11 medium under constant aeration and mixed by using filtered air (filter of 0.2 µm) with a continuous illumination of 50 µE m^{-2} s^{-1}, provided by fluorescent white tubes, at a controlled temperature of 22 ± 1°C.

2.3 Experimental set-up and cultivation

This study aimed to obtain the first data about growth performance and chemical and biological characteristics of algae consortia previously isolated from organic wastes (Su et al., 2021), using a waste stream, i.e. pig slurry derived product (NFP), as culture medium (see Section 2.1).

To do so, batch trials were carried out in triplicate in photobioreactors (PBRs) of 0.5 L working volume. pH was set at 8 and it was maintained by using pure CO_2 injection adopting an "on-demand" modality (Travieso et al., 2006). Room temperature (25°C) and constant air flux (10 L min^{-1}) were provided as well as light that was delivered by cold fluorescent lamps at irradiance of 312 µE m^{-2} s^{-1} at PBR surface, adopting a 12h:12h photoperiod regime. NFP was used as the batch growth medium; P (7.11 mg L^{-1} of K_2HPO_4) and Fe (1.02 mg L^{-1} $(NH_4)_5[Fe(C_6H_4O_7)_2]$) were added, to provide a complete growth substrate.

2.4 Microalgae growth determination

Microalgae dry weight (DW) was determined by sampling 5 mL of algae suspension from each PBR. The samples were centrifuged at 4,000 rpm for 10 min and then washed with an equivalent volume of distilled water to remove salts. Culture samples were then filtered by 1.2 µm filter (GF/C, Whatman Ltd., Maidstone, UK), dried overnight at 80 °C and weighed. Sampling was done every day.

The specific growth rate µ (day^{-1}) was calculated from the Equation (1) (Vonshak and Tomaselli, 2006):

$$\mu = (\ln N_1 - \ln N_2)/(t_1 - t_2) \qquad [1]$$

where N_1 and N_2 are the concentrations of cells (g L^{-1}) at day t_1 and t_2.

The Nitrogen (N) taken up by biomass was calculated according to Equation (2):

$$\text{N taken up by biomass} = (TNbiomass \times DB)/\, Ninitial\,\% \qquad [2]$$

in which $TN_{initial}$ (mg L^{-1}) is the nitrogen concentration at the beginning, TN_{final} (mg L^{-1}) is the nitrogen concentration at the end of the experiment, $TN_{biomass}$ (mg kg^{-1}) is the concentration of TN in the biomass and DB (kg L^{-1}) is the dry biomass produced per L of growth medium.

The Phosphorus (P) taken up by biomass was calculated according to Equation (3) (Silva-Benavides and Torzillo, 2012):

$$\text{P taken up by biomass} = (Pinitial - Pfinal)/Pinitial\,\% \qquad [3]$$

in which $P_{initial}$ (mg L^{-1}) is the phosphorus concentration at the beginning and P_{final} (mg L^{-1}) is the phosphorus concentration at the end of the experiment.

2.5 Biochemical analysis

The total N concentration ($TN_{biomass}$, g kg^{-1} DM) was detected on biomass samples of about 0.2-0.3 g, using an elementary analyzer (Elementar Rapid max N exceed), based on the analytical method of combustion "Dumas" and equipped with a thermal conductivity detector (TCD). The crude protein contents were estimated by multiplying the total N by the conversion factor 6.25 (Mišurcová et al., 2014). Carbohydrates were estimated by subtracting the percentage of ashes, lipids and crude proteins out of 100% (Breuer et al., 2012). The ash content was determined as the residue after ignition at 550 °C overnight (Mohammady et al., 2015).

The amino acid (AA) content of algal biomass was determined by the HPLC-DAD technique, according to Salati et al., (2017), with some modifications (Mišurcová et al., 2014). Acidic hydrolysis of samples was used for the determination of lysine (Lys), histidine (His), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), valine (Val), threonine (Thr), arginine (Arg), alanine (Ala), glycine (Gly), proline (Pro), glutamic acid (Glu), serine (Ser), aspartic acid (Asp) and tyrosine (Tyr). About 0.1-0.2 g of freeze-dried samples were hydrolyzed in 10 mL of 6 Mol L^{-1} HCl for 24 h in water bath at 100 °C, followed by neutralization with NaOH. For the determination of sulfur amino acids (Met

and Cys), the samples were pre-treated with 1 mL of a mixture of 30% (v/v) hydrogen peroxide and 98% (v/v) formic acid (in the ratio of 1:9 v/v) and were subsequently hydrolyzed in the way described above. For tryptophan (Trp) determination an alkaline hydrolysis was performed: about 0.1-0.2 g of freeze-dried sample was hydrolyzed with 10 mL of NaOH 4.2 Mol L^{-1} for 16 h under N_2 flux and neutralized with HCl. The HPLC analyses (Agilent 1100 Series HPLC) were performed by automated online pre-column derivatization using an automated liquid sampler and Poroshell 120 column HPH-C18 (3.0 100 mm, 2.7 lm. P/N 695975–502). The standard preparation, derivatization process, LC method used was performed according to Agilent Pub. #5990-4547EN (Pub No. 5990-4547EN, October 8, 2009, Agilent Technologies). The primary amino acids (OPA-derivatized) were monitored at 338 nm. The secondary amino acids (FMOC-derivatized) were monitored at 262 nm. For methionine (Met) and cysteine (Cys) detection, DTDPA (3,3-dithiodipropionic acid) was used as derivatizing agent instead of FMOC. The separation was carried out under gradient elution with two mobile phases. Phase A: 10 mM NaH_2PO_4 + 10 mM $Na_2B_4O_7$ + 5 mM NaN_3, pH 8.2 adjusted with HCl 5 M, and Phase B: ACN:MeOH:water (45:45:10, v/v/v). The flow rate was 1.00 mL min^{-1}, the column temperature 40 °C and injection volume 20 µl.

Total lipids were determined using a slightly modified version of the Bligh and Dyer (1959) method. An aliquot of lyophilized biomass was mixed with 600 µL of chloroform: methanol (2:1 v/v), after well mixing 200 µL of methanol and 333 µL of deionized water were added. The mixtures were then transferred into a separator funnel and shaken for 5 min. The lipid fraction was then collected from the separator funnel and gravimetrically determined after evaporation over one night. For fatty acid compositional analysis, the microalgae oils were esterified as suggested by the Sigma Aldrich Fatty Acid Methyl Ester Preparation Protocol and used for GC-MS analysis. Chromatographic analysis was performed using an Agilent 5975C Series GC/MSD and FID as detector. Volatiles were separated using a polar capillary column Zebron ZB-FAME (Zebron, Phenomenex, USA) of 30 m x 0.25 mm (ID) and a film thickness of 0.20 µm. Injection volume was 1 µL with a split ratio of 20:1. Carrier gas was

helium at a flow rate of 1 ml min^{-1}. The temperature program was isothermal for 2 min at 100 °C, then the temperature was raised at a rate of 3 °C/min to 240 °C and kept at 240 °C for 5 min. Injection temperature was 250 °C and the transfer line to the mass spectrometer was maintained at 285 °C. The mass spectra were obtained by electronic impact at 70 eV, a multiplier voltage of 1294 V and collecting data at an m/z range of 45–300. Compounds were identified and quantified by comparing their mass spectra and retention times (RT) with those from the standards contained in the Supelco 37 Component FAME Mix provided by Supelco, Sigma Aldrich. Heptadecane was used as internal standard. The crude proteins of algae samples were determined by quantifying the Kjeldahl nitrogen, and then calculated using a nitrogen conversion factor of 4.68 as previously reported by Templeton and Laurens (2015).

2.6 Data analysis

Data were processed by one-way ANOVA (analysis of variance), the Tukey's HSD multiple comparison tests ($P < 0.05$) to compare means, and Multivariate analyses, i.e., principal component analysis (PCA), using XLSTAT version 2016.02.28451.

3. Results and discussion

3.1 Characterization of nano-filtered permeate (NFP)

NFP represented the result of nanofiltration of twice S/L separated pig slurry (see Section 2.1) that under real conditions (operating full scale plant) is then subjected to a reverse osmosis step, producing a concentrated ammonia solution to be used in agriculture as fertilizer, and clean water discharged directly to surface-water bodies (see section 2.1). Instead, in this paper, NFP was directly used as substrate to grow algae in substitution of a commercial substrate (BG-11) achieving, in parallel, the partial removal of nutrient (NH_4-N) still contained in the NFP. NFP appeared suitable for microalgae growth (Table 1) because it contained 136 ± 0 mg L^{-1} of TN, 97% of which was in the ammonium (132

± 2 mg L^{-1}) form (Table 1), readily available to algae (Salbitani and Carfagna, 2021) and at a concentration avoiding any toxicity (Collos and Harrison, 2014). Additionally, the multistep separation carried out resulted in low turbidity of the NFP, which was optimal for algal growth (Table 1). Lastly, NFP contained many other useful nutrients, of which the concentrations were not far from those reported for BG-11 (Table 1), except for the low TP content (0.5 ± 0 mg L^{-1}) that potentially can cause low algae growth capacity (Richmond, 2008). Thus, P was extra-added, giving a final content similar to that of the BG-11 substrate.

Table 1. NFP chemical characterization vs. BG-11 nutrient solution

		NFP	BG-11
pH		8.5 ± 0	7.4
TN	mg L^{-1}	136 ± 0	247
NH_4-N	mg L^{-1}	132 ± 2	19
COD	mg L^{-1} O^2	77 ± 4	-
P	mg L^{-1}	7.61[a]	7.11
Na	mg L^{-1}	249 ± 3	414
Mg	mg L^{-1}	5.4 ± 0.1	7.39
K	mg L^{-1}	188 ± 8	17.95
Ca	mg L^{-1}	9.7 ± 0.2	9.81
Fe	mg L^{-1}	1.42[b]	1.42
B	mg L^{-1}	0.5 ± 0.1	0.50
Al	mg L^{-1}	0.6 ± 0.0	n.p.[e]
Cr	ug L^{-1}	4.7 ± 0.6	n.p.
Co	ug L^{-1}	4.8 ± 1.2	10
Cu	ug L^{-1}	30.7 ± 0.4	30
Zn	ug L^{-1}	57 ± 16	50
Se	ug L^{-1}	5.2 ± 0.4	n.p.
Mo	ug L^{-1}	19 ± 5	150
Cd	ug L^{-1}	7[c]	n.p.
Pb	ug L^{-1}	6.5 ± 2	n.p.
As	ug L^{-1}	u.d.l[d]	n.p.
Mn	ug L^{-1}	u.d.l	500
Ni	ug L^{-1}	u.d.l	n.p.

[a] P content in NFP was of 0.5 ± 0, p was added getting final content of 7.61 mg L^{-1}
[b] Fe content in NFP was of 0, Fe was added getting final content of 1.42 mg L^{-1}
[c] the other replicates are under detection level
[d] u.d.l refers to under detection level
[e] not present

3.2 Microalgae consortia growth

The algae consortia (AC), previously characterized for the presence of both eukaryotes and prokaryotes (Su et al., 2021) (Table 2), can be classified as follows: a *Chlorella*-dominated consortium (AC_1), a *Tetradesmus*-dominated consortium (AC_6), *Chlorella* & *Synechocystis* (Cyanobacteria) co-dominated consortia (AC_2, AC_3, AC_4, AC_5, AC_9 and AC_11), *Tetradesmus* & *Synechocystis* co-dominated consortia (AC_7 and AC_10), a *Chlorella* & *Tetradesmus* co-dominated consortium (AC_12) and, finally, a low algae presence consortium (AC_8).

The specific growth rates (μ) of the twelve ACs were in the range 0.18 ± 0.01 (AC_11) - 0.58 ± 0.06 d^{-1} (AC_12) (Table 2), with an average of 0.34 ± 0.14 d^{-1} (n=12): these values are in line with those reported for autotrophic microalgae, i.e. 0.2-0.7 d^{-1} (Kim et al., 2013). In particular, growth rates for *Chlorella* sp. and *Tetradesmus* sp. were reported, on average, as 0.45 ± 0.19 d^{-1} (n =11) (Arbib et al., 2014; Ledda et al., 2015; Liu et al., 2011; Ruiz-Martinez et al., 2012; Tam and Wong, 1996; Tejido-Nuñez et al., 2019) and 0.27 ± 0.11 d^{-1} (n = 10) (Gonçalves et al., 2019; Massa et al., 2017; Nadzir et al., 2018; Rajabi Islami and Assareh, 2019; Tang et al., 2018; Tejido-Nuñez et al., 2019), respectively. Taking into consideration the specific growth rates reported in Table 2 and the results of Tukey's HSD test, the ACs can be classified as follows: AC_1 = AC_10 = AC_12 > AC_2 = AC_3 = AC_4 = AC_5 =AC_6 = AC_7 = AC_8 = AC_9 = AC_11.

The AC_12, which was co-dominated by *Chlorella* (39.6%) and *Tetradesmus* (32.6%), probably gained significant competitive advantage regarding growth performance, reaching the highest growth rate (0.58 ± 0.03 d^{-1}), indicating that the co-culture of *Chlorella* and *Tetradesmus* promoted biomass growth. Algae co-culture has been reported to benefit the culture performance due to the ability of different species to utilize nutrients (Ray et al., 2021) and light during growth (Silaban et al., 2014). However, AC_1 and AC_10, which showed similar growth rates to AC_12 (Table 2) did not show the co-dominance of eukaryotic algae. In these cases, it may be that the prokaryotic community provided a positive effect on algae growth (Acien Fernandez et al. 2018; Lian et al., 2018). For example,

Synechocystis co-cultivated in consortium with *Chlorella* sp. could have promoted cell growth (Pachacama et al., 2016) explaining good growth performance of AC_10.

In conclusion, AC_1, AC_10 and AC_12 were the best performing ACs, representing good candidates for biomass production using NH_4-rich wastewaters. Since these ACs were isolated directly from organic wastes (Su et al., 2021), they represent well adapted algae-microbial consortia able to survive in substrates rich in nutrients and under adverse conditions. Moreover the co-cultivation of multiple microalgae species and/or bacteria provides not only the advantage of promoting and benefitting the growth of each other, but it also reduces the frequency and extent of culture crashes caused by viral, bacterial or fungal infections or predation by protozoa, especially in outdoor conditions (Lam et al., 2018).

Table 2. Algae consortia main genus composition and growing performance

	Eukaryotes		Prokaryotes		μ
	Algae genus %[a]	Main eukaryotic genus %[a]	Algae genus %[b]	Main prokaryotic genus %[b]	d^{-1}
AC_1	Chlorella 99.1%	n.f.[c]	n.f.	*Paludisphaera* (Planctomycetota) 36%	0.55 ± 0.04a[e]
AC_2	*Chlorella* 8.4%	*Nuclearia* 40.6%; *Vahlkampfia* 30.7%; *Colpoda* 15.6%	*Synechocystis* 35.9%	*Truepera* (Deinococcata) 21%	0.22 ± 0.03b
AC_3	*Chlorella* 85%	-	*Synechocystis* 19.6%	*SM1A02* (Planctomycetota) 36.8%	0.25 ± 0.04b
AC_4	*Chlorella* 76.4%	*Colpoda* 10.3%	*Synechocystis* 27.9%	*SM1A02* (Planctomycetota) 34.5%	0.31 ± 0.12b
AC_5	*Chlorella* 30.6%	*Colpoda* 36.1%; *Nuclearia* 17.7%	*Synechocystis* 84.8%	n.f.	0.29 ± 0.04b
AC_6	*Tetradesmus* 85.4%	*Colpoda* 9%	n.f.	Others[d] 61%	0.31 ± 0.02b
AC_7	*Tetradesmus* 42.6%	*Colpoda* 34.8%	*Synechocystis* 21.4%	*Chloronema* (Chloroflexi) 22.9%	0.24 ± 0.02b
AC_8	*Scenedesmus* 8.1%; *Chlorella* 6.3%	*Colpoda* 69.3%	n.f.	*SM1A02* (Planctomycetota) 42.5%	0.28 ± 0.08b
AC_9	*Chlorella* 82.3%	*Vermamoeba* 11.9%	*Synechocystis* 35.4%	*SM1A02* (Planctomycetota) 34.3%	0.31 ± 0.02b
AC_10	*Tetradesmus* 98.4%	n.f.	*Synechocystis* 54.2%	n.f.	0.52 ± 0.06a
AC_11	*Chlorella* 34.5%	*Cyclidium* 34.1%	*Synechocystis* 9.2%	*SM1A02* (Planctomycetota) 57.7%	0.18 ± 0.01b
AC_12	*Chlorella* 39.6%; *Tetradesmus* 32.6%	*Vermamoeba* 9.4%	*Synechocystis* 3.6%	*Sandaracinus* (Proteobacteria) 29.8%; Others[c] 52.5%	0.58 ± 0.06a

[a] Genus composition in microalgae consortium eukaryotic community
[b] Genus composition in microalgae consortium prokaryotic community
[d] not found
[c] Others refers to undetectable composition in prokaryotic community
[e] Means followed in the same column by the same lower-case letter are not statistically different ($p < 0.05$) according to Tukey test.

3.3 Nutrient mass balance

This work aimed at testing the growth ability of algae-microbial consortia isolated from organic wastes when a pig slurry-derived wastewater (NFP) was used as substrate, having a double purpose: i. recovering nutrients and substituting for synthetic substrates (e.g., BG-11) and ii. removing nutrients, thus cleaning the waste-derived streams. In the previous section, results demonstrated that ACs were able to grow on NFP, indicating that it was a suitable growth medium. In this section, the ability of ACs to remove nutrients from NFP is reported and discussed, considering that not only nutrient removal is important to clean NFP but that also nutrient uptake is important for algae growth and biomass composition, as will be discussed in the following sections.

Nutrients availability plays an important role in governing algae-microbial consortia growth and composition (Brennan and Owende, 2010; Ledda et al., 2016). This point is very important in the case of nutrient recovery from waste streams. Nitrogen is crucial for the growth of microorganisms (Li et al., 2014) and it is one of the main constituents of vital macromolecules, such as amino acids and proteins, pigments (chlorophyll, phycocyanin etc.), DNA, RNA, etc.(Markou et al., 2015).

The AC cultures showed a notable ability to assimilate nitrogen (Table 3). On average, 61 ± 15 % (n=12) of initial N ($TN_{initial}$) was taken up by algal biomass. Furthermore, the three fastest growing cultures, i.e. AC_1, AC_10 and AC_12, displayed N uptakes of 54 ± 0 %, 62 ± 0 % and 53 ± 11 % $TN_{initial}$, respectively, along with an average of 56 ± 4 % $TN_{initial}$ (n=3). These values were higher than those previously reported for microbial communities growing on wastewater, i.e. 44 ± 6 % $TN_{initial}$ (n=6) (Delgadillo-Mirquez et al., 2016; Prathima Devi et al., 2012; Su et al., 2011) and those reported for pure *Chlorella*, i.e. 39.7 ± 12.7 % $TN_{initial}$ (n=8) (Ledda et al., 2015; Markou, 2015).

The culture of consortia would affect the metabolism of the algae, resulting in different behaviours in nutrient removal (Chen et al., 2017) and explaining the variability registered (Table 3).

Table 3. Nitrogen and phosphorus mass balance, and biochemical compositions

AC	$TN_{initial}$[a]	TN_{final}[b]	$N_{biomass}$[c]	N taken up by biomass	$P_{initial}$[d]	P_{final}[e]	P taken up by biomass	Proteins	Lipids	Carbohydrates
	mg L^{-1}	mg L^{-1}	g kg^{-1} DM[f]	% $TN_{initial}$[a]	mg L^{-1}	mg L^{-1}	% $P_{initial}$[d]	g kg^{-1} DM	g kg^{-1} DM	g kg^{-1} DM
AC_1	136 ± 0	35 ± 6	41 ± 0	54 ± 0	7.61 ± 0	0.49 ± 0.01	94 ± 0	257 ± 0g[g]	119 ± 1fg	596 ± 4
AC_2	136 ± 0	23 ± 1	74 ± 0.6	65 ± 1	7.61 ± 0	1.13 ± 0.16	85 ± 2	460 ± 4b	105 ± 7g	405 ± 9
AC_3	136 ± 0	12 ± 3	90 ± 0.2	59 ± 0	7.61 ± 0	0.72 ± 0.37	91 ±5	561 ± 1a	152 ± 10cde	254 ± 11
AC_4	136 ± 0	20 ± 4	68 ± 0.4	78 ± 0	7.61 ± 0	0.40 ± 0.14	95 ± 2	422 ± 3c	177 ± 3b	359 ± 6
AC_5	136 ± 0	45 ± 13	43 ± 0.1	76 ± 0	7.61 ± 0	0.47 ± 0.08	94 ± 1	266 ± 0g	153 ± 9bcde	565 ± 9
AC_6	136 ± 0	29 ± 4	49 ± 0.7	65 ± 1	7.61 ± 0	0.45 ± 0.17	94 ± 2	305 ± 5f	173 ± 11bc	486 ± 12
AC_7	136 ± 0	3.4 ± 0.5	53 ± 0.7	46 ± 0	7.61 ± 0	0.54 ± 0.03	93 ± 0	334 ± 4e	128 ± 8efg	512 ± 9
AC_8	136 ± 0	38 ± 2	67 ± 0.4	53 ± 0	7.61 ± 0	0.51 ± 0.11	93 ± 1	420 ± 2c	177 ± 9b	369 ± 10
AC_9	136 ± 0	35 ± 7	64 ± 0.3	87 ± 0	7.61 ± 0	0.36 ± 0.08	95 ± 1	398 ± 2cd	178 ± 8b	406 ± 8
AC_10	136 ± 0	31 ± 1	61 ± 0.1	62 ± 0	7.61 ± 0	0.43 ± 0.08	94 ± 1	382 ± 7d	156 ± 9bcd	425 ± 9
AC_11	136 ± 0	27 ± 5	55 ± 0.3	29 ± 0	7.61 ± 0	1.37 ± 0.37	82 ± 5	341 ± 2e	135 ± 4def	494 ± 5
AC_12	136 ± 0	21 ± 1	44 ± 0.6	53 ± 11	7.61 ± 0	0.46 ± 0.01	94 ± 0	273 ± 22g	230 ± 15a	472 ± 27

[a]Initial TN concentration of culture medium at the start of the experiments.
[b]TN concentration of culture medium in the end of the experiments.
[c]TN concentration in AC biomass.
[d]Initial P concentration of culture medium at the start of the experiments.
[e]P concentration of culture medium in the end of the experiments.
[f]DM refers to dry matter.
[g]Means followed in the same column by the same lower-case letter are not statistically different ($p < 0.05$) according to Tukey test.

Phosphorus, although it makes up less than 1% of the biomass, is essential for algal growth, given that it is involved in many cellular processes (Morales-Amiral et al., 2015). The removal of inorganic phosphorus in algal culture results from two phenomena: biological assimilation and chemical precipitation as insoluble phosphate (Vonshak et al., 2002). Phosphorus content in algal biomass was not measured. However, since the pH of the cultures never reached over 8, i.e., the pH value at which the precipitation of phosphate occurs, it is conceivable that phosphates were mostly removed by active uptake rather than being precipitated as insoluble phosphate salts (Silva-Benavides and Torzillo, 2012). In this context, the P uptake measured for all ACs was very high, i.e. on average of 92 ± 4 % $P_{initial}$ (n=12), with results being higher than those of the range reported for *Chlorella* (63% - 75% $P_{initial}$) grown on anaerobic digestate of dairy manure (Wang et al., 2010), but comparable with data reported for algae growth on wastewater, i.e. 97% $P_{initial}$ (Markou, 2015).

3.4. Biochemical composition of AC biomasses

Microalgae gained interest because of their capacity to accumulate important amounts of useful biochemical molecules such as proteins, lipids, carbohydrates and pigments, compared to any other biomasses (Safi et al., 2013). Therefore, biochemical composition detection is important in characterizing ACs growing on NFP.

Average proteins content (Table 3) was, on average basis, of 368 ± 90 g kg^{-1} DM (n=12) and contents for the fastest growing cultures, i.e. AC_1, AC_10 and AC_12, were of 257 ± 0, 382 ± 7 and 273 ± 22 g kg^{-1} DM proteins, respectively.

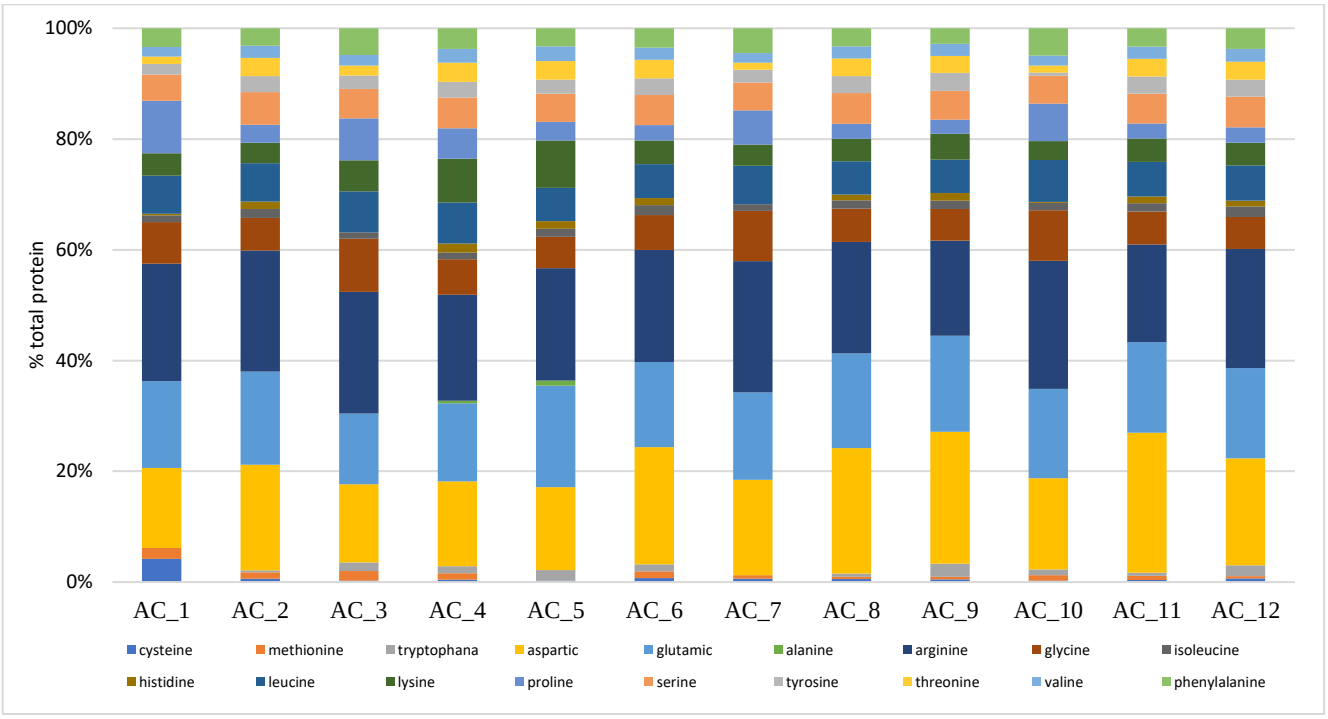
These contents were lower than those reported in the literature for *Chlorella* sp., i.e. 440 ± 129 g kg^{-1} DM (n = 7) (Ansari et al., 2017; Ajala and Alexander, 2020; Michelon et al., 2016; Nordin et al., 2019; Jiang et al., 2016) and *Tetradesmus* sp. i.e., 522 ± 173 g kg^{-1} DM (n = 2), both cultivated in different waste streams (Ajala and Alexander, 2020; Buono et al., 2016) containing more N than that of NFP (N of 136 ± 0 mg L^{-1}) used in this work, i.e. N of 594 ± 453 mg L^{-1} and of 314 ± 90 mg L^{-1}, respectively.

These figures can explain the lower protein content characterizing ACs. The fact that *Chlorella* cultivated on agricultural wastewater containing ammonia as N source (100-150 mg L^{-1}) similar to that of NFP, showed a protein content in line with those of ACs , i.e. 327 ± 150 g kg^{-1} DM (Gu et al., 2021; Markou, 2015; Zhu et al., 2019) seems to confirm that total N affected total protein content.

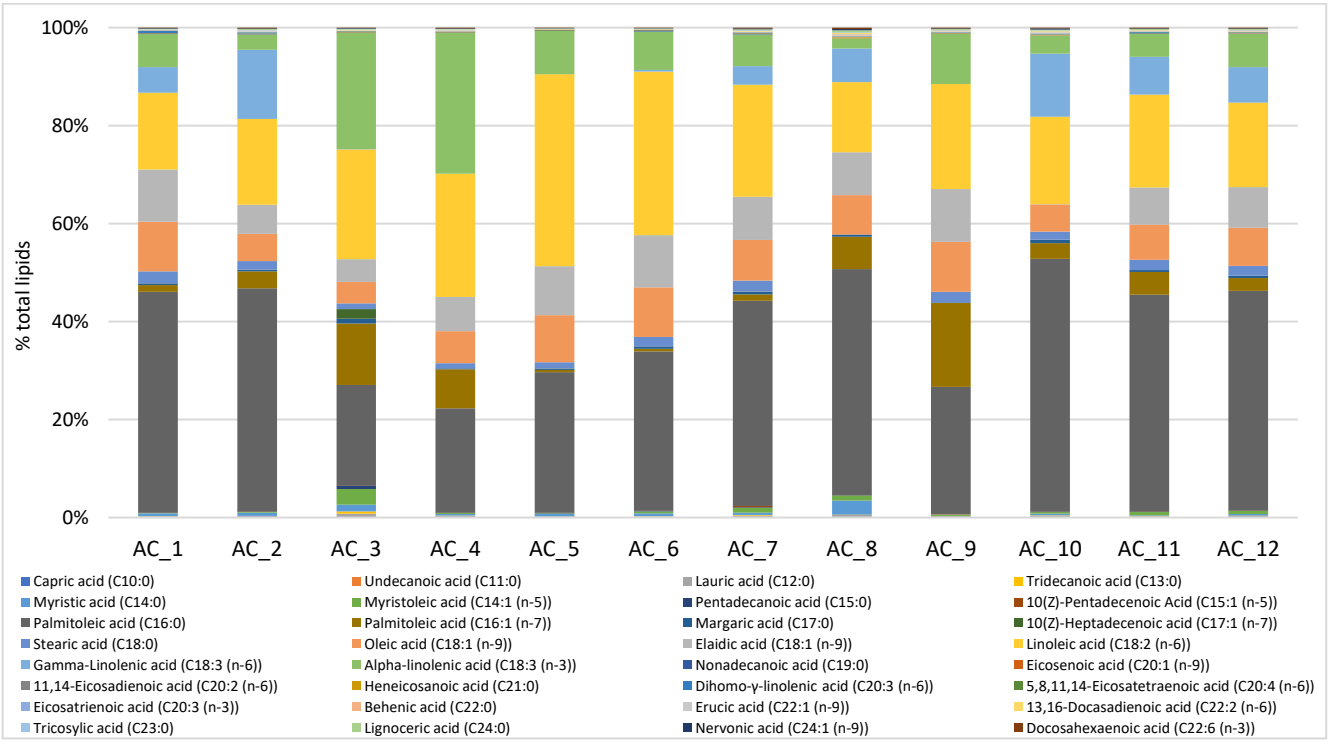
Lipids are one of the major constituents of microalgal biomass and typically their content was reported to be in the range 50 - 500 mg kg^{-1} DM depending on species and cultivation conditions (Breuer et al., 2012; Hu et al., 2008). Lipids content found in this work was, on average, of 145 ± 33 g kg^{-1}DM (n = 12), in line with data reported for *Chlorella* sp., i.e. 144 ± 98 g kg^{-1} DM (n = 9) (Ansari et al., 2017; Arif et al., 2021; Becker, 2007; Michelon et al., 2016; Nordin et al., 2019), and the data reported for *Tetradesmus* sp., i.e. 216 ± 47 g kg^{-1} DM (n = 4) (Arif et al., 2021; Massa et al., 2017; Nordin et al., 2019) (no significant differences - ANOVA, $P< 0.05$). The lipid content was, on average, higher than that reported for cyanobacteria i.e. 99 ± 56 g kg^{-1} DM (Becker, 2007), indicating that probably mixed culture (e.g. AC_10) led to lower lipid content than monospecies culture (Table 3). It is worth mentioning that between the three fastest growing ACs, the AC_12 achieved relatively high lipids contents, i.e., 230 ± 15 g kg^{-1} DM, i.e. probably the algae co-culture was positive for lipid content (Table 2 and 3).

Carbohydrate content in algal cells ranged from 40 to 640 g kg^{-1} DM depending on different algae species and cultivation conditions (Lage et al., 2018). In this work carbohydrates content in ACs presented an average of 445 ± 95 g kg^{-1} DM (n=12) (Table 3), much higher than data reported for *Chlorella* sp., i.e. 270 ± 84 g kg^{-1}DM (n = 9) (Ansari et al., 2017; Arif et al., 2021; Becker, 2007; Michelon et al., 2016; Nordin et al., 2019) and *Tetradesmus* sp., i.e. 244 ± 37 g kg^{-1} DM (n =4) (Arif et al., 2021; Massa et al., 2017; Nordin et al., 2019) (ANOVA, $p <0.05$). The three fastest growing ACs, namely AC_1, AC_10 and AC_12, revealed carbohydrates contents of 596 ± 4, 425 ± 9 and 472 ± 27 g kg^{-1} DM, respectively.

a



b



c

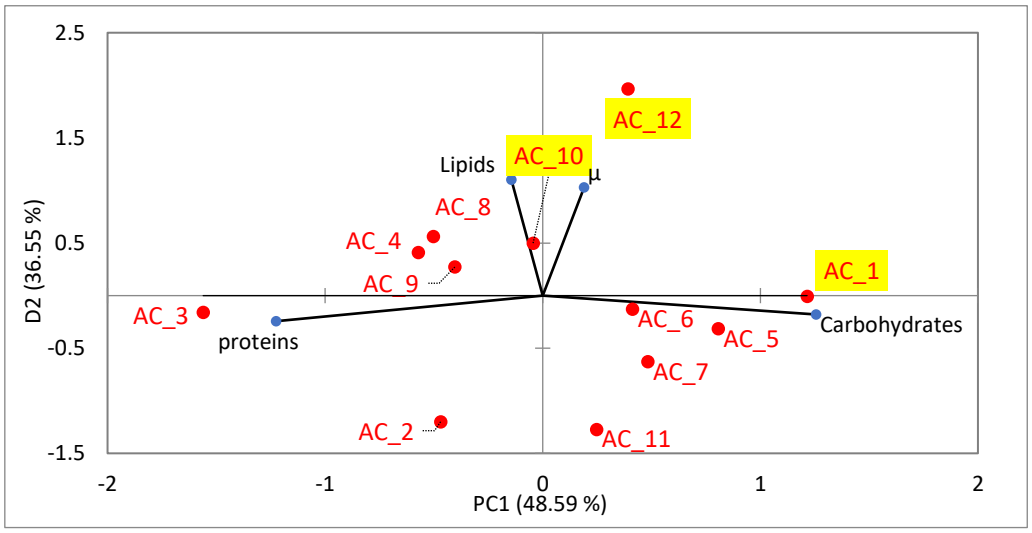
		AC_1	AC_2	AC_3	AC_4	AC_5	AC_6	AC_7	AC_8	AC_9	AC_10	AC_11	AC_12
SFAs	% total lipids	49.2	49.2	26.3	23.7	31.3	36.4	46.4	50.6	29.1	55.4	47.8	48.7
MUFAs	% total lipids	22.4	15.5	27.0	22.0	20.5	21.8	20.0	24.9	38.8	9.6	20.3	19.6
PUFAs	% total lipids	28.4	35.4	46.7	54.4	48.2	41.8	33.7	24.1	32.3	35.2	32.0	31.8

Figure 1. Amino acid speciation (a), fatty acid speciation (b) and summary of fatty acid compositions (c)

The data discussed above and reported in Figure 1 indicate a great variability among ACs for their biochemical composition, depending, above all, on ACs' characteristics since all the other variables affecting biochemical composition would be reduced by the similar growing conditions adopted throughout the experiments (Fleurence et al., 2018; Marsham et al., 2007). Multivariate analysis (Principal component analysis - PCA) was performed (Figure 2) to understand the relationship between AC growth rate and biochemical compositions (growth rate, proteins, lipids and carbohydrates contents). Two main factors (PCs) representing the 85.14 % of the total variance (48.59 % on PC1 and 36.55 % on PC2 of total variance) were found (Figure 2a). Generally, PC1 represented carbohydrates and protein contents, while PC2 was more related to lipids content and growth rate (μ). In particular, PCA suggested that to higher μ corresponded higher lipid contents, in agreement with Safi et al. (2014); while on the contrary, proteins and carbohydrate contents were opposed to each other (Dong et al., 2016). With reference to the best-performing ACs (AC_1, AC_10 and AC_12) it seemed that since their μ were similar, biochemical composition was the driver in differentiating ACs, i.e., AC_1 was characterized for the highest carbohydrate content, AC_10 for highest protein contents and AC_12 for highest lipid contents (Table 3).

In general, from the results discussed, it appears that ACs contain less protein and more carbohydrates than pure algae cultures. High levels of biomass carbohydrates contents have been reported as a result of the co-cultivation of algae with bacteria (González-González and De-Bashan, 2021). Choix et al (2012) demonstrated that the co-cultivation of two *Chlorella* strains (*C. vulgaris* and *C. sorokiniana*) with *A. brasilense* raised the positive effect of bacteria on carbohydrate production. In addition, the low N concentration in NFP compared to the N content of synthetic substrates or other wastewaters could explain the low protein content. Generally, algae grown on N-rich wastewater can produce larger quantities of extracellular proteins due to a higher N uptake leading to high protein content (Wang et al., 2014).

a



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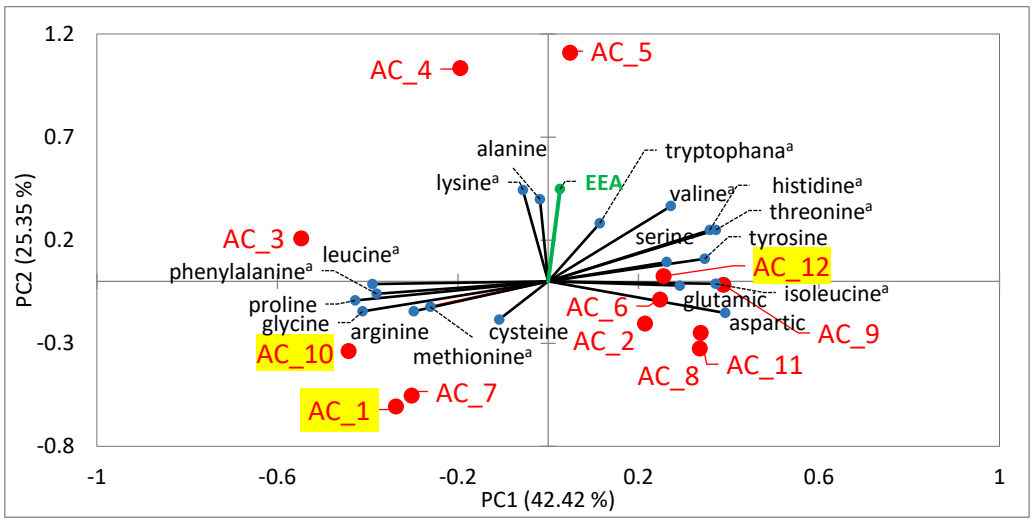

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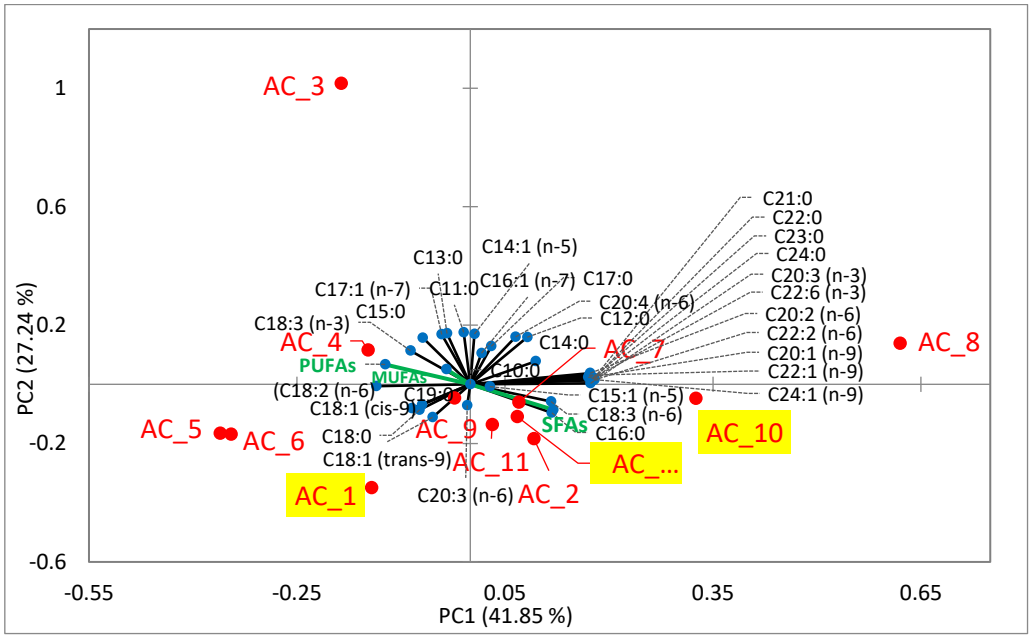

Figure 2. Principal component plots for microalgae-microbial consortia (AC) vs. specific growth rate and biochemical compositions (a), vs. amino acids (b) and vs. fatty acids (c)

3.5 Amino acids (AA) and fatty acids (FA) speciation

3.5.1 Amino acids (AA)

Eighteen amino acids (AA) were identified by analyzing all the biomasses produced (Figure 1a). The most abundant amino acids in all ACs were Arg, Asp and Glu followed by Leu, Gly, Ser, Lys, Pro, and Phe (Table S1). The essential amino acids (EAAs), which include His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val, were detected in all ACs' cultures. These data confirmed the high nutritional value of ACs (Fleurence et al., 2012).

The most abundant AAs in all ACs reported as average (n=12) values (g 100 g^{-1} crude protein) were as follows: non EAAS: Arg (20.7 ± 1.9) > Asp > (18.6 ± 3.7) > Glu (16 ± 1.4) > Gly (6.9 ± 1.4) = Ser (5.3 ± 0.3) = Pro (4.6 ± 2.3), and EAAS: Leu (6.7 ± 0.6) = Lys (4.9 ± 1.6) = Phe (3.7 ± 0.7) as EAAs. These data were mostly in line with the data reported for *Chlorella* sp. (g 100 g^{-1} crude protein) (n = 5), i.e. Arg (7.2 ± 0.9), Asp (9.7 ± 0.6), Glu (11 ± 1.5), Gly (6.3 ± 0.9), Ser (5.5 ± 1.3), Pro (4.6 ± 1.2), Cys (0.9 ± 0.7), Tyr (4.7 ± 1.7), Ala (8.6 ± 0.8), as NEAAs; Leu (8.2 ± 1), Lys (7.1 ± 1), Phe (5.2 ± 0.5), Met (1.4 ± 0.5), Trp (1.8 ± 0.9), Ile (3.6 ± 1.7), His (2.5 ± 1.3), Thr (5.2 ± 0.3) and Val (5.5 ± 1.3) as EAAs (Becker, 2007; Gorgônio et al., 2013; Maruyama et al., 1997; Safi et al., 2013; Shalaby, 2017). The higher Arg, Asp and Glu contents found in this work might be the reason leading to lower EAAs content, i.e. 24.3 ± 2.8 g 100 g^{-1} crude protein: this value is comparable with average contents of EAAs in *Chlorella* sp. of 36.2 ± 3.7 g 100 g^{-1} crude protein (n = 5). Lower EAAs content for ACs was probably due to low N content of the substrate (NFP) leading to low protein content and as consequence to lower EAAs content: this latter might be affected negatively because the synthesis of EAAs substantially exhibited slower accumulation compared to that of nonessential AAs (Lu et al., 2019). In addition it has been reported for algae that when the N source is mainly represented by ammonium, algal cells assimilate quickly the NH_4^+ avoiding toxicity, leading to net synthesis of Glu (Grosse et al., 2019; Hellebust and Ahmad, 1989; Wu et al., 2016). Arginine, as well, has been reported to be preferentially produced by algae when ammonium represents the N source (El-Sheekh et al., 2003).

Knowledge of AA composition (with particular reference to EAAs) has a great importance in establishing the nutritional value of algae-microbial consortia (Mišurcová et al., 2014). Thus, in order to evaluate AA speciation vs. ACs, PCA was performed (Figure 2b). Two main factors (PCs), which accounted for 67.77 % of the total variance, i.e., PC1 42.42 % and PC2 25.35 % of the total variance, were determined (Figure 2b). Generally, most ACs were located in the lower part of PC2, indicating low EAAs amount content except for AC_4 and AC_5. Notably, AC_5, with most abundant EAAs (30.3 g 100 g^{-1} of crude protein) showed, also, a quite high protein content (422 ± 3 g kg^{-1} DM) which makes this algae consortium of interest for further application, apart from its low growth rate (μ = 0.31 ± 0.12 d^{-1}).

With reference to the best performing ACs, it can be seen that AC_1 showed an AAs composition similar to that of AC_10, in particular for Pro, Gly and Met contents (Figure 2b) that were more abundant than those of AC_12. However, AC_12 showed significantly more abundant EAAs, namely His, Thr, Val and Ile. AC_12 displayed also the second most abundant Trp content, i.e., 50% more than that reported for AC_10, while Trp was not found in AC_1 (Figure 1a). Trp has often been found to be the limiting EAA, when assessing the nutritional value of algal proteins (Mišurcová et al., 2014), in most algae species (Dawczynski et al., 2007; Volkmann et al., 2008).

In any case, the three most performing ACs showed relatively low EAAs content, representing 20.9 (AC_1), 22.5 (AC_10) and 25.1 (AC_12) g 100 g^{-1} of crude protein, and even for AC_1 and AC_10 EAAs contents were lower than ACs average, confirming that higher growth limits protein content as well as EAA contents.

3.5.2 Fatty acids

In this work, thirty-two different fatty acids, grouped into saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) were identified in all ACs (Figure 1b). SFAs, PUFAs and MUFAs were, on average basis (n=12) of 41.2 ± 10.6 %, 37 ± 8.6 %

and 21.9 ± 6.6 % of total lipids content (Figure 1c). These figures are in line with data reported for *Chlorella* sp., i.e. SFAs of 38.1 ± 12.5 %, PUFAs of 39 ± 12.9 % and MUFAs of 22.8 ± 6.3 % of total lipids (*n*=12), respectively (Arif et al., 2021; Rohit and Venkata Mohan, 2018; Liu et al., 2011; Melo et al., 2017), as well as in agreement with the results reported for *Tetradesmus* sp., SFAs 45.2 ± 3.3 %, PUFAs 39 ± 15.3 % and MUFAs 10.5 ± 6.6 % of total lipids (*n*=4) (Arif et al., 2021; Han et al., 2019; Sharma et al., 2020).

Major SFAs were palmitic acid (C16:0) (37.4 ± 10.4 % of total lipids) > stearic acid (C18:0) (5.2 ± 4.9 % of total lipids), whereas linoleic acid (C18:2) (22.2 ± 7.1 % of total lipids) was dominant among the unsaturated fatty acids, followed by alpha-linolenic acid (C18:3) (9.4 ± 7.9 % of total lipids) > oleic acid (C18:1 *cis*-9) (7.8 ± 1.9 % of total lipids) = elaidic acid (C18:1 *trans*-9) (7.8 ± 3.0 % of total lipids) in average (*n*=12). These figures agreed with literature that reported C16:0 being the most abundant in microalgal consortia (Sharma et al., 2020) as well as in *Chlorella* sp. (Anto et al., 2019). Mahapatra et al. (2014) reported that the microalgae consortia collected from municipal wastewater contain major contributions from SFAs: C16:0 (42.3% of total lipids) > C18:0 (25.7% of total lipids), followed by unsaturated C18:1 (10.9 % of total lipids) > C18:2 (around 5% of total lipids). Comparing data of this work with the literature, it appears that less C18:0, but more unsaturated fatty acids such as C18:2 and C18:3 were found in ACs' biomass. Among the three best performing cultures (AC_1, AC_10 and AC_12), C16:0 was the most abundant unsaturated fatty acid, with an average content of 47.3 ± 3.2 % of total lipids (*n*=3), which was more similar to the value reported by Mahapatra et al. (2014) than the average content of all the ACs (n=12).

The difference of FA composition along with the compositions of SFAs, MUFAs, PUFAs are shown in Figure 3c. PC1 and PC2 explained 69.09 % of total variance, i.e., 41.85 % and 27.24 %, respectively. SFAs, MUFAs and PUFAs displayed significant differences between ACs. Higher SFAs led to less PUFAs and MUFAs. However, MUFAs did not show much variance between ACs and FAs as they located near the center. The major PUFAs detected in all cultures were C18 and C20, as well as C22

and C24 but in small amounts. In fact, C16 and C18 account for 96.2 ± 2.5 % of total lipids (n=12) were found in this work. Regarding the three best performing ACs, i.e. AC_1, AC_10 and AC_12, they showed a positive relation with C16:0 and C18:1 (Figure 2c), which is more interesting for the production of biofuel than for the nutritional values (Mathimani et al., 2018). In this latter case, ω6:ω3 (unsaturated fatty acids) ratio becomes important, being the optimum in the range 4:1-1:1 and ratios below 10:1 promoting pathogenesis of many diseases (Martins et al., 2013). AC_1 and AC_12 showed a ω6:ω3 ration of 3.1, and 3.6 which make them interesting from a nutritional point of view.

4. Conclusion

Twelve microalgae-microbial consortia isolated from organic wastes were tested for their ability to growth on nanofiltered swine manure (NFP). *Chlorella*-dominated consortia (AC_1), *Tetradesmus* & *Synechocystis* co-dominated consortia (AC_10) and *Chlorella* & *Tetradesmus* co-dominated consortia (AC_12) exhibited the highest specific growth rates. These ACs showed remarkable ability to take up nitrogen and phosphorus, indicating that NFP represents a good substrate for them, and that microalgae-microbial consortia isolated from organic wastes are well adapted to growing on this waste-derived substrate, producing useful biomass. Nutrient recovery and biomass production represent a real example of the circular economy to be implemented at local level by using adapted microbial populations.

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Chapter IV

Phaeodactylum *tricornutum* cultivation under mixotrophic conditions with glycerol supplied with ultrafiltered digestate: a simple biorefinery approach recovering C and N

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Phaeodactylum *tricornutum* cultivation under mixotrophic conditions with glycerol supplied with ultrafiltered digestate: a simple biorefinery approach recovering C and N

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Abstract

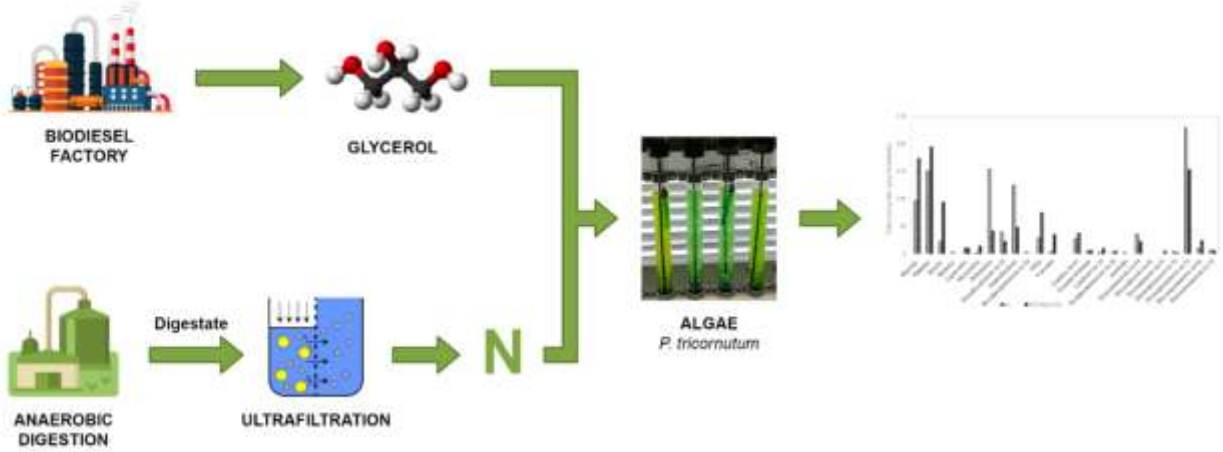
Phaeodactylum *tricornutum* was cultivated mixotrophically in batch mode providing glycerol as C source, i.e. 0.02, 0.03 and 0.04 Mole L^{-1} glycerol, and ultrafiltered digestate (UF) as N source. Biomass productivity, biomass composition, N efficiency use, and total energy balance were recorded and compared to autotrophic condition. Under mixotrophic conditions, biomass productivity of *P. tricornutum* increased by 1.29 and 1.60 times (0.03 Mole L^{-1} and 0.04 Mole L^{-1} glycerol) with respect to autotrophic condition.

Algae protein content declined as glycerol concentration increased in opposite to carbohydrate content. Lipid content did not change but unexpectedly, a lower unsaturated fatty acid in mixotrophic culture was observed than that of autotrophic culture. Mixotrophic conditions offered a higher energy recovery efficiency (EF_t) than autotrophic condition (5.7% in 0.04 Mole L^{-1} glycerol and 4.2% in autotrophic trial, respectively). Additionally, the efficiency of glycerol conversion into biomass (EF_{gly}) increased with glycerol dose getting 22.8% for 0.04 Mole L^{-1} glycerol.

Key words: Energy Balance; Glycerol; Mixotrophy; N recovery; Phaeodactylum *Tricornutum*

Abbreviation

Abbreviations	Word
Gly	Glycerol
UF	Ultrafiltered Digestate
C	Carbon
N	Nitroge
P	Phosphorus
TS	Total Solids
COD	Chemical Oxygen Demand
TOC	Total Organic Carbon
TKN	Total Kjeldahl Nitrogen
$N\text{-}NH_4^+$	Ammonia Nitrogen
BOD_5	Biochemical Oxygen Demand
Cb	biomass concentration
μ	growth rate
Dp	Daily biomass productivity
Nre	Nitrogen removal
No	Nitrogen concentration at the beginning
Nf	Nitrogen concentration at the end
Sr	Reactor surface exposed to light
h	Hours of light exposure
DW	Dry Weight
TN	Total nitrogen
TP	Total phosphorus
PUFAs	Polyunsaturated fatty acids
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
RT	Retention times
PAR	Photosynthetically active radiation
Iav	Average irradiance



Graphic Abstract

1. Introduction

Microalgae convert inorganic N and P, carbon dioxide, light and water into useful organic compounds through photosynthesis, producing especially protein, carbohydrates and lipids (D'Imporzano et al., 2018). Because of the high lipid content, algae represent promising feedstocks for biofuels production (Leite et al., 2013). Anyway, Delrue et al., (2012) identified three major bottlenecks of microalgae biodiesel production consisting in (i) lipid accumulation by microalgae, (ii) cultivation steps and (iii) downstream processes. These problems were confirmed by Chisti et al., (2013) which suggested producing biofuels from microalgae in an economically sustainable way only for certain niche fuels such as jet fuel.

On the other hand, algal lipids also have high nutritional value, containing fatty acids ranging from C12 to C24, often with monounsaturated fatty acids and polyunsaturated fatty acids (C16 and C18 fatty acids such as docosahexaenoic acid, eicosapentaenoic acid, arachidonic acid and so on) and storage lipids, e.g., triacylglycerols (Calinoiu et al., 2014).

P. tricornutum has been included among potential candidates for biodiesel production (Silaban et al., 2014) due to its high lipid content. Nevertheless, this algal species has also been reported to be a potential source of polyunsaturated fatty acids (PUFAs), mainly eicosapentaenoic acid (EPA) since this represents up to 40% of the total fatty acids (Hamilton et al., 2016; Rebolloso Fuentes et al., 2000). Furthermore, *P. tricornutum* represents a source of carotenoids (Silaban et al., 2014) for human consumption.

Currently, photoautotrophic cultivation is the main modality for microalgae production, unless light availability limits biomass production. Because the excess CO_2 stimulated photosynthesis but blocked the metabolization of organic substrate (Sforza et al., 2012) a right amount of CO_2 supply must be provided, representing about 10-20% of the total algae production costs (Bhatnagar et al., 2011). Culturing algae heterotrophically by using organic substrates (e.g., agroindustry by-products) as the C source contributes towards achieving higher biomass production (Wu et al., 2012) than autotrophy,

while also reducing the costs. The combination of autotrophy with heterotrophy, i.e. mixotrophy, represents an innovative methodology (D'Imporzano et al., 2018) which is able to take advantage of both the two approaches mentioned above if secondary C sources are available, i.e., organic C sources (e.g. wastes and by-products) and CO_2 (e.g. CO_2 from anaerobic digestion or industrial processes). Moreover, taking into consideration that algae production requires a huge amount of both water and nitrogen and that N and P fertilizers cost increased dramatically in recent years, the recovery of UF as nitrogen source represents a promising possibility to produce algal biomass reducing total cost （Veronesi et al., 2017).

P. tricornutum can grow in mixotrophic conditions in the presence of diverse organic compounds such as acetate, starch, glycine, glucose, fructose and glycerol (Wang et al., 2020; Penhaul Smith et al., 2020; Cer et al., 2000; Cerón-García et al., 2013; Cerón García et al., 2005; Wang et al., 2012; Liu et al., 2009; Morais et al., 2009), (Wang et al., 2020)producing high value marketable compounds such as proteins, essential amino acids, carotenoids and fatty acids with particular emphasis on ω3, ω6 (Rebolloso Fuentes et al., 2000). With respect to autotrophy and heterotrophy, mixotrophic growth combines the use of both organic and inorganic C source, and light as energy, to attain high biomass concentration lowering algae cost production (Perez-Garcia et al., 2011) and increasing the quality of biochemical components (Pahl et al., 2010).

Therefore the organic nutrient for potential use on a commercial scale for mixotrophic cultures has to be easily sterilisable, able to promote good growth favouring the synthesis of the desired product but, above all, it should be inexpensive (Cerón García et al., 2005).

Glycerol represent a by-product coming from biodiesel production (Rastegari and Ghaziaskar, 2015) and by now it is used for different purpose. For example pharmaceutical use (Yuan and Li, 2012), biogas production (Paulista et al., 2020) and chemical production (Lari et al., 2018) were reported. Glycerol has been proposed, also, producing microalgae for different purpose, i.e. biodiesel production (Pyle et al., 2008), biomolecules (Khanra et al., 2017) and chemicals such as Docosahexaenoic acid

(DHA) production (Yang et al., 2012), because it is a useful low-cost C and energy source, producing algae (Perez-Garcia et al., 2011). Therefore, using glycerol as by-products to produce new goods, i.e. biorefinery approach (Behera et al., 2019), represents a reality moving to circular economy approaches (Ubando et al., 2020).

It has been shown, also, that glycerol it is the most effective C sources for *P. tricornutum* promoting both mixotrophic growth and lipids production, resulting in the highest biomass and EPA productivities (Cerón García et al., 2005). Cerón García et al. (2013), investigated the mixotrophic growth of *P. tricornutum* by comparing fructose and glycerol as organic carbon source, found that glycerol promoted significantly higher biomass productivity than fructose. Hence, glycerol seems to be a serious candidate to be considered for promoting the mixotrophic growth of algae.

Nutrients represent a not-to-be neglected cost of producing algae biomass, i.e., 4-8% (depending on different microalgae cultivation modes) of the total cost including major equipment, power, labour costs and so forth (Acién Fernández et al., 2012; Norsker et al., 2019). Therefore, the use of recovered nutrients from secondary streams such as animal slurry/digestate is becoming a common methodology (Vaneeckhaute et al., 2017), combining the nutrient recovery with manure cleaning (Shi et al., 2018). According to Matassa et al. (2015), approximately 34% of total N entering the agricultural system is ultimately present in animal waste and municipal wastewater. Hemalatha et al. (2019) studied the potential and feasibility of microalgae in treating dairy wastewater for the generation of bio-based products in a biorefinery framework, showing that 90% of organic carbon removal with simultaneous removal of nutrients. Thus, nutrient recovery technologies have substantial implications for the sustainability of biorefineries while providing an alternative to current unsustainable approaches to fertilizer production.

Previous work (Veronesi et al., 2017; Abu Hajar et al., 2016) indicated a good outcome from the use of ultrafiltrate (UF) from digestate treatment, since this adds N in the form of ammonia to the culture medium. Ledda et al. (2016) introduced the use of UF in a bio-refinery frame, concluding that N

recovered was useful as secondary material in producing algae. Moreover, they demonstrated that the integration of anaerobic digestion and microalgae production, is technically feasible.

Although previous paper reported the use of glycerol as C source for microalgae (e.g.,Veronesi et al., 2015) production and the recovery on nutrients (N and P) by digestate pretreatment (e.g., Ledda et al., 2016), no data have been reported discussing the join effect in replacing both C and nutrient with secondary streams. In this way attesting the validity of such approach generating scientific data, can be useful for further studies on environmental and economic advantage coming from this biorefinery approach in producing algae biomass.

This work represents an attempt to assess the ability of the alga *P. tricornutum* to grow mixotrophycally using glycerol as the main C source and UF as an additional N source with an objective of a biorefinery approach for recovering C and N, in experiments which provided a comparison with the autotrophic condition, tested different C doses and gave insight into macromolecular comparisons of the products.

2. Materials and Methods

2.6 Microalgae strain

The microalga used in these trials was the diatom *P. tricornutum* strain SAG1090-1a, acquired from Sammlung von Algenkulturen, Pflanzenphysiologisches Institut (Universität Göttingen, Germany). Algae inoculum was maintained in 500 mL Erlenmeyer flasks in f/2 medium (Guillard, 1962; Robert, 1975) under constant aeration and mixing by using filtered air (filter of 0.2 µm) with a continuous illumination of 50 µE m^{-2} s^{-1}, provided by fluorescent white tubes, at a controlled temperature of 22 ± 1°C.

2.2. Culture medium

The culture medium used during batch trials was f/2. This is a widely used enriched seawater medium designed for growing coastal marine algae. The nutrient compounds in this recipe is showed in Table

S1. For mixotrophic cultivation, an aliquot of the carbon substrate (glycerol - Gly) was added to f/2 medium (see below). f/2 and f/2 C-enriched medium were autoclaved at 120 °C for 90 min before use. Earlier studies showed the glycerol concentration chosen in this work is reasonable and nontoxic (Cerón García et al., 2000; Morais et al., 2017).

Table S1. f/2 medium solution recipe

Component	Stock Solution ($g \cdot L^{-1}$ dH_2O)	Quantity Used	Concentration in Final Medium
$NaNO_3$	75	1 mL	8.82×10^{-4}
$NaH_2PO_4 \cdot H_2O$	5	1 mL	3.62×10^{-5}
$Na_2SiO_3 \cdot 9H_2O$	30	1 mL	1.06×10^{-4}
Trace metals solution	(See following recipe)	1 mL	/
Vitamins solution	(See following recipe)	0.5 mL	/
$FeCl_3 \cdot 6H_2O$	/	3.15 g	1.17×10^{-5}
$Na_2EDTA \cdot 2H_2O$	/	4.36 g	1.17×10^{-5}
$MnCl_2 \cdot 4H_2O$	180.0	1 mL	9.10×10^{-7}
$ZnSO_4 \cdot 7H_2O$	22.0	1 mL	7.65×10^{-8}
$CoCl_2 \cdot 6H_2O$	10.0	1 mL	4.20×10^{-8}
$CuSO_4 \cdot 5H_2O$	9.8	1 mL	3.93×10^{-8}
$Na_2MoO_4 \cdot 2H_2O$	6.3	1 mL	2.60×10^{-8}
Thiamine · HCl (vitamin B_1)	/	200 mg	2.96×10^{-7}
Biotin (vitamin H)	1.0	1 mL	2.05×10^{-9}
Cyanocobalamin (vitamin B_{12})	1.0	1 mL	3.69×10^{-10}

Ultrafiltered digestate (UF) was added to provide an additional N source to sustain the expected algal growth increase because of glycerol addition.

The ultrafiltered digestate was sampled from a biogas plant equipped with a full-scale digestate treatment unit (membrane-based treatment unit). The process is characterized by sequential integration of solid/liquid separations, ultrafiltration, reverse osmosis and cold ammonia stripping, as described by Ledda et al. (2013). Samples were immediately stored at 4 °C and characterized upon arrival. Glycerol was obtained from Merck (Merck KGaA, Darmstadt, Germany).

2.3 Microalgae growth culture conditions

2.3.1 Experimental design

Batch trials were carried out in six photobioreactors (PBRs) of 0.5 L working volume. The pH was set at 8 and it was maintained by using pure CO_2 injection adopting an "on-demand" modality (Travieso et al., 2006). Room temperature (25°C) and constant air flux (10 L min^{-1}) were provided as well as light that was delivered by cold fluorescent lamps at an irradiance of 312 µE m^{-2} s^{-1} at the PBR surface, adopting a 12h:12h photoperiod regime. The culture medium was f/2 and f/2 enriched medium with added ultrafiltered digestate (UF) and different concentrations of glycerol (Gly) to assess the effect of carbon addition and use of recovered fertilizers (UF).

Batch trials were performed in five conditions: i. trial 1 (Trial A), performed with standard f/2 medium in autotrophic conditions; ii. trial 2 (Trial A-UF), i.e., Trial A with added UF, this trial was performed to attest the effect of adding UF under autotrophic conditions, i.e., $N\text{-}NH_4^+$ under the autotrophic condition; iii) trials 3,4 and 5 (Trial UF-Gly 0.02, 0.03, 0.04), i.e., Trial A-UF with the added glycerol source at 0.02, 0.03 and 0.04 Mole L^{-1} of glycerol, respectively. Glycerol has been widely tested previously producing microalgae (Table 1), and concentration used were in the range 0.01-0.1 Mole L^{-1} of glycerol. Cerón García et al. (2005), indicated that glycerol concentrations higher than 0.1 Mole L^{-1} of glycerol were inhibitory, suggesting testing lower concentration.

The starter inoculum 10% (v/v) having 0.3 g L^{-1} of microalgae was placed into the reactors and culture medium was added to start the trials. Batch experiments were carried out in triplicate.

2.3.2 Monitoring cultures

Microalgae dry weight (DW) was determined by sampling 10 mL of algae suspension from each PBR. The samples were centrifuged at 2,683 x g for 10 min and then washed with an equivalent volume of distilled water to remove salts. Culture samples were then filtered through a 1.2-µm filter (GF/C,

Whatman Ltd., Maidstone, UK) and dried overnight at 80 °C. The presence of contaminants, mainly bacteria, was verified by centrifuging the supernatant after algae separation at centrifugal force of 24,149 x g for 20 min, and a bacterial pellet was then observed by light microscope at 100x Zeiss Axio Scope.A1 (Carl Zeiss Group, Oberkochen, Germany). Quantitative determination of bacterial biomass was not performed. Sampling was performed every 2 days.

2.4 Analytical methods

Total Nitrogen, ammonia nitrogen ($N\text{-}NH_4^+$) and chemical oxygen demand (COD) were determined using fresh material according to *standard methods* for wastewaters characterization (APHA, AWWA, WEF, 2005). All the other analytical evaluations were performed on freeze-dried biomass. Total Kjeldahl Nitrogen (TKN) was determined according to *standard methods* (APHA, AWWA, WEF, 2005). Total phosphorus (TP) was determined by inductively coupled plasma mass spectrometry (ICP-MS, Varian, Fort Collins, USA) according to the 3051A and 6020A EPA methods (EPA, 2007).

Total lipids were determined using a slightly modified version of the Bligh and Dyer (1959) method. An aliquot of freeze-dried sample mixed with 5 mL of chloroform: methanol (2:1 v/v) was ultra-sonicated (VWR USC300T, VWR International, Radnor, USA) for 15 min at 45 kHz, the mixtures were then transferred into a separator funnel and shaken for 5 min. The lipid fraction was then separated from the separator funnel and gravimetrically determined after solvent evaporation using a rotary evaporator (Büchi R110, Büchi Labortechnik AG., Flawil, Switzerland). Fatty acid profile quantification was performed by transesterification according to the method described by Rodríguez Ruiz et al. (1998) and quantification by GC-MS. Samples were esterified as suggested by the Sigma Aldrich Fatty Acid Methyl Ester Preparation Protocol. Chromatographic analysis was performed using an Agilent 5975C Series GC/MSD and FID as detector. Molecules were separated using a polar capillary column Zebron ZB-FAME (Zebron, Phenomenex, USA) of 30 m x 0.25 mm (ID) and a film thickness of 0.20 µm. Injection volume was 1 µl with a split ratio of 20:1. Carrier gas was helium

at a flow rate of 1 ml min^{-1}. The temperature program was isothermal for 2 mins at 100 °C, then the temperature was raised at a rate of 3 °C/min to 240 °C and kept at 240 °C for 5 min. Injection temperature was 250 °C and the transfer line to the mass spectrometer was maintained at 285 °C. The mass spectra were obtained by electronic impact at 70 eV, a multiplier voltage of 1294 V and collecting data at an m/z range of 45-300. Compounds were identified and quantified by comparing their mass spectra and retention times (RT) with those from the standards contained in the Supelco 37 Component FAME Mix provided by Supelco, Sigma Aldrich. Heptadecane was used as internal standard.

The crude proteins of algae samples were determined by quantifying the Kjeldahl nitrogen, and then calculated using a nitrogen conversion factor of 4.68 as previously reported by Templeton and Laurens (2015). The ash content was determined by the residue after ignition at 550 °C overnight.

Carbohydrates were estimated by subtracting the percentage of ashes, lipids and crude proteins out of 100% (Molina Grima et al., 1997; Wilkie and Mulbry, 2002). The specific growth rate μ (day^{-1}) was calculated from the Equation (1) (Vonshak and Tomaselli, 2006):

$$\mu = (\ln N_1 - \ln N_2)/(t_1 - t_2) \qquad [1]$$

where N_1 and N_2 are the concentrations of cells (g L^{-1}) at day t_1 and t_2.

Daily biomass productivity (*Dp* as mg L^{-1} d^{-1}) during the culture period was calculated by the Equation (2):

$$Dp = (N_1 - N_2)/(t_1 - t_2) \qquad [2]$$

Nitrogen removal (*Nre* %) was calculated according to Equation (3):

$$Nre = (No - Nf)/No\,\% \qquad [3]$$

in which *No* is the nitrogen concentration at the beginning and *Nf* is the nitrogen concentration at the end of the experiment.

C_b indicates the final concentration of biomass.

Light intensity as photosynthetically active radiation (PAR) was detected by a light meter with LI-190 quantum sensor (LI-COR, Lincoln, Nebraska, USA). Energy as PAR supplied to the reactor (E_1 as kJ) was calculated by Equation (4):

$$E_1 = PAR \times Sr \times h \quad [4]$$

in which PAR is Photosynthetically Active Radiation provided to the reactor, Sr is the reactor surface exposed to light and h are the hours of light exposure.

Energy provided as organic carbon and energy in the produced biomass (combustion enthalpy) was calculated on the base of biochemical composition according to Lehninger (1985).

All analysis parameters were carried out triplicate. The statistical significance of the results was evaluated by one-way ANOVA (analysis of variance) and Tukey's HSD multiple comparison tests ($P < 0.05$) using SPSS software (SPSS statistical software).

3. Results and Discussion

3.1 By-products characterization

The chemical features of the organic streams used for the mixotrophic trials in the experiment are shown in Table 2. Ultrafiltrate from digestate showed low carbon (C) content but it was rich in both N (under the ammonia form) and P. In addition, the BOD_5/COD ratio (Table 2) suggested that C contained was poorly degradable. Glycerol however was characterized for its high carbon content that was easily degradable (high BOD_5/COD) and low nutrient content.

Table 1. Comparison of mixotrophic performances of *P. tricornutum* cultivated using different carbon source according to literature and this work.

Carbon Source	Amount	Culture Condition	Highest Eicosapentaenoi Acid (EPA) productivity ($mg\ L^{-1}\ d^{-1}$)	Highest biomass concentration ($g\ L^{-1}$)	Reference
Glycerol	0.02-0.04 Mole L^{-1}	Batch	-	4.96	This work
Glucose	0.5-5 g L^{-1}	Batch	-	1.16	Wang et al, 2012
Starch				0.83	
Acetate				0.89	
Glucose	0.1 Mole L^{-1}	Fed Batch	18.3	3.4	Sevilla et al, 2004
Fructose	0.005-0.02 Mole L^{-1}	Batch	-	3.50	Cerón García et al, 2006
Glucose				2.20	
Mannose				1.05	
Lactose				0.77	
Glycerol				7.04	
Acetate	0.005-0.1 Mole L^{-1}	Batch	-	1.15	Cerón García et al, 2004
	0.5-5 g L^{-1}		4.98	1.79	
Starch	0.005-0.1 Mole L^{-1} 0.005-0.02 Mole L^{-1}		4.09	2.18	
Lactate					
Glycine			4.99	2.46	
Glucose	0.5-5 g L^{-1}		6.55	2.01	
Glycerol	0.005-0.1 Mole L^{-1}		8.56	2.99	
Fructose	0.02 Mole L^{-1}	Fed Batch	40	8.0	Cerón García et al, 2013
Glycerol	0.1 Mole L^{-1}			14.0	
Glycerol	0-0.1 Mole L^{-1}	Batch	33.5	2.4	Cerón García et al, 2000
Glycerol	0-0.1 Mole L^{-1}	Batch	-	1.3	Morais et al, 2016

Table 2. Chemical characteristics of substrates used for mixotrophic trials

	pH	TS	COD	TOC	TKN	$N\text{-}NH_4^+$	P tot	BOD_5	BOD_5/COD
		$g\ kg^{-1}$	$g\ O_2\ L^{-1}$	$g\ L^{-1}$	$mg\ L^{-1}$	$mg\ L^{-1}$	$mg\ L^{-1}$	$g\ O_2\ L^{-1}$	
UF[a]	8.4±0.2	3.1±0.2	1.49±0.00	0.56±0.00	1,376±33	1,155±49	28.7±0.8	0.54±0.03	0.36±0.02
Gly	6.8±0.1	990±72	1,680±154	737±1	udl[b]	udl	udl	1,307±360	0.78±0.22

[a]UF: Ultrafiltrate, Gly: Glycerol.

[b]udl: under detection limit.

3.2. Growth and yield

Results obtained indicated that there were no significant differences in the biomass concentration (C_b) at the end of the trials between Trials A, A-UF and UF-Gly 0.02. Trial UF-Gly 0.03 showed a small but statistically significant increase in the biomass production with respect to Trial A and A-UF (Figure 1, Table 3), while the addition to the culture of more glycerol (Trial UF-Gly 0.04) allowed the consolidation of this increase (Figure 1, Table 3) which was 1.6 times higher than that of control (Trial A), in agreement with previous findings reported in the literature (Cerón García et al., 2000; Morais et al., 2017; Villanova et al., 2017).

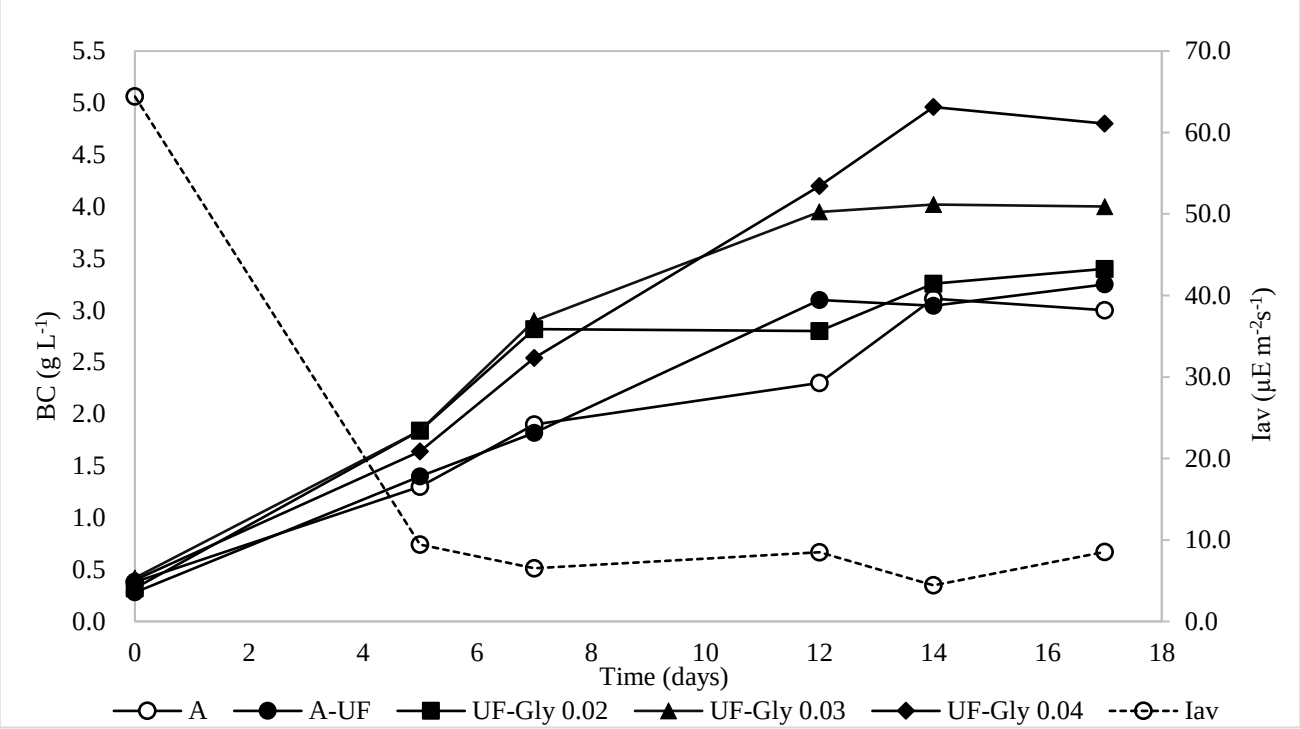


Figure 1. Biomass productivity (BC) for P. tricornutum cropped under mixotrophic (Gly) and autotrophic (A) condition and Irradiance profile (Iav) measured during batch trials.

Table 3. Growing parameters detected during autotrophic and mixotrophic trials of *P. tricornutum*

	C_b	µ	D_p	Carbon in the culture media	Carbon in the outlet culture media
	g L^{-1}	d^{-1}	g $L^{-1}d^{-1}$	g L^{-1}	g L^{-1}
A	3.11±0.08a[a]	0.13 ±0.01a	0.16±0.01a	0	0.52±0.02a
A-UF	3.25±0.15a	0.14±0.011ab	0.17±0.01a	0.06	0.67±0.01b
UF-Gly 0.02	3.4±0.1a	0.14±0.02ab	0.18±0.02a	1.4	0.66±0.01b
UF-Gly 0.03	4.02±0.01b	0.15±0.01b	0.22±0.01a	2.58	0.69±0.01b
UF-Gly 0.04	4.96±0.11c	0.16±0.04a	0.26±0.04a	3.68	0.81±0.02c

[a]Means followed in the same column by the same lower-case letter are not statistically different ($p < 0.05$) according to Tukey test.

These results suggest that in the first phase of the trials, the low biomass concentration allowed exponential algal growth while, over a certain biomass concentration, the light became the limiting factor and a plateau phase in the algal growth was reached (day 14). Light availability in autotrophic culture measured as the average irradiance inside the photobioreactor (Iav), as proposed by Acién Fernández et al. (1997), decreased rapidly as the biomass concentration increased (Figure 1). In particular for the period 12^{th} -17^{th} day, the net photosynthesis in terms of algae growth that can be modelled at 10-µE $m^{-2}s^{-1}$, was close to 0.005 d^{-1}. Thus, the further increase in the biomass concentration due to light was very low, and the average irradiance was close to the compensation point.

On the contrary under mixotrophic growth, the plateau phase was delayed because carbon (almost completely used up during the trial) (Table 3) further sustained algae growth when light became the limiting factor. This result was corroborated by the fact that added C was almost completely consumed during the trial (Table 3).

Data obtained in this work can be discussed in comparison with data from the literature. Table 1 summarizes the mixotrophic performances of *P. tricornutum* cultivated using different carbon sources. A number of studies have reported the ability of the obligate photoautotrophic microalga *P. tricornutum* to grow mixotrophically in the presence of different organic substrates. Although mixotrophy is a promising option for enhancing the productivity of many metabolites, its potential depends on the product of interest. In this case, according to Table 1, glycerol has been shown to be one of the most effective carbon sources for promoting mixotrophic growth with significant amounts of biomass or biomass productivity. The highest biomass concentration obtained in this work was 4.96 g L^{-1} at 0.04 Mole L^{-1} glycerol addition, which was a very good result if compared to the highest biomass production reported by Cerón García et al (2005) (7.04 g L^{-1}) but using glycerol at 0.1 Mole L^{-1} (Table 1). The same Authors found that glycerol concentrations higher than 0.1 Mole L^{-1} were inhibitory for algal growth, while the other C-sources tested had barely any impact on growth relative to controls in batch culture.

3.4 Nutrient Mass Balance

The recovery of nutrients from a waste stream (UF) to grow algae represents an interesting topic, reducing the cost for fertilizers, cleaning waste streams and promoting a circular economy approach in algae production. For this purpose, mass balances of N (Table 3) were performed during the trials in order to understand whether N supplied was used by the algae, allowing both biomass growth on organic C and N recovery as proteins.

The highest absolute amount of N converted into biomass, i.e., 210 mg L^{-1} (Table 4), was recorded for Trial A-UF. This result appeared interesting if we take into consideration that this trial did not provide a higher biomass production than Trial A. Probably a luxury uses of N occurred due to the higher amount of available N, as the biomass composition seemed to suggest (Figure 2). About 187 mg L^{-1} of N was missing from the balance due to the volatilization of N. Zimmo et al. (2000), found that approximately 40% of initial nitrogen was lost in algae-based batch likely because of N volatilisation. Nevertheless, this amount exceeded the total ammonia provided, suggesting that probably part of the $N\text{-}NO_3^-$ dosed was fixed as organic C in microbial biomass and subsequently converted into ammonia because of biomass degradation, allowing an extra quota of ammonia to be volatilized. This observation was confirmed by the fact that all trials performed with the addition of organic C showed consistently higher N losses than Trial A-UF, because of the presence of organic C which stimulated heterotrophic microbial growth and the subsequent mineralization of microbial bodies with the production of ammonia (Table 3). Guerra Renteria et al. (2019), studying the co-culture of *Chlorella vulgaris* and *Pseudomonas aeruginosa* in the presence of external glucose, demonstrated that the amount of nitrogen utilised by microbes was higher than that up taken by microalgae.

Therefore, nitrogen balance seemed to suggest that i. UF addition allowed increasing N availability, supporting algae growth and more protein production (Figure 2), while conversely, ii. UF addition in combination with glycerol did not stimulate N algae uptake rates, which were in line with those of the

control (Trial A), but it promoted N losses probably because of microbial N-uptake and successive N-mineralization to ammonia.

These figures found confirmation in both the algae macromolecular composition which showed for trials with glycerol a protein content reduction proportional to algae growth ($r = 0.99$, $P < 0.05$, $n = 4$) and protein content that was always lower than that of Trial A-UF.

The nitrogen lost in those trials with UF seemed most likely because of N volatilization since the N provided by UF was under this form. However, the amount of N missing in the balance was also relevant in the trial A, where only nitrate was provided. However, the application of organic C has been reported promoting microbial activity (Mohanty et al., 2020), so that nitrate was used to produce, also, microbial biomass that successively degraded producing ammonia that contributed to N volatilization. In this way, indirectly, data for missing N (Table 4) gave an idea of the microbial N turnover outlining the competition for nitrogen between algae and bacteria, i.e. competition was higher when organic C was supplemented because it stimulated microbial biomass production. It can be concluded that in the presence of glycerol, microbial growth was stimulated competing with algae for N, this latter switching the metabolism to carbohydrates accumulation (Figure 3) due to N starvation. So adding extra N in combination with the C source was not favourable for algae, which were not able to take advantage of it due to microbial competition.

3.5 Biochemical characterization

The protein content (w/w percentage) of algae decreased (Figure 2) with the increase of glycerol concentration due to N limitations as previously discussed and reported in the literature. On the other hand, different authors (Cer et al., 2000; Heredia-Arroyo et al., 2010; Villanova et al., 2017) reported that the response of algae metabolism to glycerol supplementation mimics the response to nitrogen limitation, promoting lipid accumulation and fatty acid composition modification.

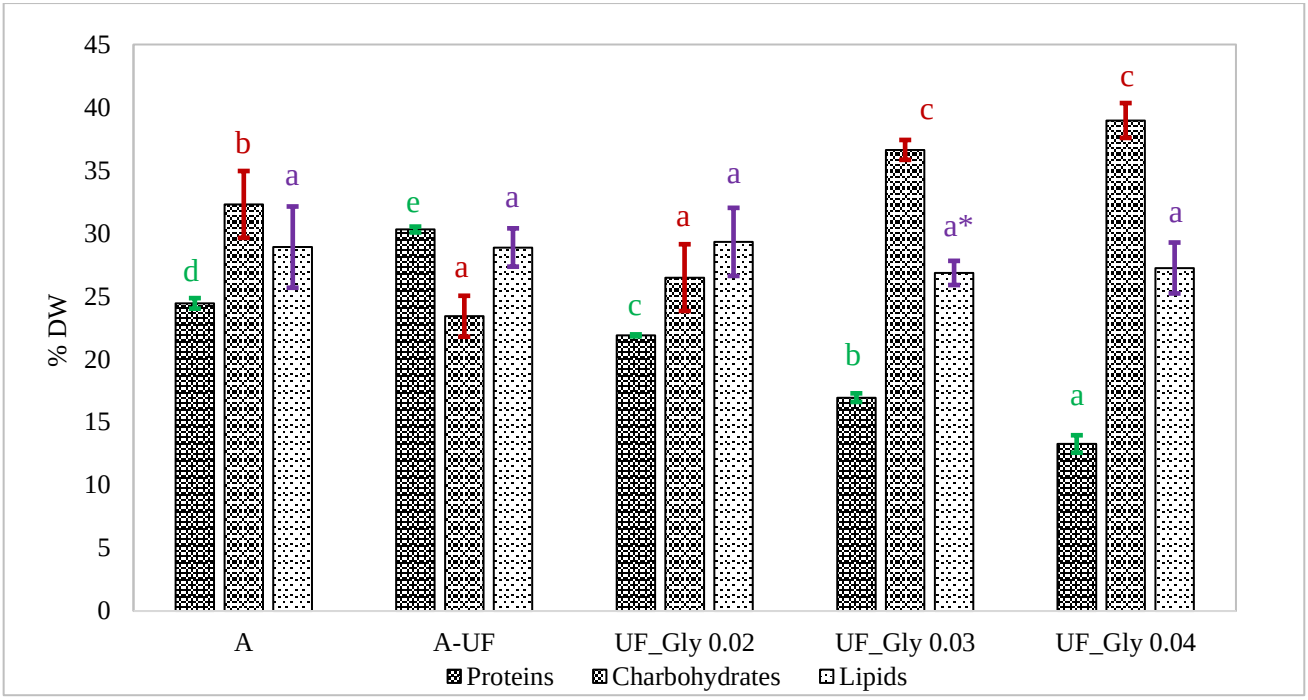


Figure 2. Biochemical composition of samples grown in mixotrophy and control (Means followed by the same lower-case letter are not statistically different ($p < 0.05$) according to Tukey test).

Lipid contents (as w/w percentage) obtained in the trials did not vary with the addition of UF and/or glycerol, but globally increased as more biomass was produced (compare Figure 2 and Table 3). As regards to the fatty acid profile (Figure 3), the EPA was the most abundant fatty acid produced in autotrophy (Trial A), followed by palmitic and palmitoleic acids. This result agreed with that of Wang et al. (2012), which reported by analysing the fatty acid profiles of *P. tricornutum* yielded under mixotrophic-batch condition and using various C sources, that the main fatty acids were palmitoleic, palmitic acids and EPA (Figure 3). Contrarily, under mixotrophic conditions (Trial UF-Gly 0.04) the EPA relative content decreased and the relative amounts of palmitic and myristic acid increased. Mixotrophic culture resulted in a relative decrease in omega 3 and polyunsaturated fatty acids and in an increase of the saturated fraction in this work.

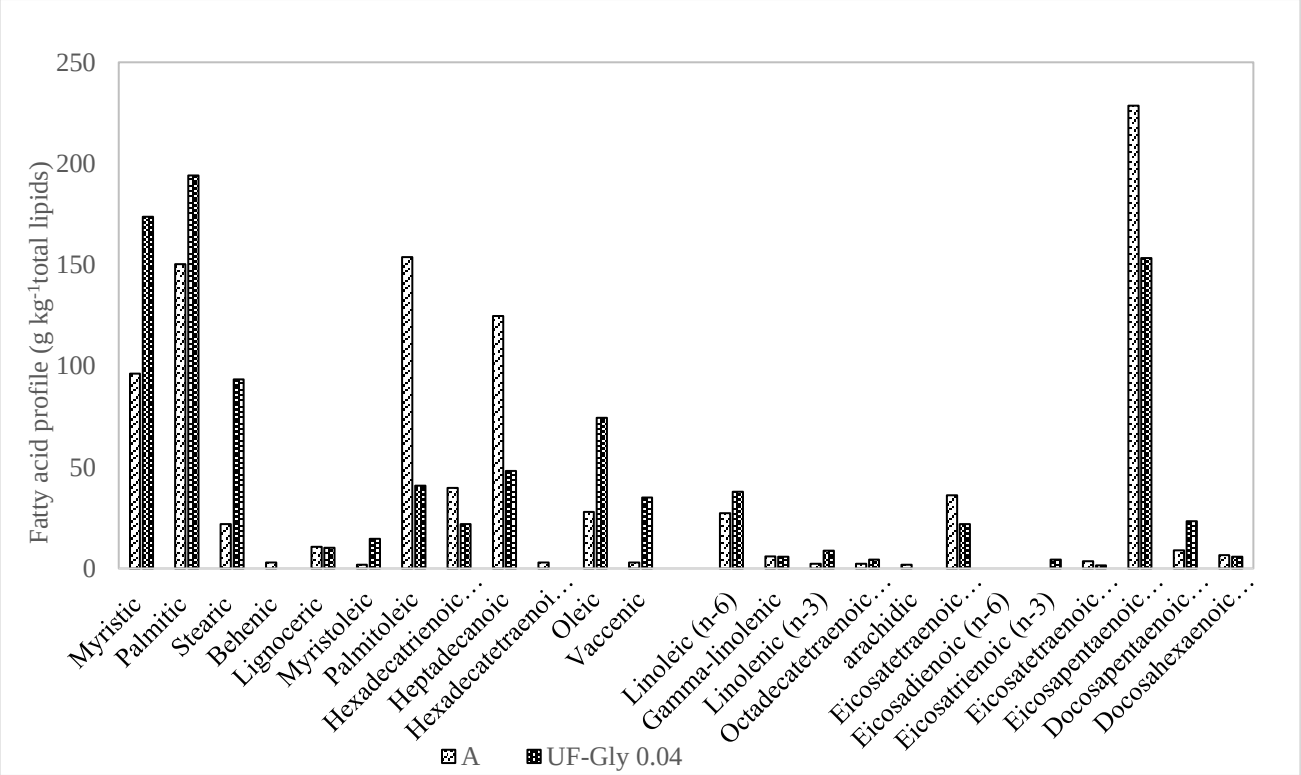


Figure 3. Fatty acids profile for biomass cropped under autotrophic (Trial A) and mixotrophic (UF-Gly 0.04 Mole L^{-1}) condition.

In this way, it was of interest reporting biodiesel properties taking into consideration the effect of mixotrophy on lipid profile: mixotrophy changing fatty acid profile affected greatly biodiesel characteristics that seemed far from standard reported for biodiesel (Table S2).

The effect of mixotrophy on the lipids profile has been studied previously and contrasting data have been reported. Hamilton et al. (2016) studying the potential production of EPA and Docosahexaenoic acid (DHA) by transgenic *P. tricornutum* under autotrophic, heterotrophic and mixotrophic conditions, found that EPA levels decreased in mixotrophic conditions with 1% w/w addition of glucose compared to photoautotrophic conditions. However, Patel et al. (2019) investigated the growth of *P. tricornutum* on birch and spruce hydrolysates compared to autotrophic cultivation and glucose synthetic media, discovered that EPA concentration in the biomass for mixotrophic conditions (in different glucose concentration) were always higher than that corresponding to the autotrophic condition. It is worth

noting that the highest EPA concentration was reached at 256 mg L^{-1} in the spruce hydrolysates (heterotrophic) rather than in mixotrophic or autotrophic conditions.

Table S2. Biodiesel properties

Properties	Unit	Autotrophic A[a]	Mixotrophic UF-Gly 0.04[a]	Biodiesel ASTM D6751-12	Biodiesel EN 14214:2012
Saturated Fatty Acid (SFA)	%	27.5	47.1		
Mono Unsaturated Fatty Acid (MUFA)	%	45.0	31.9		
Poly Unsaturated Fatty Acid (PUFA)	%	27.9	5.3		
Degree of Unsaturation (DU)	/	100.8	42.5		
Saponification Value (SV)	mg g^{-1}	205.1	175.8		
Iodine Value (IV)	/	92.5	39.6		120
Cetane number (CN)	/	52.1	68.5	47	51
Long Chain Saturated Factor (LCSF)	/	3.7	8.6		
Cold Filter Plugging Point (CFPP)	°C	-5.0	10.5		
Cloud Point (CP)	°C	2.9	5.2		
Pour Point (PP)	°C	-3.7	-1.2		
Allylic Position Equivalent (APE)	/	60.7	19.8		
Bis-Allylic Position Equivalent (BAPE)	/	31.3	8.6		
Oxidation Stability (OS)	h	6.9	27.7	3	8
Higher Heating Value (HHV)	/	39.6	33.2		
Viscosity (Kinematic Viscosity)	$mm^2 s^{-1}$	3.8	3.2	1.9-6	3.5-5
Density	g cm^{-3}	0.9	0.7		0.86-0.9

[a]Biodiesel properties were calculated taking into consideration fatty acid profile form this work and BiodieselAnalyzer© software which is freely available in the public domain (http://www.brteam.ir/analysis/).

Indeed the carbon source can influence the lipids content and fatty acids composition: Cerón García et al (Mirón, 2006) found the EPA level of *P. tricomutum* increased 2-fold for culture fed with glycerol in comparison to the control. The study of Cerón García et al. (Cero et al., 2004) found that EPA productivity increased by 3% after adding 0.1 Mole L^{-1} glucose in the culture. Moreover, the microalgal oils from *P. tricornutum* contained mainly monounsaturated fatty acids (51-62%) and saturated fatty acids (approximately 27%). This suggests high oxidative stability which is also consistent with the findings of this work (Figure 3). On the other hand, mass balance performed indicated that both EPA and DHA amounts for each batch were almost constant (0.22 g L^{-1} for EPA and 0.07 g L^{-1} for DHA) and the increase of both biomass and lipids content registered for mixotrophic batch (UF-Gly 0.04 Mole L^{-1}), corresponded to the highest production of stearic acid (0.44 g L^{-1}).

A number of factors have been reported to influence the algae lipids profile and its total content, i.e. N limitations, light level and carbon supply in the medium (Yeesang and Cheirsilp, 2011).

Probably higher biomass concentration (Table 3) characterizing the trials performed with glycerol caused a different distribution of the light supplied to each cell, leading to differences in biochemical composition, such as previously reported (Khotimchenko and Yakovleva, 2005; Solovchenko et al., 2008). Recently, Cointet et al. (2019) investigated the impact of N repletion and depletion as well as the light intensity (30, 100, and 400 μmol photons m^{-2} s^{-1}) on algae growth, photosynthetic performance and macromolecular contents of three marine microalgae species, i.e. *Entomoneis paludosa*, *Nitzschia alexandrina*, and *Staurosira sp*, reporting that under high light and N limitation, the photosynthetic apparatus was negatively impacted, forcing cells to reduce their growth and to accumulate lipids and/or carbohydrates. However, increasing lipids content under stressful conditions did not increase the production of lipids of interest, such as probably happened to the UF-Gly 0.04 trial where, N limitation, high C content and less average light intensity, lead to highest total production of stearic acid, but stable EPA and DHA production.

The carbohydrate content displayed a remarkable increase in the mixotrophic trials indicating that the addition of glycerol to the culture media switched the metabolism to carbohydrate production and storage. Carbohydrates content linearly correlated with glycerol addition and algae growth ($r = 0.96$, $p < 0.05$, $n = 4$ and $r = 0.94$, $p < 0.05$, $n = 4$, respectively). Negative linear correlation with protein content ($r = 0.99$, $p < 0.05$, $n = 4$) seemed again to confirm that N starvation due to microbial competition caused the switch of metabolism under mixotrophic conditions to carbohydrates accumulation.

Villanova et al. (Villanova et al., 2017) reported partially analogous findings, i.e., an increase in carbohydrate metabolism to yield products such as xylose, trehalose, and mannitol by glycerol addition, although they observed, also, a DHA increase due to glycerol addition.

3.6 Energy Balance

The energy efficiency was calculated considering the total energy supplied to the system (radiant plus chemical energy; E_1 and E_c), the energy content in the microalgae biomass (E_b) and the biomass yield, describing thus the conversion efficiency of the total energy into biomass (EF_t), the conversion efficiency of the glycerol chemical energy into biomass (EF_{gly}), and the biomass yield on the energy supplied to the culture (Y_{SE}).

Autotrophic conditions (Trials A and A-UF) resulted in an EF_t value of 4.2-4.3%, with these values within the range reported for *P. tricornutum* performance. Under outdoor conditions (unfortunately no lab-scale data are available), Quelhas et al. (Quelhas et al., 2019) recorded an EF_t of 2.1% working with an irradiance (2,000 μE m^{-2} s^{-1}), much higher than that used in this work.

Table 5. Energy balance for trials performed

Trial	E_l[a]	E_c[b]	E_{tc}[c]	E_b[d]	EF_t[e]	EF_{gly}[f]	Y_{SE}[g]	Y_c[h]
	kJ	kJ	kJ	kJ	% (kJ)	% (kJ)	g kJ^{-1}	g kJ^{-1}
A	753	-	753	31.6	4.2	-	0.0021	-
A-UF	753	0.9	754	32.2	4.3	-	0.0022	-
UF-Gly 0.02	753	14.6	767	32.4	4.2	5.3	0.0022	0.0098
UF-Gly 0.03	753	44.7	797	38.3	4.8	14.8	0.0025	0.0101
UF-Gly 0.04	753	67.3	820	47	5.7	22.8	0.0030	0.0137

[a]E_l: light energy (as PAR) supplied to the reactor during the all trial calculated as PAR supplied to the reactor * reactor surface exposed to light.
[b]E_c: chemical energy supplied by glycerol.
[c]E_b: energy content in the algae biomass.
[d]EF_t: conversion efficiency of the total energy into biomass.
[e]EF_{gly}: conversion efficiency of the glycerol chemical energy into biomass.
[f]Y_{SE}: biomass yield on the supplied energy to culture.
[g]Y_c: biomass yield on the chemical energy supplied by glycerol.

Mixotrophic culture allowed higher biomass production in comparison to autotrophic culture, thanks to the addition of chemical energy supplied by glycerol. Therefore, the EF_t increased with the increase of organic carbon concentration in the medium (Table 5) when in excess of 0.02 Mole L^{-1}; thus UF-Gly 0.03 and UF-Gly 0.04 showed the highest values, i.e., 4.8 ± 0.01% and 5.7 ± 0.13%, respectively.

EF_{gly} increased, as well as EF_t with C concentration, passing from 5.3 ± 0.21 % (Trial UF-Gly 0.02) to 22.8 ± 0.62 % (Trial UF-Gly 0.04). These results seemed to suggest the possibility that an initial energetic effort was needed to shift the metabolic pathways from fully autotrophic conditions to mixotrophy, after that the energy efficiency conversion increased a lot, i.e., a C threshold concentration able to trigger the metabolic pathway could be indicated at about 1.4 g L^{-1} of carbon (0.02 Mole L^{-1} of glycerol).

Unfortunately, data for *P. tricornutum* grown in mixotrophy were not available. However, previous data on energy efficiency of organic carbon transformation into biomass reported for the green alga *Chlorella*, i.e., 32% (Salati et al., 2017) and *Scenedesmus* i.e., 45% (Ren et al., 2014), were higher than those reported in this work.

The biomass yield on the supplied chemical energy (Y_C) can provide an evaluation of the efficiency by which the supplementary glycerol was used to produce biomass in mixotrophic conditions. For Trial UF-Gly 0.04 the Y_C value was of 0.014 g kJ^{-1}, which was higher than those reported in previous studies, i.e., 0.0041-0.009 g kJ^{-1} (Cerón García et al., 2005; Villanova et al., 2017) and in line with data reported for *Chlorella* by Salati et al. (Salati et al., 2017), i.e., 0.015 -0.02 g kJ^{-1}.

3.7 Economic implication

The lab scale of the work does not allow a thorough economic analysis, but give the opportunity to identify some points of interest and perspectives in the cultivation of microalgae in mixotrophy. The production data and the energy balance show that the addition of glycerol causes an increase in production, compared to the autotrophy, of 0.5 kg of biomass for each kg of glycerol supplied.

If we consider the main cost of production in autotrophy (electricity) the production cost of a kg of microalgae is around 3 euros kg^{-1}, (Acien et al 2012) in the hypothesis to save fertilizers costs using recovered nutrients. The glycerol cost is 250 euro Mg^{-1}(according to market quotation in North Italy in 2020), and thus the increase in production due to the addition of glycerol has a marginal cost of 0.4

euro per kg of algae biomass (the only cost being the supply of glycerol). Given these numbers, through the simple analysis of production costs and marginal costs, the advantage of mixotrophy appears evident. The addition of glycerol and the increased production of biomass does not cause a larger volume of medium to be moved, but simply turn into a more concentrated culture, thus leaving the fixed operating costs of autotrophy unchanged. The increase in production due to the addition of glycerol would bring the average operating costs to 1.7 Euros kg^{-1}, maximizing the production investment already made with autotrophic production.

4. Conclusions and future prospects

Mixotrophic cultivation of *P. tricornutum* using glycerol as the C source allowed increased biomass production under batch conditions in comparison with autotrophy. Ultrafiltrate addition allowed increasing N availability, supporting algal growth and more protein production but it did not stimulate N uptake by algae under mixotrophic conditions.

Biomass analysis indicated that the specific protein content decreased with the increase of glycerol concentration in the medium, conversely an increasing trend was found for carbohydrate storage. It is interesting to note that lipids content did not show statistical differences between these trials. However, an increase of the total amount of saturated fatty acid along with a decline in unsaturated fatty acid in mixotrophic culture was observed.

Finally, the efficiency of the transformation of energy into biomass rose from 4.02% in autotrophy to 5.70% at the highest dose of glycerol with the increase of carbon content in the medium. A similar trend was observed for the efficiency of the mixotrophic pathway with a sharp increase of 17.7% (from 5.3% in trial UF-Gly 0.02 to 23.0% in trial UF-Gly 0.04, respectively).

Combining microalgae cultivation and wastewater treatment is a promising aspect that has attracted widespread attention due to its potential economic and environmental benefits (Ubando et al., 2020). This work provides implications for the biorefinery approach of *P. tricornutum* utilising waste in terms of cultivation nutrition supply. It was also demonstrated that *P. tricornutum* can be utilized for the

treatment of nutrient-rich wastewater. However, it remains to be examined by further investigation on the ecological circumstances about the possible influences on lipid production because the coexistence of mixotrophs algae with bacteria. Furthermore, due to the fact that different cultivation factors play important roles on the algae biomass protein, lipids and carbohydrates content, so it is advisable to involve metabolic engineering approaches for the production of different classes of biomass in diverse microorganisms (Majidian et al., 2018).

It is also worth mentioning that large-scale cultivation should be conducted and performed for in-depth investigation in the future looking into the results from the sustainability perspective analysis (Majidian et al., 2018) as a fact that *P. tricornutum* has been reported as potential candidates for biodiesel production due to its high lipid content (Silaban et al., 2014). So under the consideration of economic feasibility, exergoeconomic and exergoenvironmental analyses as well as life cycle assessment (Rosen, 2018) have become more popular for measuring the real cost and the environmental impact of an engineering process. Exergoeconomic and exergoenvironmental analyses provide useful insights for improving the cost effectiveness and environmental friendliness of engineering processes by in different operating conditions. Furthermore, critical evaluation of the operational, environmental and biological parameters of large-scale microalgae cultivation biotechnologies can be compared and further discussed for the sustainability on the commercial implementation (Behera et al., 2019).

Acknowledgements

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Chapter V

Conclusion

Conclusion

This Ph.D dissertation firstly aimed to investigate the presence of self-adapted algae-microbial consortia (AC) in various different organic wastes rich in mineral nutrients and organic carbon, as the first step for a subsequent use of these consortia to produce algae biomasses. As a results, twelve ACs were isolated from sixteen organic wastes. *Chlorella* and *Tetradesmus* were the most represented microalgae among twelve ACs in combination with prokaryotic communities, i.e. Cyanobacteria, Proteobacteria and Planctomycetota. *Chlorella* showed a great adaptation to strong environmental condition and it dominated those organic waste characterized for adverse condition for algae development. On the contrary, *Tetradesmus* dominated those organic waste characterized for mild condition prevailing on *Chlorella*.

Further, growth ability and nutrients recovery ability of twelve ACs isolated from organic wastes when a pig slurry-derived wastewater (NFP) was used as growth substrate in autotrophic cultivation, were evaluated. Three best performant ACs, namely *Chlorella*-dominated consortia (AC_1), *Tetradesmus* & *Synechocystis* co-dominated consortia (AC_10) and *Chlorella* & *Tetradesmus* co-dominated consortia (AC_12) characterized by high growth rates, i.e., 0.55 ± 0.04, 0.52 ± 0.06 and 0.58 ± 0.03 d^{-1}, respectively. Comparable nutrients recovery ability was demonstrated of all ACs, 61 ± 15 % $TN_{initial}$ (n=12) and 92 ± 4 % $P_{initial}$ (n=12) were taken up averagely. Nevertheless, biochemical composition suggested that these ACs showed high carbohydrates and lipid contents but low protein and essential amino acid contents, probably because of low N concentration of NFP. However, the three ACs with high growth rates are recommended as good candidate for further applications.

Finally, the ability of the alga P. *tricornutum* to grow mixotrophically using glycerol as the main C source and a waste stream as an additional N source were demonstrated with higher biomass production, i.e., 1.6 times higher biomass concentration (UF-Gly 0.04) than that in autotrophic condition. Specific

protein content decreased with the increase of glycerol concentration in the medium, conversely an increasing trend was found for carbohydrate storage. Lastly, mixotrophic conditions showed higher energy recovery efficiency than autotrophic conditions (5.7 % in 0.04 Mol L^{-1} glycerol and 4.2 % in autotrophic trial, respectively).