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Agroindustrial Wastewater Treatment with Simultaneous Biodiesel Production in Attached Growth Systems Using a Mixed Microbial Culture

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Abstract: The use of cyanobacteria in biological wastewater treatment technologies can greatly reduce operation costs by combining wastewater bioremediation and production of lipid suitable as biodiesel feedstock. In this work, an attached growth system was employed to achieve the above-mentioned dual objective using a mixed microbial culture dominated by *Leptolyngbya* and *Limnothrix* species in diverse heterotrophic consortia. Kinetic experiments on different initial pollutant concentrations were carried out to determine the ability of the established culture to remove organic load (expressed by d-COD, dissolved-Chemical Oxygen Demand), N and P from agroindustrial wastewaters (dairy, winery and raisin). Biomass and oil productivity were determined. It was found that significant removal rates of nutrients were achieved in all the wastewaters examined, especially in that originated from winery in which the highest d-COD removal rate (up to 97.4%) was observed. The attached microbial biomass produced in winery wastewater contained 23.2% lipid/biomass, wt/wt, which was satisfying. The growth in the dairy wastewater yielded the highest attached biomass productivity (5.03 g m^{-2} day^{-1}) followed by the mixed effluent of winery-raisin (4.12 g m^{-2} day^{-1}) and the winery wastewater (3.08 g m^{-2} day^{-1}). The produced microbial lipids contained high percentages of saturated and mono-unsaturated fatty acids (over 89% in total lipids) in all substrates examined. We conclude that the proposed attached growth photobioreactor system can be considered an effective wastewater treatment system that simultaneously produces microbial lipids suitable as biodiesel feedstock.

Keywords: *Leptolyngbya*; Limnothrix; wastewater treatment; biodiesel; attached systems

1. Introduction

One current challenge for ecological engineering is to develop economically feasible technologies to treat wastes (liquid or solid) as a biomass source and, ideally, transform them into useful byproducts. Various physicochemical treatment methods demand large amounts of energy, chemicals, and

manpower. On the contrary, the biological treatment of wastewaters is considered to be a more environmental friendly and cost-effective approach. Few studies have showed that biological treatment using algal/cyanobacterial-bacterial consortia can efficiently remove pollutants from wastewaters [1]. In addition, the use of microalgae and cyanobacteria can aid environmental mitigation as they produce lipids suitable for second and third generation biofuels [2,3]. Therefore, applications such as wastewater treatment and biofuel production can be combined [4]. In these combined systems effluents are considered as a source of nutrients rather than as waste material, while the biomass produced may be converted into energy.

A review of the literature shows that until a few years ago research on wastewater treatment using algae focused mainly on municipal and dairy wastewater treatment using suspended microalgae under aseptic conditions [5,6]. However, media sterilization in a large scale for production of low-value commodities, such as biofuel, is not a practical and economical solution [7]. On the other hand, the coexistence of microorganisms in wastewater treatment systems has been widely investigated in an attempt to simulate natural processes. Specifically, the use of algal-bacterial cultures in sustainable and cost-efficient biosystems of municipal and agroindustrial wastewater treatment has increased over the past few years [8–10]. The selection of microorganisms is a significant issue to handle, especially considering that algal-bacterial consortia should be able to grow in harsh environmental conditions. Usually, microalgae and bacteria form aggregates and settle quickly due to gravity and their large size [11–13]. This biomass bioflocculation contributes to a less costly and simpler biomass harvesting method, avoiding additional steps such as centrifugation, filtration or coagulation.

Although most previous research focused on suspended algae growing mainly in ponds [14,15], in recent years research has concentrated on the use of attached systems, either as axenic cultures or as attached consortia [16,17] (Table 1). Immobilizing microalgae in receptive matrixes alleviates harvesting problems and high operation costs providing efficient removal of nitrogen and phosphorus from several wastewaters [14,18]. Attached growth processes have been examined for both nutrient uptake from wastewater [19–21] and lipid production [22]. Compared to standard suspended photobioreactors, attached cultivation systems lead to higher biomass production (naturally concentrated biomass), are more feasible at a large scale, have better light distribution within the reactor, have lower water consumption and improved operation control [23]. The feasibility of nonsuspended algae cultivation, is dependent on inexpensive and environmentally friendly substrate and support material [24]. According to the literature various support materials have been tested for non-suspended cultivation (including carrageenan, chitosan, alginate, nylon, cotton, glass slides, stainless steel) (Table 1) [8,14,17,18,25,26]. However, the majority of the above, and in particular the polymeric matrices (like Teflon, silicon, Plexiglas or acrylic), are costly and nonresistant during long-term operation periods thus making their application in large-scale systems debatable. The proposed attached growth system, using a transparent glass bioreactor, remains viable for longer periods of time allowing light penetration across the whole photobioreactor (PBR). In fact, the salts coating the surface of the glass rods enable better adherence conditions for biofilm formation and the glass rods are hard-wearing and do not need to be replaced. Biofilm protects cells from biocides, predators and harsh conditions (extreme pH or temperature values), helping them to remain viable for longer periods of time. Biofilms contain different types of microorganisms, e.g., bacteria, fungi and microalgae [27]. Microalgal biofilm formation is a complex process [28] while the adhesion mechanism is not yet clearly understood [29,30]. It is believed that hydrophobic reactions are driving forces for biofilm formation on hard substrates [31]. During biofilm formation, cells produce extracellular polymeric substances that build the matrix and hold the biofilm together. These substances comprise various chemical groups that function as binding sites (e.g., phosphate groups or carboxyl groups) [32].

Numerous studies have dealt with the treatment of wastewaters (mainly municipal and domestic wastewaters) using algal biofilms, also namely attached growth systems, however, only a few have focused on biofuel production [33]. The current treatment system used raw agroindustrial wastewaters with coproduction of biodiesel leading to reduced cost. As seen in Table 1, the majority of studies aimed

at nutrient removal only (without examining the possibility of biodiesel production) and the initial d-COD concentrations used were much lower than those examined in the present study. It should also be noted that most of these works used common microalgae (e.g., *Chlorella* sp., *Scenedesmus obliquus*, *Nitzschia palea*) under aseptic conditions.

To the best of our knowledge research has not been carried out on growing cyanobacteria-based flocs on support materials for treating raw agroindustrial wastewaters coupled with production of biodiesel. The purpose of this work was to develop an attached growth system and a robust mixotrophic microbial consortium able to grow on agroindustrial wastewaters and efficiently remove organic matter and nutrients. Next-generation sequencing (NGS; Illumina MiSEq Sequencing) was used to reveal the bacterial taxa comprising of the substrate's consortia. Biomass productivity and maximum oil content were also calculated to investigate the ability of this system to produce biodiesel.

Table 1. Synoptic literature review of the conditions and yields of microalgae-based attached systems using different growth substrate.

Substrate	Pretreatment of Substrate	Culture Conditions/Support Material	Culture Species	Initial d-COD (mg L^{-1})/%Removal	% Lipid Content in Total Dry Biomass (Attached)	% Nutrient Removal					C/N	Biomass Productivity (g m^{-2} day^{-1})/Growth Rate (day^{-1})	References
						NH_4^+	NO_3^-	TN	PO_4^{3-}	TP			
Nutrient medium BG-11	-	Indoor Outdoor /Glass with filter paper	*Scenedesmus obliquus*	-	47.9 -	-	-	-	-	-	-	5.2/- 50–80/-	[34]
Nutrient medium F2	-	Continuous /Cotton Cloth	*Chlorella vulgaris*	-	-	-	40	-	-	43	-	0.719/-	[35]
Nutrient medium BG-11 Artificial seawater	Autoclaved	Semi-continuous / Stainless steel mesh	*Leptolyngbya* sp.	-	16–21	-	-	-	-	-	-	2.012/- 1.87/-	[36]
Municipal wastewater	Enriched with $NaNO_3$	Continuous/plastic sheets PVC	*Nitzschia sp.*, green filaments	-	-	-	100	-	98	-	-	7.7/-	[37]
Municipal wastewater	Autoclaved	Semi-continuous /Marble slab	*Leptolyngbya* sp.	428 /-	18.2–24.8	100	100	-	-	100		2.93/0.369	[38]
Municipal wastewater	-	Continuous / Concrete slab	*Phormidium autumnale*, *Pseudanabaena* sp., *Chrococcus* sp., *Scenedesmus acutus* *Cymbella minuta*	-	-	-	-	-	-	97	-	12.21/-	[39]
Municipal wastewater	Secondary treatment	Bench/PVC Medium/PVC Pilot/aluminum wheel with cotton cords	Mixed culture biofilms: *Chlorella*, *Scenedesmus*, *Pediastrum*, *Nitzschia*, *Navicula*, *Crucigenia*, *Synedra*, *diatoms*	-	11.2–13.8	-	-	- 76	-	88 23	-	5.5/- 20/- 31/-	[17]
Municipal wastewater	Screening, grit removal	Batch /transparent PVC	Filamentous blue-green, Bacteroidia, Flavobacteria, Beta/Gamma-proteobacteria	190.9 / 98.2	-	100	-	78.8	-	64.8	-	10.9/-	[40]
Municipal wastewater	Sand and grease trap, Sieved	Batch / Plexiglas	Mixed microalgae and aerobic bacteria flocs	-	14.1	-	-	~55	-	~60	4.22	18.4/-	[13]
Domestic wastewater	Sand filter	Continuous /Polycarbonate wall	*Scenedesmus obliquus*	Total: 143/73 Soluble: 59/43	-	94	-	66	99	96	1.44	2.5/-	[41]

Table 1. *Cont.*

Substrate	Pretreatment of Substrate	Culture Conditions/Support Material	Culture Species	Initial d-COD (mg L^{-1})/%Removal	% Lipid Content in Total Dry Biomass (Attached)	% Nutrient Removal					C/N	Biomass Productivity (g m^{-2} day^{-1})/Growth Rate (day^{-1})	References
						NH_4^+	NO_3^-	TN	PO_4^{3-}	TP			
Domestic wastewater and centrates	Anaerobically digested mixed sludge, primary sedimentation	Continuous/thick foam PVC	Mixed algal bacterial culture	TOC: 76 mgL^{-1}/50 180 mgL^{-1}/86	-	100	-	30–80	77–90	-	1.2–3.1	0.5–3.1/-	[42]
Synthetic wastewater		Batch/cylindrical glass rods	*Limnothrix* sp.	Carbohydrates < 4.5 mg L^{-1}	21 (24.14)	-	80.9	-	98.54	-	-	1.11/-	[43]
Mixture of settled swine and sewage	Screened through 2-mm mesh	Continuous / Acrylic plastic ponds	*Chlorella vulgaris*, aerobic bacteria	298 / 90.6	-	73.7	-	-	91.7	77.8	-	37.2–39.2 / -	[44]
Dairy manure effluent	Raw Anaerobically digested	Outdoor / Turf scrubber raceways	*Rhizoclonium hieroglyphicum*, *Microspora willeana*, *Ulothrix ozonata*, *Oedogonium sp.*	- -	- -	- -	- -	60–90 -	- -	70–100 -	9–12 4–6.5	25/- -	[21]
Dairy manure wastewater	Filtration	Semi-continuous/polystyrene foam	*Chlorella sp.*	-	9	98.7	-	79	80	93	-	2.57/-	[22]
Dairy manure effluent	Anaerobically digested dairy	Indoor/ATS	*Rhizoclonium hieroglyphicum*	-	7.7	-	-	-	-	-	-	21/-	[20]
		Outdoor/ATS		-	6	-	-	-	-	-	-	7.6/-	
Swine manure effluent	Raw dairy Raw swine manure effluent	Indoor/ATS		-	7.5	-	-	-	-	-	-	21.3/-	
		Outdoor/ATS		-	9.9	-	-	-	-	-	-	14.6/-	
		Indoor/ATS		-	9.3	-	-	-	-	-	-	10.7/-	
Swine slurry	Centrifuged	Continuous /PVC transparent tube	*Chlorella sorokiniana*, bacterial community from swine manure	-	-	94		94–100		70–90		-	[45]
Swine manure	Rotary screen through 0.15 mm, Diluted	Continuous outdoor /Flexible white PVC	Mixed algal-bacterial consortium	1220 2417 / 76	-	96	-	28 69	-	<10	-	21.3–27.7/-	[46]
Dairy wastewater	Aerobically	Batch/ cylindrical glass rods	Mixed *Leptolyngbya/Limnothrix*-based *consortium*	3075/93.6 2420/65.5	16.1 (11.5) 16.1 (19)	- -	87.5 49.5	70.5 73.4	83.2 68.4	- -	141.6 61.3	2.89/0.460 5.03/0.925	This study
Winery wastewater		Batch/ cylindrical glass rods	Mixed *Leptolyngbya/Limnothrix*–based consortium	4675/7.4 2385/95.8	21 (23.2) 19.6 (10.9)	- -	54.6 77.7	80 87.7	34.2 38.3	- -	186 243	1.61/0.530 3.08/0.683	This study
Mixed wastewater		Batch/ cylindrical glass rods	Mixed *Leptolyngbya/ Limnothrix*– based consortium	5090/91.1 1930/91.5	16.2 (17.4) 18.6 (11.5)	- -	79.6 90.5	87 97.1	87.4 52.9	- -	175.5 34.8	4.12/0.536 1.23/0.420	This study

ATS: Algal turf scrubbers.

2. Materials and Methods

2.1. Wastewater Samples

The dairy wastewater (DWW) used in this study (aerobically pretreated secondary cheese whey and washing waters; pH: 4.5–6, d-COD: 43000 ± 2000 mg L^{-1}, Total Kjeldahl Nitrogen, TKN: 1.1 g L^{-1}) was taken from a local cheese factory (Papathanassiou cheese factory, Agrinio, western Greece) [47]. Winery and raisin wastewaters were taken from a local winery (Grivas winery, Agrinio, western Greece) and a raisin processing factory of the Agricultural Cooperatives Union-Aeghion, respectively. The winery wastewater (WWW, (pH: 3.5–5, d-COD: 80,000–90,000 mg L^{-1}, Total Kjeldahl Nitrogen, TKN: 0.7–2.72 g L^{-1}) was received after washing of the fermentation tanks, barrels and bottles, while the raisin wastewater (RWW, pH: 6–7, d-COD: 1600–9000 mg L^{-1}, Total Kjeldahl Nitrogen, TKN: 0.03–0.05 g L^{-1}) was obtained after washing the storage tanks and the raisins prior packaging. All wastewaters were filtered and stored at −20 °C until use.

2.2. Biological Material and Culture Conditions

Initially a microbial mat taken from the sewage wastewater treatment plant of Agrinio city (from secondary treatment unit) was cultivated (wastewater used as media) under steady conditions (T = 28 ± 2 °C, continuous illumination (24/24): fluorescent lamps 200 μmol m^{-2} s^{-1}, 25–29 W m^{-2}) and stirred using centrifugal mini-pumps of flow rate 380 L h^{-1} capacity. A mixed population was developed which was autotrophically cultivated (stock culture) under the same conditions in aquariums (rectangular glass tanks with a total volume of 10 L) containing (in g L^{-1}): $MgSO_4 \cdot 7H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.05; K_2HPO_4, 0.108; KH_2PO_4, 0.056, and KNO_3, 0.2; at pH 7.2 ± 0.3.

Experiments using dairy wastewater (DWW), winery wastewater (WWW) and a mixture of raisin wastewater (RWW) and WWW (mixed wastewater, MWW) as substrates were performed. DWW, WWW and MWW substrates were diluted with tap water at different rates leading to various initial pollutant concentrations (experimental sets A, B and C) (Table 2). For all the MWW sets conducted constant ratio of RWW: WWW by 85%:15%, respectively. All experiments were conducted in duplicate. 700 mL of stock culture was inoculated in each batch experiment containing 56 ± 11.9 mgL^{-1} dry biomass. Initially, the pH was regulated between 7 and 7.5. However, during the bioprocesses pH increased from 7 to 9. It should be mentioned that this range of pH is suitable for heterotrophic and autotrophic metabolism.

Table 2. Characterization of all types of wastewater used as growth medium for a microbial population dominated by cyanobacteria species (DWW: dairy wastewater, WWW: winery wastewater, MWW: mixed winery-raisin wastewaters).

Experimental Set	Initial d-COD (mg L^{-1})	Initial Concentrations (mg L^{-1})				Initial Biomass Concentration (mg L^{-1})	C:N	N:P
		NO_3^-	TN	PO_4^{3-}	Total Sugars			
DWW-A	4081 ± 54.8	13.64 ± 0.3	59.22 ± 4.9	26 ± 0.62	713.25 ± 52.8	276 ± 14.14	68.92	2.3
DWW-B	3075 ± 257.7	10.4 ± 1.1	21.72 ± 4.1	13.1 ± 0.49	302.1 ± 46.25	268 ± 2.83	141.6	1.66
DWW-C	2420 ± 106.7	7.85 ± 0.18	15 ± 3.4	8.48 ± 2.24	618.3 ± 103.2	390 ± 14.14	161.3	1.76
WWW-A	4675 ± 109.6	11.03 ± 0.1	25.12 ± 9.8	5.8 ± 0.3	89.21 ± 0.1	65.71 ± 10.7	186.12	4.33
WWW-B	3806 ± 74.3	8.56 ± 0.1	33.12 ± 4.5	2.8 ± 0.07	80.5 ± 2.5	64 ± 2.03	114.9	11.8
WWW-C	2385 ± 43.4	5.4 ± 0.007	9.82 ± 2	5.5 ± 0.007	41.3 ± 0.47	59 ± 2.83	243	1.8
MWW-A	5091 ± 270.3	18.35 ± 0.4	28.9 ± 5.3	15.5 ± 0.64	190.3 ± 6.93	202 ± 19.8	175.5	1.9
MWW-B	4116.2 ± 61.5	8.07 ± 0.3	16.48 ± 0.04	5.1 ± 0.17	112.13 ± 4	105 ± 24.04	249.5	3.24
MWW-C	1927.5 ± 409.4	16.95 ± 0.1	55.5 ± 4.85	11.25 ± 0.12	56.82 ± 4.16	79 ± 1.4	34.77	4.83

2.3. Microscopy Analysis of Microbial Communities

Samples were collected from the 5–6 days old autotrophic attached growth. Fresh and Lugol preserved subsamples were examined under an inverted epifluorescence microscope (Nikon Eclipse TE 2000-S, Nikon, Tokyo, Japan) with a microscope camera (Nikon DS-L1). The cyanobacterial taxa composition was determined using taxonomical keys and papers.

2.4. DNA Extraction and Amplicon Sequencing

Samples were collected from both the autotrophic attached culture and the untreated samples of dairy and winery wastewater. Subsamples of ca. 50 mL were filtered using 0.2 µm nucleopore filters and stored at −20 °C until further molecular analysis. The DNA collected from each filter was isolated using the MoBio PowerWater Isolation Kit according to the manufacturer's instructions and the V3-V4 region of the 16S rRNA gene (approximately 465 base pairs) was amplified according to the SD-Bact-0341-bS-17: 5′-CCTACGGGNGGCWGCAG-3′ and S-D-Bact-0785-a-A-21: 5′-GACTACHVGGGTATCTAATCC-3′ primers [48]. PCR reactions and the barcode amplicon sequencing process were performed by the Mr. DNA Company [49]. Briefly, the PCR products were purified using calibrated Ampure XP beads and the purified products were used to prepare the DNA libraries following the Illumina MiSeq DNA high-throughput library preparation protocol. DNA library preparation and sequencing was performed at Mr. DNA [49] on a MiSeq following the manufacturer's guidelines. The produced reads were processed using MOTHUR v 1.34.0 software and following the standard operating procedure [50,51]. Forward and reverse reads were joined and the barcodes were removed. Reads < 200 bp, with homopolymers > 8 bp and with ambiguous base calls were removed from downstream analysis. The remaining reads were dereplicated to the unique sequences and aligned independently against the SILVA 128 database [52]. The reads suspected for being chimeras were then removed using UCHIME software [53]. The remaining reads (between 13,706 and 22,575 in the three samples examined) were clustered into Operational Taxonomic Units (OTUs) at 97% sequence similarity threshold. Singletons were removed as they were likely erroneous sequencing products [54]. One-hundred-and-fifteen OTUs were produced in total, and were taxonomically classified using BLASTN [55] on the SILVA 128 database [52]. Sequences were submitted to GenBank-SRA under the accession number SRR6491174.

2.5. Experimental Setup

In this research, the photobioreactors (PBRs) used were glass aquariums equipped with 36 cylindrical glass rods (of 0.5 cm in diameter each rod). The dimensions of the aquariums were 29 × 10 × 15 cm (length × width × height). A schematic presentation of the reactor is available in Economou et al. [43]. The surface area of each rod was 19.04 cm^2, providing a sufficient surface area for microbial growth and attachment. In addition, the transparent glass rods allowed light penetration across the whole PBR. Also, the use of a supporting metallic grid placed on the surface of the aquarium kept in vertical position all glass rods. This configuration allowed the easy removal of each single rod from the PBRs and therefore biomass harvesting. The flow rate of substrate medium was adjusted in 50 L h^{-1} (Dilution rate D = 14.2 h^{-1}) to allow cell attachment to the rods and PBR walls. The illumination was continuous, suitable for microalgal growth [41,56] and was provided at a distance of about 25 cm from the PBR's surface.

2.6. Analytical Procedures

Samples (grab samples) of constant volume of aquarium wastewater were collected on a daily basis and analyzed for various parameters. Attached and suspended microbial biomass was harvested from each batch experimental run. For suspended biomass determination 100 mL of culture (for each sampling) was centrifuged at 4100 rpm for 20 min. Additionally, the microbial mass attached to the supporting rods was harvested by scraping two randomly selected glass rods

for each sampling. At the end of each experimental set the biomass attached to the PBRs' walls was also harvested. Following centrifugation, aliquots of the supernatants were separated and collected for chemical analysis. After harvesting, suspended and attached biomass was dried at 105 °C and then gravimetrically determined. The supernatant after centrifugation was collected for dissolved oxygen demand (d-COD), orthophosphate (PO_4^{3-}), NO_3-N, NO_2-N, and Total Kjeldahl Nitrogen (TKN), measurements, according to APHA [57]. DuBois et al. [58] was used for total sugars measurements. Biomass productivities P (mg DW L^{-1} day^{-1}) were calculated from the variation in biomass concentration through time according to Gonçalves et al. [10]. Nutrient removal efficiencies and the maximum specific growth rate (μ) of the mixed culture were determined according to Tsolcha et al. [1]. Concentration of the total biomass was the sum of suspended and attached biomass in each experiment set and was correlated with TSS [59].

2.7. Lipid Extraction/Fatty Acid Analysis

The extraction of lipids from dry biomass cells was performed according to Folch's method using a mixture of chloroform: methanol (2:1, *v*/*v*) as solvent [60]. The extract was then washed with 0.88% (w/v) KCl solution to remove non-lipid components and dried over anhydrous Na_2SO_4. Finally, the solvent was removed by evaporation and the produced oil was gravimetrically determined as a percentage of the dry cell weight (% DCW) [61]. The fatty acid profile of the produced oil was determined as fatty acid methyl esters (FAME), following AFNOR method [62]. Both total lipid extraction method and the fatty acid analysis procedure that were used in this study is described analytically at Tsolcha et al. [1].

2.8. Statistical Analysis

Results were reported as means ± standard deviation (SD). The statistically significant differences of biomass production, lipid content and physicochemical parameters were analyzed using one-way analysis of variance (ANOVA) at significance of ($p < 0.05$).

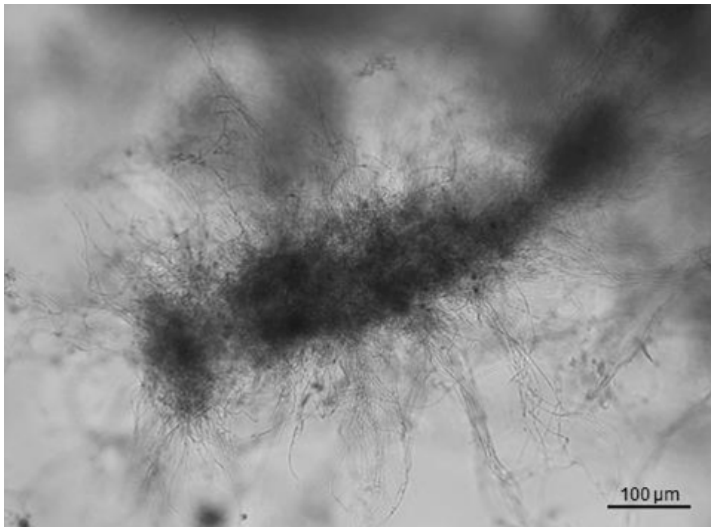
3. Results and Discussion

3.1. Consortia Analyses

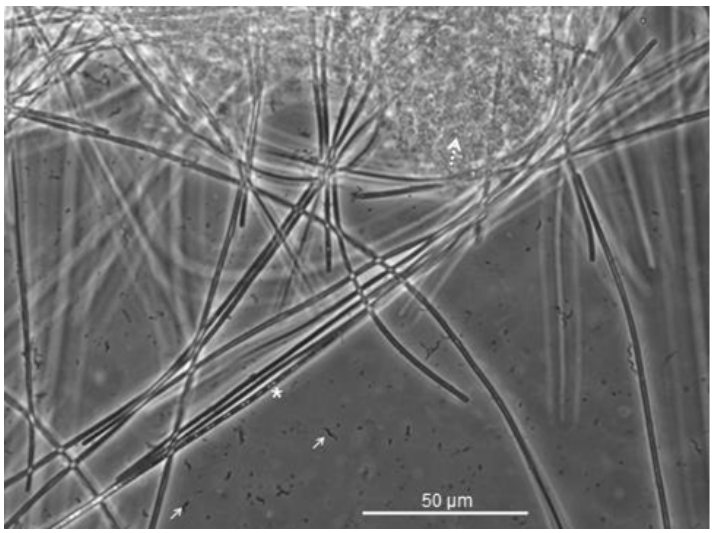
Microscopic analysis (Figure 1) showed aggregates of cyanobacterial trichomes associated with attached colonies of heterotrophic bacteria and large planktonic bacterial cells. The trichomes exhibited the morphological features of the genera *Leptolyngbya* and *Limnothrix* (Figure 1b). Cells of *Limnothrix* were characterized by small polar gas vacuoles [63], while the trichomes of *Leptolyngbya* and *Limnothrix* without gas vacuoles were dominant. The intrageneric taxonomic classification of the genus *Leptolyngbya* is difficult because of its simple morphology and minute dimensions, while molecular analysis has resulted in the identification of new genera (e.g. *Nodosilinea*) of the very large heterogenous genus *Leptolyngbya* [64]. The molecular analysis showed that of the most abundant OTUs, one was closely related to *Leptolyngbya* sp. (OTU005) and another to *Limnothrix planctonia* (OTU008) (Table 3).

Amplicon sequencing revealed 115 prokaryotic OTUs in the three samples examined (Stock culture, DWW, WWW). The rarefaction curves calculated approached a plateau in all cases, indicating a sufficient coverage of the existing prokaryotic diversity in all samples (data not shown). Overall, the majority of the detected OTUs belonged to the high-level taxonomic groups of firmicutes (39% of the total OTUs), followed by proteobacteria (38%), bacteroidetes (10%) and cyanobacteria (5%). On the other hand, the most dominant taxonomic groups in terms of relative abundance were bacteroidetes, comprising of 32% of the total number of reads, followed by firmicutes (30%), proteobacteria (18%) and cyanobacteria (14%). Of the 12 most abundant OTUs, each comprising >1% of the total number of reads in all samples (Table 3), two were attributed to cyanobacteria and represented the bulk of cyanobacterial abundance. OTU005 had a *Leptolyngbya* sp.-related clone as

its closest relative, but on the top 10 hits of BLAST searches, it was also found to be closely affiliated to the new genus *Nodosilinea*-related clones of the very large heterogenous genus *Leptolyngbya* [64]. OTU005 was the fourth most dominant OTU overall and was especially abundant in the stock culture, accompanied by a *Limnothrix*-related OTU (OTU008). These two OTUs were the most dominant OTUs in terms of relative abundance in the stock culture (along with a proteobacteria-related OTU) (Table 3). It is noteworthy that the most abundant OTU (OTU001) in the entire dataset, comprising nearly 78% of the number of reads in the DWW, was attributed to a Bacteroidetes taxon (Table 3). The second dominant OTU in the DWW, OTU003, was closely affiliated to the Firmicutes *Lactobacillus delbrueckii*, a well-known lactic acid bacterium which can be used for solid-state fermentation [65]. The dominant OTU in the WWW, OTU002, was closely affiliated to the Firmicutes *Pediococcus parvulus*, a taxon of wine origin [66] important for metabolic-engineering strategies aiming to improve exopolysaccharide production in the food industry [67]. Of the dominant OTUs detected in the WWW, OTU021 was closely affiliated to the Firmicutes *Oenococcus oeni*, a taxon that holds major importance in oenology where it is the primary bacterium involved in completing malolactic fermentation [68].



(a)



(b)

Figure 1. (**a**) Micrograph of *Leptolyngbya* aggregation of trichomes, part of the biofilm, as seen by phase contrast light microscopy; (**b**) Micrograph of part of a trichome aggregate showing details of *Leptolyngbya* and *Limnothrix* (asterisk) trichomes and heterotrophic bacteria (free bacteria are indicated by the thin arrows and the attached bacterial colony is indicated by a thick arrow).

Table 3. List of the overall dominant Operational Taxonomic Units (OTUs) in all samples (with relative abundance > 1% of the total number of sequences in all samples), their high taxonomic affiliation, their closest relative based on BLAST searches against the SILVA 128 database, the isolation source of the strain, and their relative abundance (%) in the stock culture and the two treatments.

OTUs	Putative Taxonomic Affiliation	Closest Relative (% Similarity) [Accession Number]	Isolation Source	Stock Culture	Dairy Wastewater	Winery Wastewater
OTU001	Bacteroidetes	Prevotellaceae sp. (99%) [JF806757]	Sewage from bioreactor	0.15	77.9	0.37
OTU002	Firmicutes	*Pediococcus parvulus* (99%) [MF540542]	Calabrian sourdough	0.15	0.47	57.4
OTU003	Firmicutes	*Lactobacillus delbrueckii* (99%) [CP023139]	Complete genome	0.01	14.3	0.52
OTU005	Cyanobacteria	*Leptolyngbya* sp. (98%) [FJ410906]	Industrial estate	21.0	0.10	0.08
OTU006	Proteobacteria	*Lysobacter brunescens* (99%) [KC157043]	Lake	18.4	0.08	0.09
OTU008	Cyanobacteria	*Limnothrix planktonica* (99%) [KP726241]	Freshwater	16.5	0.11	0.07
OTU010	Bacteroidetes	Uncultured clone (99%) [FJ377379]	Unknown	10.4	0.08	0.02
OTU013	Bacteroidetes	Uncultured clone (95%) [GU074246]	Groundwater	7.76	0.04	0.03
OTU009	Proteobacteria	*Acinetobacter baumannii* (99%) [KY114513]	Environmental sample	0.01	0.09	4.75
OTU011	Firmicutes	*Dialister* sp. (99%) [KM396274]	Human feces	0	3.73	0.03
OTU016	Bacteroidetes	*Fluviimonas pallidilutea* (99%) [KU991470]	Surface water	5.12	0.03	0.01
OTU021	Firmicutes	*Oenococcus oeni* (99%) [KY561609]	Red wine	0.01	0.02	2.93

3.2. Microbial Growth

A series of batch kinetic experiments was carried out using unsterilized wastewaters obtained from local production plants at different seasons and times of day. These experiments determined the ability of the cyanobacterial-based culture to remove nutrients and simultaneously produce biomass and lipids. Following addition of the inoculum into the bioreactor, a mixed consortium established forming biofilm on the glass rods and PBR walls, indicating that the added species (cyanobacterial-bacterial) may have a synergistic relationship. The cyanobacterial-bacterial flocs that developed during wastewater treatment are shown in Figure 1. The formed cooperative system that is probably supported by binding mechanisms led to the formation of settleable biomass as also recorded by Gutzeit et al. [11]. Many studies confirm the existence of positive interactions between microalgae/cyanobacteria and bacteria that enhance wastewater treatment and biomass production [9,40,44].

The maximum attached biomass productivity recorded for the experimental sets DWW-C and MWW-A, reaching the values of 5.03 and 4.12 g m^{-2} day^{-1}, respectively. Specific growth rate values ranged from 0.217 to 0.925 day^{-1} (Table 4), which are values higher than those previously recorded for attached *Leptolyngbya*-based cultures (0.369 day^{-1} by Singh and Thakur [38] using municipal wastewater as substrate), as well as suspended growth *Leptolyngbya*-based cultures (0.24–029 day^{-1} using winery substrate or 0.16–022 day^{-1} using mixed winery-raisin substrate by Tsolcha et al. [1]. It should be mentioned that autotrophic experiments performed with chemical media containing minerals with the same initial N:P ratio used in the DWW and MWW experiments, presented lower biomass productivities of between 1 and 2.2 g m^{-2} day^{-1} (data not shown). These values are in line with the maximum areal biomass productivity recorded in the mesh incubator autotrophic experiments of *Leptolyngbya* sp. (2.01 g m^{-2} day^{-1}) by Singh et al. [36]. The *Limnothrix* sp. examined by Economou et al. [43] showed a total biomass productivity of about 1.11 g m^{-2} day^{-1} in an attached growth system similar to that used in this work. The production of biomass achieved in attached growth systems is closely related to the selected species as well as the prevailing microbial interaction (mutually beneficial or harmful effects) and specific applied conditions, including nutrient concentration, light intensity, pH, flow of medium, and substrate properties [17,69]. Mixed culture biofilms usually present the highest biomass productivity rates that can reach up to 30 g m^{-2} day^{-1} (Table 1). The mixed culture used in this study showed higher biomass productivity rates compared to those of related axenic cyanobacterial cultures. It is probable that the added heterotrophic bacteria (contained in wastewaters) enhanced biomass productivity as also observed by Bai et al. [70].

Cyanobacteria are known for their tolerance to harsh environmental conditions. However, a significant advantage of several filamentous cyanobacteria compared to nanosized microalgae is their easy harvest from the culture medium due to their shape and larger size [71]. Thus, expensive harvesting techniques such as centrifugation, flocculation or filtration are avoided [72,73]. The microbial culture used in this work which consisted of filamentous cyanobacteria forming aggregates, showed a natural tendency to settle and to attach itself to the immobilized materials (rods and PBR walls), thus facilitating harvesting (Figures S1 and S2). Bacterial colonies were seen to attach onto the surfaces of the filamentous cyanobacteria (Figure 1b), as also observed by Zamalloa et al. [45].

Table 4. Values of nutrient removal, oil content, biomass productivity and specific growth rate for each set of experiments (DWW: dairy wastewater, WWW: winery wastewater, MWW: mixed winery and raisin wastewaters).

Experimental Set	Removal Rate %				d-COD Removal %	Maximum % Oil Content		Biomass Productivity		Specific Attached Growth Rate (day^{-1})
	NO_3^-	TN	PO_4^{3-}	Total Sugars		Total	Attached	Total mg $(L\ day)^{-1}$	Attached g $(m^2\ day)^{-1}$	
DWW-A	53.7 ± 0.3	89.3 ± 0.1	80.8 ± 0.6	85.2 ± 0.36	88.5 ± 0.2	14.8 ± 3.57	15.3 ± 0.88	292.8	2.74	0.217
DWW-B	87.5 ± 0.6	79.3 ± 2.5	83.2 ± 3.2	78.1 ± 4.9	93.6 ± 1.4	16.1 ± 0.43	11.5 ± 1.45	118.1	2.89	0.46
DWW-C	49.4 ± 9	73.4 ± 1.7	68.4 ± 8.3	85.2 ± 2.3	65.5 ± 0.95	16.1 ± 0.52	19 ± 1.98	249.5	5.03	0.925
WWW-A	54.6 ± 4.5	80 ± 2	34.2 ± 5.15	16.5 ± 4.64	97.4 ± 0.73	21 ± 1.49	23.2 ± 0.32	98.9	1.61	0.53
WWW-B	37.8 ± 2.34	83.2 ± 0.1	10.2 ± 0.02	32 ± 1.94	95 ± 1.12	16 ± 0.64	18.7 ± 3	79.56	1.3	0.333
WWW-C	77.7 ± 0.63	87.7 ± 0.6	38.3 ± 25	44.4 ± 5.05	95.8 ± 0.78	19.6 ± 0.3	10.9 ± 3.8	90.7	3.08	0.683
MWW-A	79.6 ± 0.23	87 ± 0.13	87.4 ± 0.7	40.1 ± 1.37	91.1 ± 0.61	16.2 ± 1.13	17.4 ± 3.27	230.73	4.12	0.536
MWW-B	55 ± 9.2	77.8 ± 4	60.2 ± 2.1	49 ± 0.41	89 ± 4.6	9.8 ± 0.05	8.9 ± 2.1	175.25	2.7	0.587
MWW-C	90.53 ± 0.3	97.1 ± 0.09	52.9 ± 1.97	41.9 ± 4.13	91.54 ± 0.4	18.6 ± 2	11.5 ± 1.7	113	1.23	0.42

Regarding algal/cyanobacterial attachment substrates, research has shown higher growth rates on cellulose-based natural polymer surfaces than synthetic polymer surfaces [18]. For commercial use a suitable support material should be inexpensive, weightless, thin, long-lived, water resistant, easy to inoculate, and able to maintain enough algal/cyanobacterial cells/colonies/filaments for a new round of re-growth after harvesting [34]. Among existing support media, glass reactors provide widespread light distribution. It should also be mentioned that the presence of bacteria enhances the adhesion of microalgae to glass surfaces. In this study, significantly higher attachment to glass surfaces was observed at pH 9 compared to pH 7 or 6 (optical observation), as also noted by Tosteson and Corpe [74] and Sekar et al. [25]. Additionally, the use of glass as an immobilized material allowed faster biomass growth when diluted substrates were used (DWW-C and WWW-C in Figure 2, Figure S3). The system tested ensured adequate light penetration and easy biomass harvest, as well as high surface area provided by both the glass rods and the walls of the PBR.

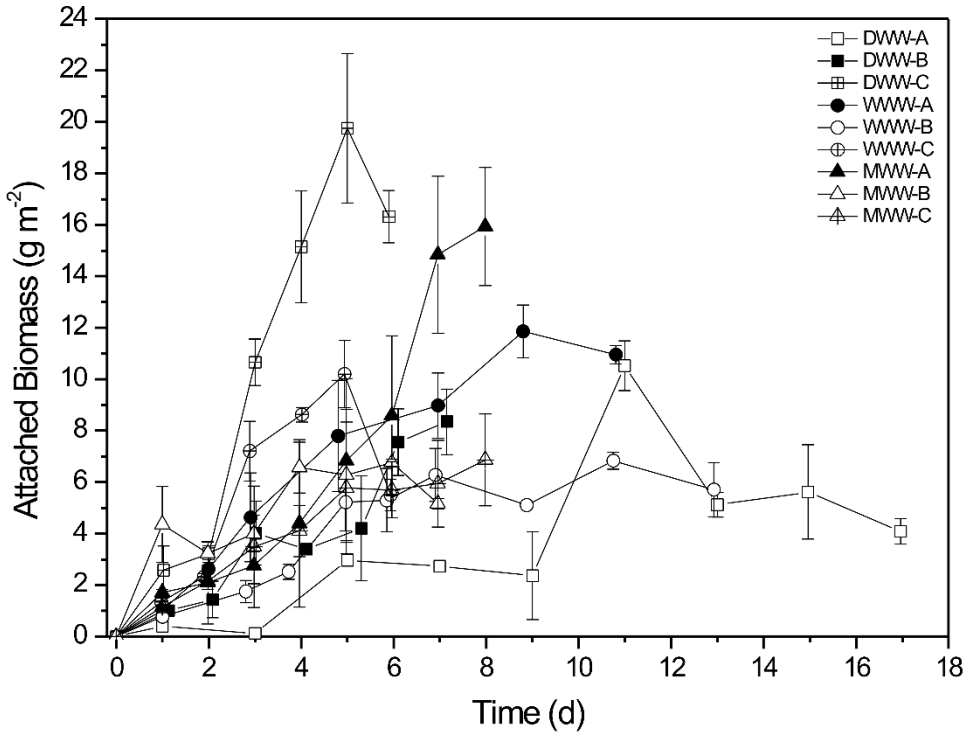


Figure 2. Profile of attached biomass production through time using different dilution ratios (A, B, C) of wastewater as growth medium [DWW: dairy wastewater, WWW: winery wastewater, MWW: mixed (winery and raisin) wastewater].

3.3. Removal of Nutrients and Organic Load from Wastewaters

Liquid effluents from agroindustry contain high organic content with high levels of proteins, nitrogen, phosphorous, dissolved sugars and minerals. The specific mixed microbial culture of this study was able to remove both organic and inorganic pollutants from these agricultural wastewaters by mixed autotrophic, mixotrophic and heterotrophic metabolism. According to the literature, mixotrophic cultivation has several advantages over single photoautotrophic or heterotrophic modes as it provides higher biomass and lipid productivities, as also observed in the experiments of this study. Indeed, the nutrient uptake by the microbial consortium growing in the tested substrates was higher than that observed by the single cultures (cyanobacteria/algae) used as control, thus proving the synergistic effect of microalgal-bacterial consortia [11].

It should be mentioned that the use of undiluted agroindustrial wastewater (dairy, winery, and raisin as raw sources), showed initial d-COD concentrations inhibitory for autotrophic growth. Dilution was also considered necessary to allow light penetration across the bioreactor. Regarding the

mixed experiments, raisin and winery wastewaters were combined because it was necessary to dilute the winery wastewater with a wastewater (such as raisin) that contained lower organic load, nutrient and ion concentrations as well as lower turbidity and color intensity [1]. The purpose of mixing was to avoid (as much as possible) the use of fresh water for dilution.

In all sets of conducted experiments initial organic load values were between 1930 to 5090 mg d-COD L^{-1}. In most experiments, high d-COD removal rates were performed with values between 65.5 and 97.4%. Specifically, the WWW experimental series presented d-COD removal rates higher than 95% (Table 4). In most experimental sets d-COD removal was achieved within 6–7 days, with the exception of sets with high initial d-COD concentrations (over 3500 mg L^{-1}) (Figure 3). The existence of residual dissolve organic matter is mainly attributed to the presence of slowly biodegradable organic matter and carbon in some colloidal form [75]. Significant differences in d-COD removal rates (p = 0.94143) were not observed between the experimental groups DWW, WWW and MWW. In addition, significant differences were not observed (p = 0.1136) in sets DWW-B, WWW-A and MWW-C. In these experimental sets were observed the highest d-COD removal rates (p = 0.1136). It is worth mentioning that Godos et al. [46] (Table 1) using mixed cultures for agroindustrial wastewater treatment achieved d-COD removal efficiencies of up to 76% (initial concentration < 2420 mg d-COD L^{-1}) which are lower than the rates of the present study that reached 95.8% with similar initial d-COD concentrations (WWW-C). In fact, the d-COD removal observed in this work is among the highest recorded in the literature for similar mixed cultures in attached systems despite the high initial d-COD concentrations applied (Table 1). The d-COD removal rates achieved in this study for the WWW experimental sets (over 95% for initial concentrations between 2385–4675 mg L^{-1}) are higher than those referred by Tsolcha et al. [1] for *Leptolyngbya*-based cultures in suspended growth reactors using winery substrate (up to 85.8% for initial concentrations between 1732–2043 mg L^{-1}). Removal of total sugars reached values of up to 49% for experimental sets with low initial sugars concentrations (below 190 mg L^{-1}) and higher values (up to 94%) were recorded in sets with high initial sugars concentrations (DWW sets). The increase of total sugars observed after day 7 of cultivation (Figure 4) was probably attributed to secretion of soluble materials (e.g., polysaccharides and/or organic compounds from the algal/bacterial cells) [76].

Percentage removal efficiency of nitrate ranged from 38 to 90.5% (Figure 5) while nitrite concentration constantly was below the value of 0.2 mg L^{-1} in all experimental sets. Total nitrogen (TN) removal efficiencies (73.4–97.1%) were higher than those achieved for nitrate as nitrate assimilation is an energy-linked process and TN uptake is carried out by the entire microbial consortium. In fact, the maximum TN removal reached 97.1% for the experimental set MWW-C, which is higher than that previously reported in similar mixed attached systems (Table 1). Significant differences in nitrate removal were also noticed between the three experimental groups DWW-A-B-C (p = 0.00228), WWW-A-B-C (p = 0.00751) and MWW-A-B-C (p = 0.00161) but also between DWW-WWW-MWW (p = 0.01418). This may be attributed to the photo-dependent nitrate uptake process as a different substrate colour was observed following after each dilution. The initial concentrations of nitrogen and phosphorus used in this research were relatively higher than previous studies on attached growth systems [46], thus indicating that the treatment system presented here is effective. The remaining organic nitrogen may comprise organic matter produced during algal growth and the wastewater treatment process. Orthophosphate (PO_4^{3-}, OP) presented the highest removal rates in the experimental sets with high initial OP concentrations (Figure 6). Relatively high OP removal rates of between 68.4% and 83% were observed in all DWW experimental sets (Table 4). However, the highest OP removal rate (87.4%) was recorded in the MWW-A. Significant differences in OP removal rates were not observed between the sets DWW-A-B-C (p = 0.96866), WWW-A-B-C (p = 0.19725) and MWW-A-B-C (p = 0.71982). However, significant differences were recorded between all the experimental groups DWW-MWW-WWW (p < 0.002). The different initial OP concentrations of each wastewater (between 8.48–26 mg L^{-1} for DWW, 2.8–5.8 for WWW, and 5.1–15.5 for MWW) are likely contributed to the previously reported differences.

Various environmental factors (e.g., temperature, light intensity, initial nutrient concentration, extracellular pH, inoculation density, as well as population interactions) have significant impact on nutrient uptake rate for various microorganisms [39,56,67]. The initial C:N:P ratio as well as the microbial members comprising the consortium are of profound significance and influence the overall yields of the culture systems. Thus, each substrate (dairy, winery or mixed effluent) requires different initial biological and chemical parameters in order to achieve a self-sustaining system with the dual purpose of pollutant removal and by-products production. The highest TN (87%), orthophosphate (87.4%) and d-COD (91.1%) removal rates were observed in MWW-A, which had the lowest N/P ratio (1.9) for the MWW substrate (Tables 2 and 4). In addition, with the same low N/P ratio (close to 1.8), all three substrates showed their highest attached biomass productivity (5.03, 3.08, 4.12 g m^{-2} day^{-1} for DWW-C, WWW-C and MWW-A, respectively). It is worth mentioning that in a similar study, Godos et al. [46] used mixed microbial populations and agroindustrial wastewaters recorded lower removal values of d-COD (76%), TN (69%) and phosphorus (<10%).

In the present study, the remaining d-COD or nutrient concentrations were above the permissible limits of European legislation for discharge into an urban wastewater treatment plant (d-COD 500 mg L^{-1}) or directly into natural water bodies (d-COD 125 mg L^{-1}) [77]. Therefore, a post-treatment step will be required (such as open pond and constructed wetlands).

Precise cost data of the proposed treatment system cannot be estimated safely because pilot-scale experiments and process parameter optimization are necessary prior to scaling-up. However, expenditure includes: fixed costs (including the aquariums, glass rods, lamps, and pumps for wastewater recirculation), the operating cost (mainly the energy consumed by the light source and recirculation pumps), and the management cost (significantly high) and includes the transfer of the specific wastewaters to the treatment plant.

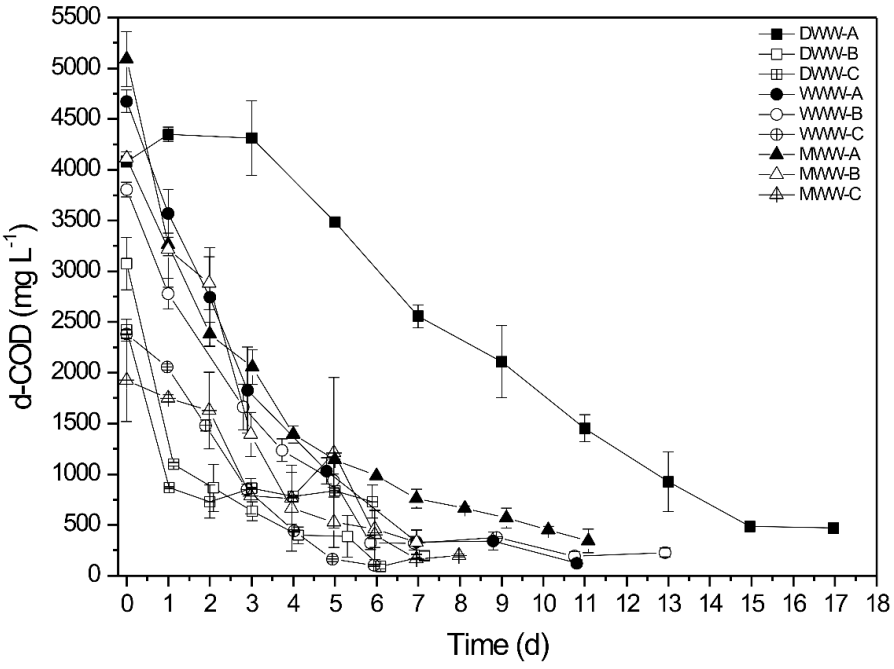


Figure 3. Profile of d-COD removal through time using different dilution ratios (A, B, C) of wastewater as growth medium [DWW: dairy wastewater, WWW: winery wastewater, MWW: mixed (winery and raisin) wastewater].

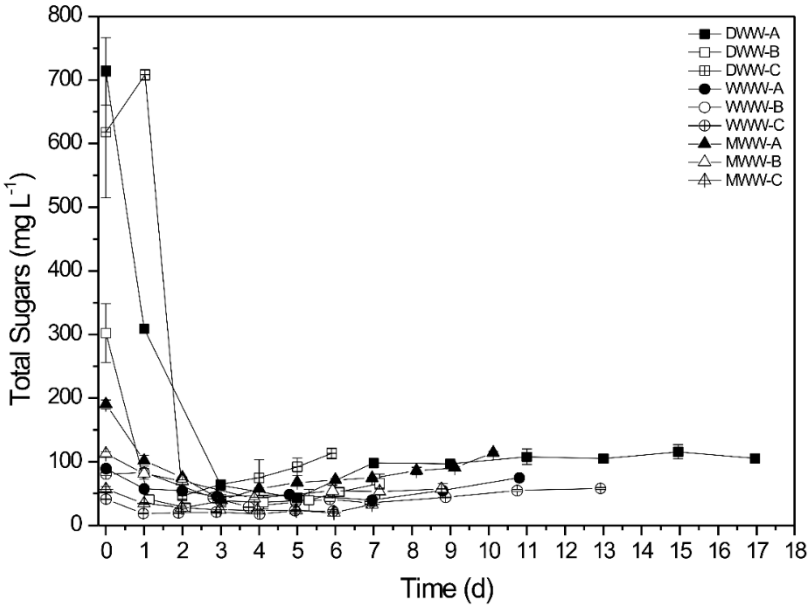


Figure 4. Profile of total sugars removal through time using different dilution ratios (A, B, C) of mixed wastewater as growth medium [DWW: dairy wastewater, WWW: winery wastewater, MWW: mixed (winery and raisin) wastewater].

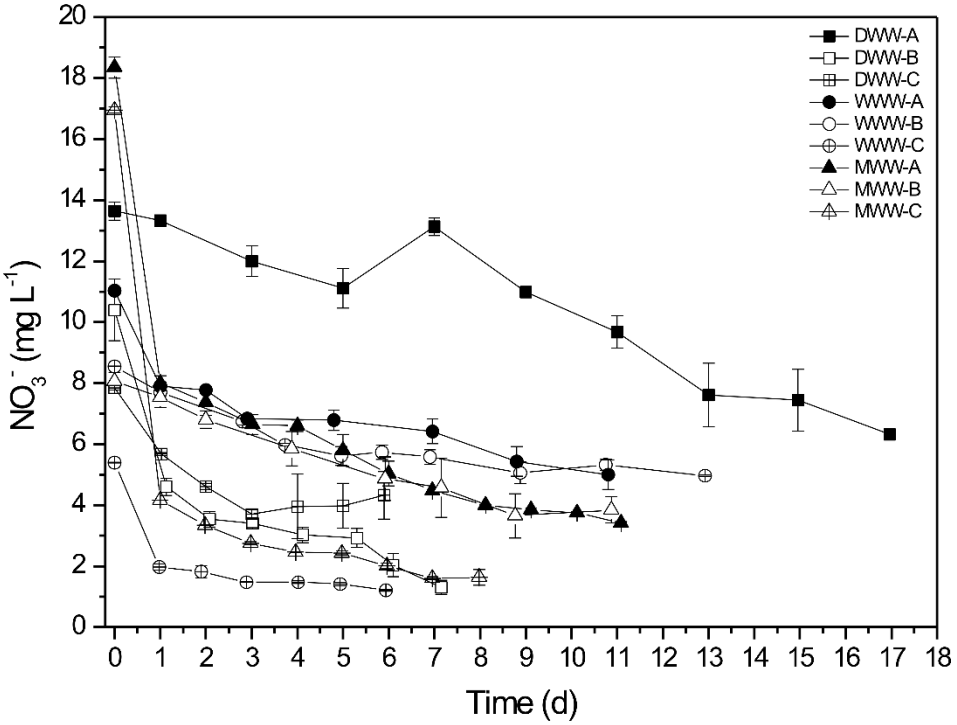


Figure 5. Profile of nitrate removal through time using different dilution ratios (A, B, C) of wastewater as growth medium [DWW: dairy wastewater, WWW: winery wastewater, MWW: mixed (winery and raisin) wastewater].

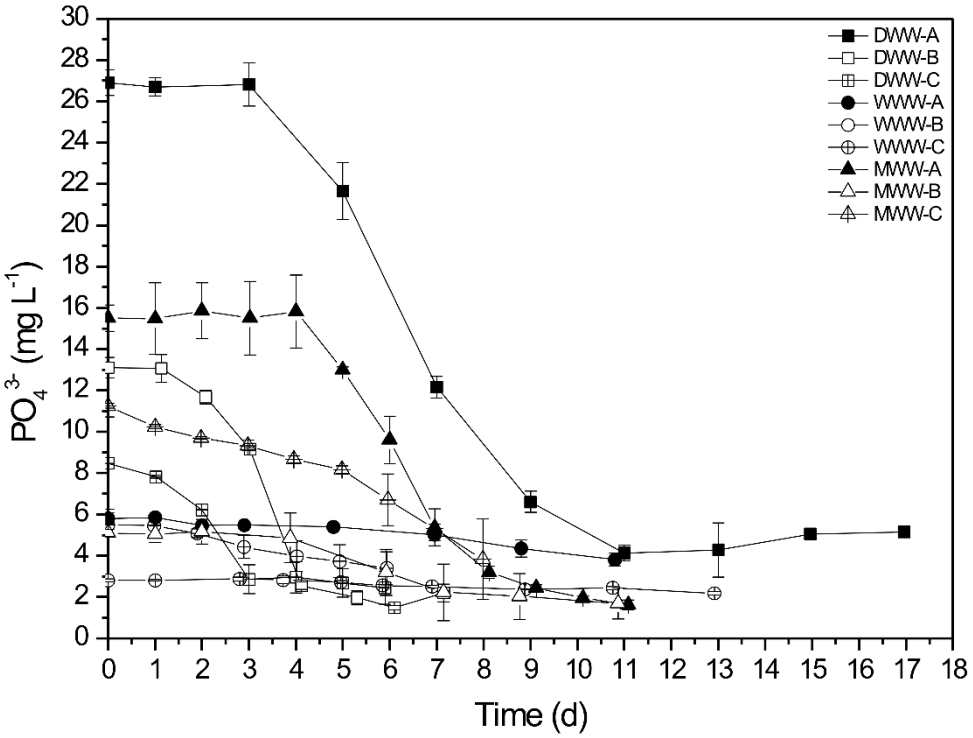


Figure 6. Profile of orthophosphate removal through time using different dilution ratios (A, B, C) of wastewater as growth medium [DWW: dairy wastewater, WWW: winery wastewater, MWW: mixed (winery and raisin) wastewater].

3.4. Lipid Production/Fatty Acid Profile

It is well known that environmental factors such as light, temperature or nutrients/minerals can change microbial lipid metabolism as a result of adaptation. The total and attached lipid contents recorded for all experimental sets were in the range of 9–23% d.w. (Table 4). The highest total lipid content (ranging from 19–21% d.w.) was recorded when using WWW as growth substrate; the substrate that also presented the lowest attached biomass productivities. This inconsistency was also noted in comparable studies treating agroindustrial wastewaters in suspended growth systems [1,78]. High total lipid content values were also recorded in MWW-C (18.6% d.w.) and MWW-A (16.2% d.w.). However, values of attached lipid content were highest in set WWW-A where they reached a maximum of 23.2% d.w. Singh and Thakur [38] were also found similar lipid contents (24.8% d.w.) for *Leptolyngbya* sp. A *Leptolyngbya*-based microbial consortium in a suspended growth system and using winery wastewater as substrate presented lower values of lipid content ranging between 7 and 11% d.w. [1]. Economou et al. [43] investigated a *Limnothrix*-based system using synthetic wastewater and the same experimental design as in the present study, and recorded a total lipid content of 21% d.w. and 24.14% d.w. in the attached dry biomass [43]. It appears that lipid production is strain and experiment-dependent. According to literature ratio of C/N/P not only affect the growth rates and nutrient uptake but also the lipid production [79]. For instance, in the present study, the two highest lipid content values occurred with N/P ratios of about 4 (21%, N/P = 4.33 for WWW-A and 18.6%, N/P = 4.83 for MWW-C). Significant differences in attached lipid content were observed between sets WWW-A-B-C (p = 0.0355) and between all experimental groups DWW-WWW-MWW (p = 0.00165). The different initial nutrient concentrations of experimental sets are likely contributed to the previously reported differences.

The reliability of microbial extracted oil for biodiesel applications depends not only on the quantity of oil produced but also on its fatty acid (FA) composition. Usually, unsaturated FAs content decreases biodiesel stability and increases NOx emission. Hence, the proposed profile should include high

amounts of saturated and monounsaturated FAs with low levels of polyunsaturated FAs. Lipid analysis was performed at the end of exponential and early stationary growth phases and the generated FA profile is shown in Figure 7. The results revealed that the cultivation conditions influence both the growth pattern and the quality of the biodiesel products. In all substrates tested the major FAs detected were: C18:1 (7–39%), C16:0 (20–23%), C16:1 (4–18%), C18:2 (7–29%) and C18:0 (2–8%), which are the most frequently detected FAs in biodiesel [80]. Specifically, C18:1, which is regarded as appropriate for biodiesel, presented the highest content in WWW (with the oiliest biomass), exhibiting the same behavior as in suspended growth systems [1]. Additionally, C18:3 content was below the value of 12% in all experiments thus indicating the profile's suitability for vehicle use according to European Biodiesel Standards EN14214 [81]. According to the literature, the perfect candidate for biodiesel also contains a small carbon chain length from C16–18, as well as saturated FAs with mono or di-unsaturation [82,83]. Therefore, the summary value of C14:0, C16:0, C16:1, C18:0, C18:1 and C18:2 was estimated in all tested substrates. The highest amounts of these FAs were recorded in the WWW substrate (85.3%), followed by MWW and DWW with 78.7% and 77.7%, respectively. Experiments with stock culture media presented FAs of 77%. It should be mentioned that in previous research with suspended Leptolyngbya-based systems the highest values of these FAs were recorded in MWW (89.13%) [1]. The change in FA profile may be a type of protecting mechanism that helps microorganisms to acclimate to changing environmental conditions. It has been previously reported that the composition of microalgal lipids can be altered by changing various physical conditions during cultivation, including feedstock [84,85]. Further research is required to find out the scalability of this culture concept and to enhance the FA content. According to literature increase of lipid content in microalgae and improvement on lipid extraction efficiency can be performed by manipulating the cultivation conditions or/and by controlling the extraction steps [86].

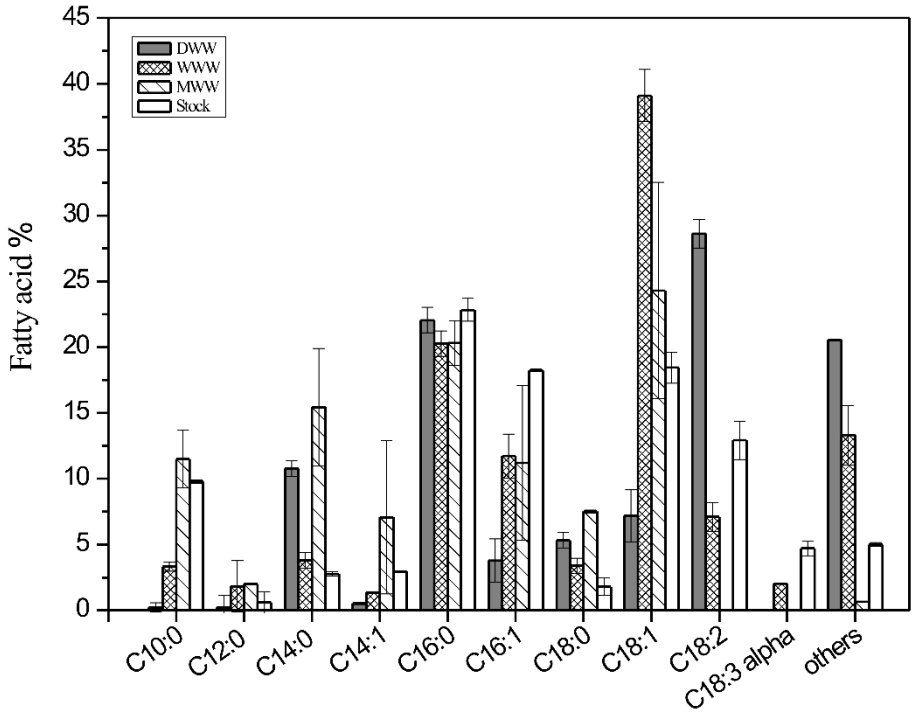


Figure 7. Fatty acid analysis of the lipids produced by the microbial consortium in attached growth systems cultivated in the all substrates (DWW: dairy wastewater, WWW: winery wastewater, MWW: mixed wastewater, Stock: synthetic medium).

The FA methyl ester values recorded here are similar with earlier recorded data in studies using *Leptolyngbya* sp. and *Limnothrix* sp. [43,87], with carbon chain sizes ranging from C12 to C18, dominated

by C16:0 and C18:1. FA profiles observed in this study indicate the suitability of the produced microbial oil for biodiesel production.

Finally, the BiodieselAnalyzer© software was used for analyzing theoretically biodiesel properties [88]. According to European standards, vehicular biodiesel should have a cetane number and an oxidation stability of a minimum of 47 and 6 h, respectively, while an iodine value lower than 120 g I_2/100 g [89]. The estimated biodiesel properties of fatty acids contained a higher cetane number (56.86–65.22) and a lower iodine value (33.03–71.06 g I_2/100 g) and a higher oxidation stability (6.71–15.54 h) as shown in Table 5.

Table 5. Theoretical biodiesel properties of the microalgal mat based on their fatty acid composition in different substrates (DWW: dairy wastewater, WWW: winery wastewater, MWW: mixed winery and raisin wastewaters, Stock: synthetic medium).

Biodiesel Properties	DWW	WWW	MWW	Stock
Saponification value (mg KOH/g fat)	165.96	197.61	212.81	205.56
Iodine value (g I_2/100 g)	62.07	65.23	33.03	71.06
Cetane number	65.22	59.24	64.52	56.86
Long chain saturated factor	4.86	3.71	5.79	3.18
Cold filter plugging point (°C)	−1.20	−4.86	1.71	−6.48
Cloud point (°C)	6.61	5.67	5.69	7.02
Allylic position equivalents	64.43	57.31	24.30	56.69
Bis-allylic position equivalents	28.63	11.12	19.25	22.316
Oxidation stability (h)	6.71	15.54	7.81	9.28
Higher heating value (mJ/kg)	30.62	36.25	35.72	35.77
Kinematic viscosity (mm^2/s)	2.68	3.26	2.94	2.93
Density (g/cm^3)	0.684	0.809	0.804	0.806

4. Conclusions

A rich in OTU's mixed microbial community, dominated by cyanobacteria and in taxon richness by bacteroidetes and firmicutes, was investigated in an attached photobioreactor system (using glass rods as support material to provide long-term operation conditions and allow light penetration) for its efficiency to remove organic and inorganic pollutants from agroindustrial wastewater effluents (dairy, winery, mixed winery and raisin effluents). The effect of initial pollutant concentrations on biomass production and lipid content was examined. High d-COD removal rates (up to 97.4%) and reduction in nitrogen (up to 97%) and phosphorus (87.4%) concentrations were observed for all substrates used. In fact, winery wastewater lead to d-COD removal rates of up to 95% in all experimental sets. Diluted dairy wastewater achieved the highest attached biomass productivity (5.03 g m^{-2} day^{-1}) and the highest specific growth rate (μ = 0.925 day^{-1}). The overall attached microbial biomass contained 10–23.2% lipids that were dominated by saturated and monounsaturated FAs thus indicating its suitability for biodiesel production. The above results indicate that the attached growth photobioreactor presented here can effectively treat agroindustrial wastewaters and simultaneously produce biomass suitable for biodiesel production, reducing significantly the cost of biodiesel production and environmental impacts. However, further research is needed to improve wastewater treatment as well as to enhance microbial growth rates and thus improving the sustainability of this technology. The most significant advantage of attached systems is that a harvesting step is more inexpensive or is even not required. Avoiding this expensive and time-consuming step deems microbial growth and lipid production more feasible. Some of the factors that need to be optimized for large-scale application of the proposed treatment system include seed culture preparation, uniform distribution of nutrients, light regime, bioreactor configuration, physicochemical parameters, biomass and lipid yield optimization and, primarily, harvesting and lipid extraction. To enhance the dominance of the cyanobacteria-based culture in field studies and large-scale treatment,

the bioaugmentation process could be applied. Addition of the specific consortium would increase the existing microbial population and guarantee the efficiency of the entire biotreatment process.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/10/11/1693/s1, Figure S1: Photographs of the experimental photobioreactors PBRs showing the gradual increase of microbial biomass in attached culture systems with dairy wastewater as growth substrate (1th to 7th day of culture), Figure S2: Visual increase of microbial biomass on glass rods in attached growth culture systems, Figure S3: Profile of suspended and total biomass production through time using different dilution ratios (A, B, C) of wastewater as growth medium [DWW: dairy wastewater, WWW: winery wastewater, MWW: mixed (winery and raisin) wastewater].

Author Contributions: Conceptualization, A.G.T. and D.V.V.; Methodology, A.G.T, D.V.V, G.A., M.M.-G. and O.N.T.; Validation, O.N.T., S.G.; Formal Analysis, C.S.A., O.N.T., S.G.; Investigation, O.N.T, S.G.; Writing-Original Draft Preparation, O.N.T., M.M.-G., S.G and A.G.T.; Writing-Review & Editing, A.G.T., D.V.V., G.A., C.S.A., M.M.-G. and O.N.T.

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