Inoculum of indigenous microalgae/activated sludge for optimal treatment of municipal wastewaters and biochemical composition of residual biomass for potential applications.

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Abstract

In this work, we analysed the effect of inoculating different ratios of autochthonous microalgae/activated sludge in municipal wastewaters to optimise the bioremediation and to investigate the potential application of the residual biomass. One of the employed microalgal strains, *Chlorella sp* CW2, was isolated from the activated sludge itself and molecularly characterised in this work for the first time, while the other one, *Chlorella sp* Pozzillo, was isolated in previous work. Microalgae/activated sludge growth curves were measured by using a UV-vis spectrophotometer and a fluorimeter to distinguish the contribution of the photosynthetic microorganisms to the total growth. The effectiveness in abating the COD, total nitrogen and total phosphorous content was assessed. Overall, the best abatement is achieved by the microalga *Chlorella sp* CW2 inoculated with activated sludge in the ratios 1:2 and 2:1, with a decrease of – 81.39%±0.56, -86.12%±0.43, -82.89%±2.66 and -82.5%±0.83, -72.66%±0,46, -97.15%±0.44 of COD, total nitrogen and total phosphorous, respectively. The residual biomass was also analysed for its content of carbohydrates, lipids, and fatty acids f their future valorisation and application. Considering the final composition of the obtained biomass and its remediation potential, the most promising sample may be the microalga *Chlorella sp* CW2 inoculated with activated sludge in the ratio 1:5.

Keywords

Wastewater treatment; Microalgae; Activated sludge; Heterotrophic bacteria; Biomass valorisation

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1. Introduction

Microalgae are unicellular and photosynthetic microorganisms able to use inorganic compounds such as carbon dioxide, nitrate and phosphate as a nutrient source. Microalgae are exploited in several fields, ranging from nutraceuticals and pharmaceutics, thanks to their content in high-value biocompounds [1], to biodiesel because of their lipid content [2] and several strategies have been proposed to enhance the productivity of their high-value biocompounds [3,4]. Across the variety of applications that have been explored in the last 20 years, a recent trend involves selecting indigenous microalgal population for exploiting it in regional applications, an approach called phycoprospecting [5]. The reason for this choice is that microalgae harvested from an environment are already adapted to its solicitation and then more resistant when employed in industrial applications. This idea was already applied to Sicily for different applications [6–8].

Several studies suggest to couple wastewater treatment and microalgal cultivation for several reasons: microalgae can well employ nutrients such as nitrogen and phosphorous to sustain their growth producing a biomass rich in high-value compounds such as lipids or carotenoids and at the same time mitigating carbon dioxide in the atmosphere [9–11]. The removal of these compounds is a priority for wastewater treatment plants since excess nitrogen and phosphorus in discharged wastewater can lead to downstream eutrophication and ecosystem damage [12]. Furthermore, the traditional wastewater treatment plants in Sicily are normally not sized to treat high nitrogen concentrations and are often highly contaminated [13]. The biological removal of nitrogen in traditional wastewater treatment plants involves additional denitrification steps to the oxidation ponds that are time-consuming and expensive. Therefore, microalgae may be well applied for the improvement of the tertiary treatment of urban wastewaters, in particular when wastewaters plants deliver in sensitive areas, in which the nitrogen and phosphorous concentrations have to be particularly low, for example because of the proximity to bathing locations. Several studies showed how microalgae in wastewater plants may work simultaneously with the heterotrophic microorganisms that are part of the activated sludge (AS) [14]. In fact, they produce oxygen that can be later used by the heterotrophic bacteria to degrade organic carbon in the matrix, reducing the need of mechanical aeration [15]. Microalgae produce compounds that are useful to the bacterial community, such as supplemental carbonic source while heterotrophic bacteria produce compounds such as vitamins useful to microalgae [16]. Remarkably, microalgae and bacteria may produce biofilm or aggregates that enhance the harvesting efficacy [17–19]. Although many studies were performed on this topic, only few of them analysed the ideal ratio between microalgae and activated sludge and the possible applications of the obtained biomass.

Furthermore, none of them addressed the topic of simultaneously reducing the COD, nitrates and phosphates concentration for delivering in sensitive areas. For this reason, in this work, we analysed the effect of inoculating two autochthonous microalgal strains and activated sludge in different ratios in municipal wastewaters in order to obtain a positive interaction between the microalgae and the heterotrophic bacteria and to identify the best combination for bioremediation purposes. One of the strains was isolated and molecularly characterised in this work for the first time. We measured the growth curves and the abatement of COD, total phosphorous and total nitrogen for each sample. We also analysed the residual biomass for its content in carbohydrates, lipids and fatty acid and suggested the best application for its valorisation.

2. Materials and Methods

2.1. Molecular identification

Chlorella sp CW2 was isolated by the combination of filtration and dilution methods. Once the individual strain was obtained, the liquid cultures were transferred into a solid medium to obtain individual colonies that were used for molecular characterization by colony polymerase chain reaction (PCR). Molecular characterization was performed by using Platinum™ II Hot-Start PCR Master Mix (Invitrogen, Thermo Fisher Scientific), the forward primer A (5′-ACC CTG GTT GAT CCT GCC AG-3′) and primerSSU-inR1 (5′-CAC CAG ACT TGC CCT CCA-3′). For the Colony PCR the following program was used: 94 °C (2 min), 25 cycles of 94 °C (15 s), 60 °C (15 s) and 68 °C (30 s) and a final 1 min extension step at 68 °C. The PCR products were run on 1% of agarose gel (RedSafe™, Chembio 0.1 µL/mL) against Thermoscientific GeneRuler 100 bp Plus DNA Ladder. The PCR products were purified and sequenced by BMR service genomics. The sequences obtained were then analysed using online bioinformatics software BLAST for the alignment of sequences and the identification of the belonging species.

2.2. Microalgal growth in sewage

Microalgae *Chlorella sp* Pozzillo (CP), previously isolated from a green bloom in a polluted marine water area [8], and microalgae *Chlorella sp* CW2 (CS), isolated in the present work, were grown in municipal sewage coming from AMAP plant of Balestrate, 90041, PA, Italy. Microalgae were inoculated in the municipal sewage in addition to Activated Sludge (AS) in different ratios (w/w) according to Table 1:

Table 1: The initial addition of algae and activated sludge.

Sample ID	Algae/activated sludge ratio	Employed microalgae
AS	0:1	-
CP 1:0	1:0	*Chlorella sp* Pozzillo
CP 5:1	5:1	*Chlorella sp* Pozzillo
CP 1:5	1:5	*Chlorella sp* Pozzillo
CP 2:1	2:1	*Chlorella sp* Pozzillo
CP 1:2	1:2	*Chlorella sp* Pozzillo
CS 1:0	1:0	*Chlorella sp* CW2
CS 5:1	5:1	*Chlorella sp* CW2
CS 1:5	1:5	*Chlorella sp* CW2
CS 2:1	2:1	*Chlorella sp* CW2
CS 1:2	1:2	*Chlorella sp* CW2

The three samples respectively of sludge and the two *Chlorella*, AS, CP 1:0 and CS 1:0, were cultivated in the sewage as control. In order to test the hypothesis that in the Activated Sludge harvested in the treatment oxidation tank there was a photosynthetic community, it was treated with a commercial herbicide (Buggy®) before inoculating it in the sewage. A pre-culture for each sample was set up by inoculating 10 mL of sample from a stock culture in 100 mL of sewage. When cells were in the late exponential phase (after about 8 cultivation days), 10 mL of the cell suspension were used to inoculate the sewage. 150 mL of culture were grown in 500 mL Erlenmeyer flasks placed in an oscillating incubator (Corning Lse) under a 127 $\mu E/m_2$ s photon flux with a photoperiod light/dark of 12 h at 27°C. Light intensity was measured with a Delta Ohm-HD 9021 quantometer equipped with a Photosynthetic Active Radiation (PAR) probe (Delta Ohm LP 9021 PAR). The algae were cultivated for 10 days. The concentration of the microalgal suspension was checked by daily measuring the absorbance at 750 nm in a spectrophotometer (Cary 60 Uv-vis, Agilent technologies) and in a fluorometer (Cary Eclipse Fluorescence Spectrophotometer, Agilent Technologies, Ex. 430 nm – Em. 670 nm) in order to distinguish the contribution of photosynthetic microorganisms. Furthermore, the cultures were observed at the end of the cultivation with an optical microscope B-800 (Optika). For each condition, a biological triplicate ($n = 3$) was performed.

2.3. Harvesting of microalgal biomass

After the growth, the cell suspension was centrifuged (3600 rpm, 10 min, NEYA 10R) and the biomass was frozen in liquid nitrogen and freeze-dried for 48 h in a bench lyophilizator (FreeZone 2.5 L, LABCONCO, US). The biomass was then stored at -20°C for further analysis.

2.4. Extraction and analysis of fatty acids

About 20 mg of lyophilized microalgae biomass was weighted in a glass tube and about 7 mg of glass beads were added. Then 5 mL of chloroform/methanol (2:1, v/v) and 1 mL of NaCl 1% were added and the mixture was vigorously mixed with a laboratory vortex and subsequently centrifuged until the formation of two phases. The lower phase (chloroform phase) was transferred in a pre-weighted tube and the solvent was evaporated under a nitrogen stream. After complete solvent evaporation, total lipids were determined gravimetrically and subsequently transesterified by adding 1 mL of sodium methoxide (1 g NaOH in 100 ml MeOH) and 1 mL of hexane and kept at 50°C for one hour. 1 μL of the upper phase was then analysed by gas chromatography using a GC 7890B System (Sigma-Aldrich, US) equipped with an FID detector and a capillary column Omegawax 250 (Sigma-Aldrich, US). The initial temperature was 50 °C, increased to 220 °C as working temperature. Total analytic time was 79.5 min and argon was used as the carrier. The quantification of lipid was performed by comparing samples chromatograms with the standard. Supelco 37-Component FAME Mix (Sigma-Aldrich, US) was used as standard. Measurements were done in triplicate (n = 3) and the average value was retained.

2.5. Total carbohydrates

Total carbohydrate content was determined according to Trevelyan et al. [20]. Briefly, 10 mg of freeze-dried biomass were suspended in 6 mL HCl 2 N and hydrolysed in a water bath for 1 h at 100 °C. Subsequently, 4 mL of a fresh anthrone solution (Sigma-Aldrich, 2 mg mL^{-1} in 95-97% H_2SO_4) were added to 1 mL of sample extract. The absorbance of each sample was read at 620 nm (Cary 60 Uv-vis, Agilent technologies). Aliquots of different glucose concentrations (0.02-0.1 mg L^{-1}) were prepared and processed in the same way as microalgal extracts, to obtain a calibration curve. Measurements were done in triplicate (n = 3) and the average value was retained.

2.6. Sewage analysis

Pre-treated sewage coming from the municipal treatment plant AMAP located in Balestrate, 90041, PA, Italy, was used. This batch was analysed for COD, total phosphorous (TP) and total nitrogen (TN) and then stored at -20°C until it was used for microalgae inoculation. After

147 microalgal growth, the sewage was filtered (11 μm, Whatman filter paper) and the above analyses
148 were repeated. The COD analysis was performed following the ISPRA Method 5135 by employing
149 cuvette test LCK 514, Hach Company. TN and TP analysis were performed by a breakdown
150 procedure following UNI EN ISO 11905-1 followed by a colorimetric reaction according to DIN
151 38405-9 for TN and UNI EN ISO 6878 for TP. For the colorimetric analysis a spectrophotometer
152 UV-VIS DR6000, Hach Company, was employed. Measurements were done in triplicate (n = 3)
153 and the average value was retained.

154 2.7. Statistical analysis

155 Data in biological triplicate were tested for statistical significance of the variations in different
156 samples via one-way ANOVA analysis. Tukey test was employed to analyse the differences
157 among samples with a confidence interval of 95% and a tolerance of 0,0001. Results are shown
158 as means and standard deviations are reported as error bars.

159 **3. Results and discussion**

160 3.1. Microorganisms' growth

161 Microalgal/activated sludge mixed cultures were analysed by reading the absorbance at 750 nm
162 and the fluorescence (ex 430-em 670) of the suspension. This technique was adopted to highlight
163 the contribution of photosynthetic microorganisms to the total growth. In fact, by measuring the
164 absorbance at 750 nm, it is possible to detect every particle present in the suspension that can
165 absorb light at that wavelength. The measurement of the fluorescence, instead, ensures that only
166 chlorophyll is measured. Although the analysis of fluorescence is adopted in microalgal field, to
167 the best of the author's knowledge this approach has been adopted in the past only in other
168 contexts [21] and not for analysing microalgal-bacterial consortia. In Figure 1 the growth curves
169 of the activated sludge (AS) are reported. Orange and blue symbols refer to AS and AS+ Herbicide
170 (AS+H) measured by absorbance method, while green and yellow symbols refer to the same
171 cultures measured by fluorescence. Firstly, after about 7-8 days of cultivation, a signal of
172 fluorescence is detected in the control containing only activated sludge (AS) (green line in Figure
173 1), meaning that in the AS, harvested in the treatment oxidation tank, there is a certain
174 photosynthetic community. To test this hypothesis, the AS was also treated with a commercial
175 herbicide (Buggy®) before inoculating it in the sewage (blue and yellow lines of Figure 1). As it
176 can be seen, the untreated sample (no herbicide) shows a similar trend of fluorescence and
177 absorbance, while the treated one shows almost no fluorescence at all. The obtained biomass was

also observed through an optical microscope, as shown in Figure 2 a and b, confirming our hypothesis. In Figure 2 a, showing the untreated biomass, it is possible to observe a good variety of photosynthetic microorganisms (e.g. some dyatoms and green algae), while only bacteria are shown in Figure 2 b. The presence of microalgae in activated sludge was also observed in the past by other researchers [22,23].

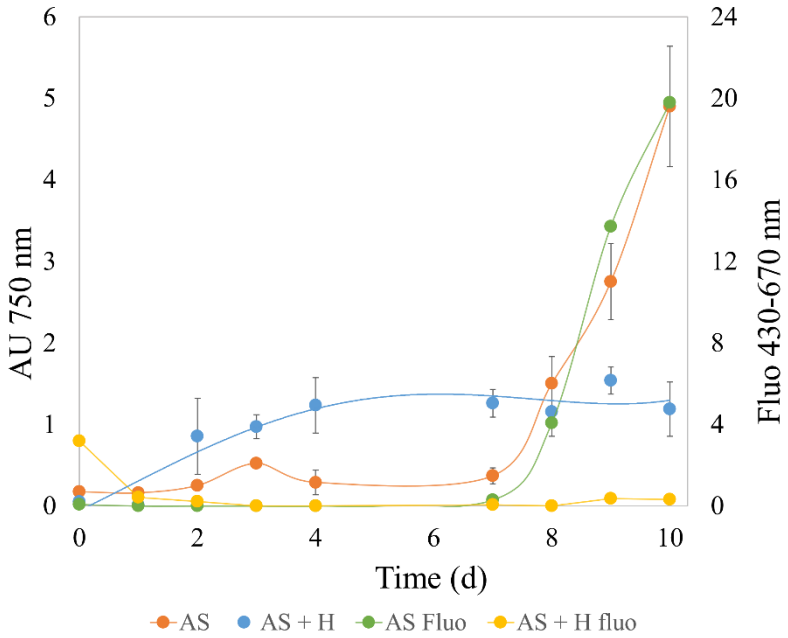

Figure 1: Growth curves of the activated sludge grown in sewage with or without a commercial herbicide: AS, Activated Sludge growth curve measured by absorbance method; AS + H, Activated Sludge + Herbicide growth curve measured by absormance method; AS Fluo, Activated Sludge growth curve measured by fluorescence; AS + H Fluo, Activated Sludge + Herbicide measured by fluorescence.

Considering the variety of the sample shown in Figure 2 a, we decided to isolate a photosynthetic strain from the AS. The reason for this choice was that, accordingly to the idea of "phycoprospecting" [5], microalgae are well-adapted to the environment in which they are isolated, and may be employed in the same environment in order to exploit their potentialities.

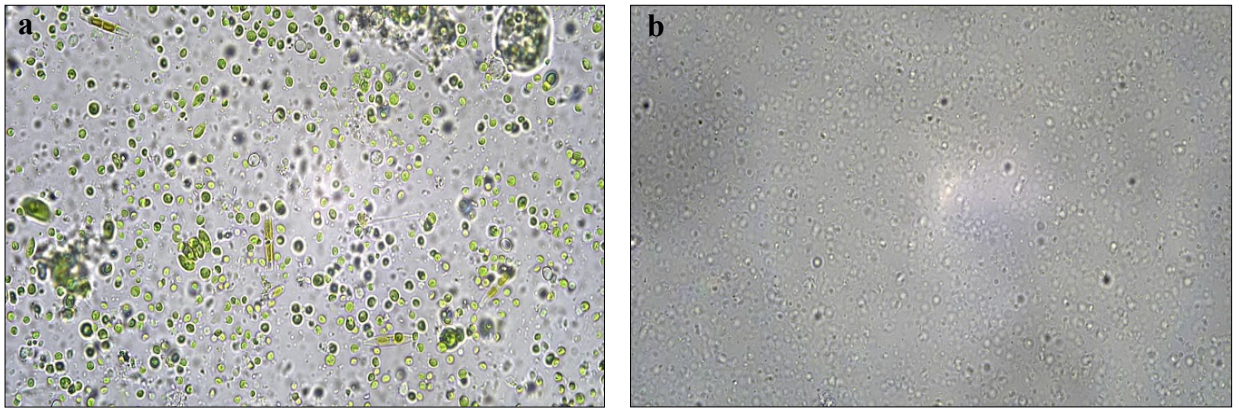

Figure 2: Optical microscope observation of the activated sludge grown in sewage a) without herbicide; b) with herbicide. Magnification: 40x.

Molecular characterization of the isolated microalga was obtained using universal primers for the amplification of 18S. The PCR using the universal primers for the amplification of 18S gave one product of the expected size (Figure 3). The obtained sequence was deposited in Genbank database: *Chlorella sp* CW2 with the accession number OM905735.

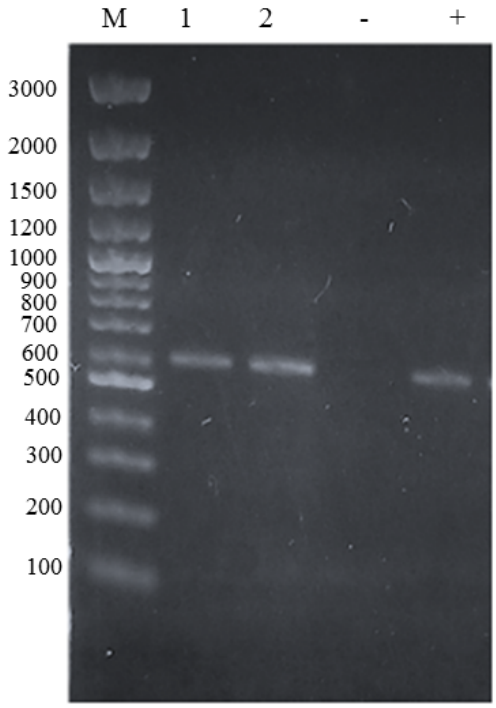

Figure 3: Gel electrophoresis of PCR product on 1% agarose gel; M: molecular marker, +: positive control, -: negative control, 1 and 2: 18S rDNA PCR product of the culture *Chlorella sp* CW2.

Table 2 shows the percentage of identity of the DNA sequence of 18S rRNA gene from *Chlorella sp* CW2 with other known *Chlorella* strains, including the *Chlorella sp* Pozzillo employed in this work.

Table 2: Identity of the DNA sequence of 18S rRNA gene from *Chlorella* sp CW2 with other known *Chlorella* strains.

Isolate species	Target	Identity	Accession
***Chlorella sp* Pozzillo**	18S ribosomal RNA gene	97%	MT259188.1
***Chlorella kessleri*, strain SAG 211-11g**	18S ribosomal RNA gene	98%	X56105.1
***Chlorella vulgaris* UMT-M1**	18S ribosomal RNA gene	98%	KJ561358.1

As it can be seen in Table 2, the percentage of identity with the three reported species is always below 98%, indicating that the isolated strain is genetically diverse from the other *Chorella* strains.

Microalgae *Chlorella sp* Pozzillo and *Chlorella sp* CW2 were then inoculated in real sewage in different ratios (w/w) to activated sludge as shown in Table 1. It is worth noting that the original concentration of *Chlorella sp* CW2 present in the activated sludge is in any case always negligible with respect to the inoculated quantities in the experiments. In Figure 4 the growth curves of the eleven inoculation samples (see Table 1) are reported. As it can be seen, microalgae without the addition of AS (blue line in Figure 4 a and c) reached higher optical densities and fluorescence compared to all the other cultivations. All the other cultures did not show marked differences in absorbance, excluding the control of only AS that seem to enter later into the exponential phase compared to the other cultures.

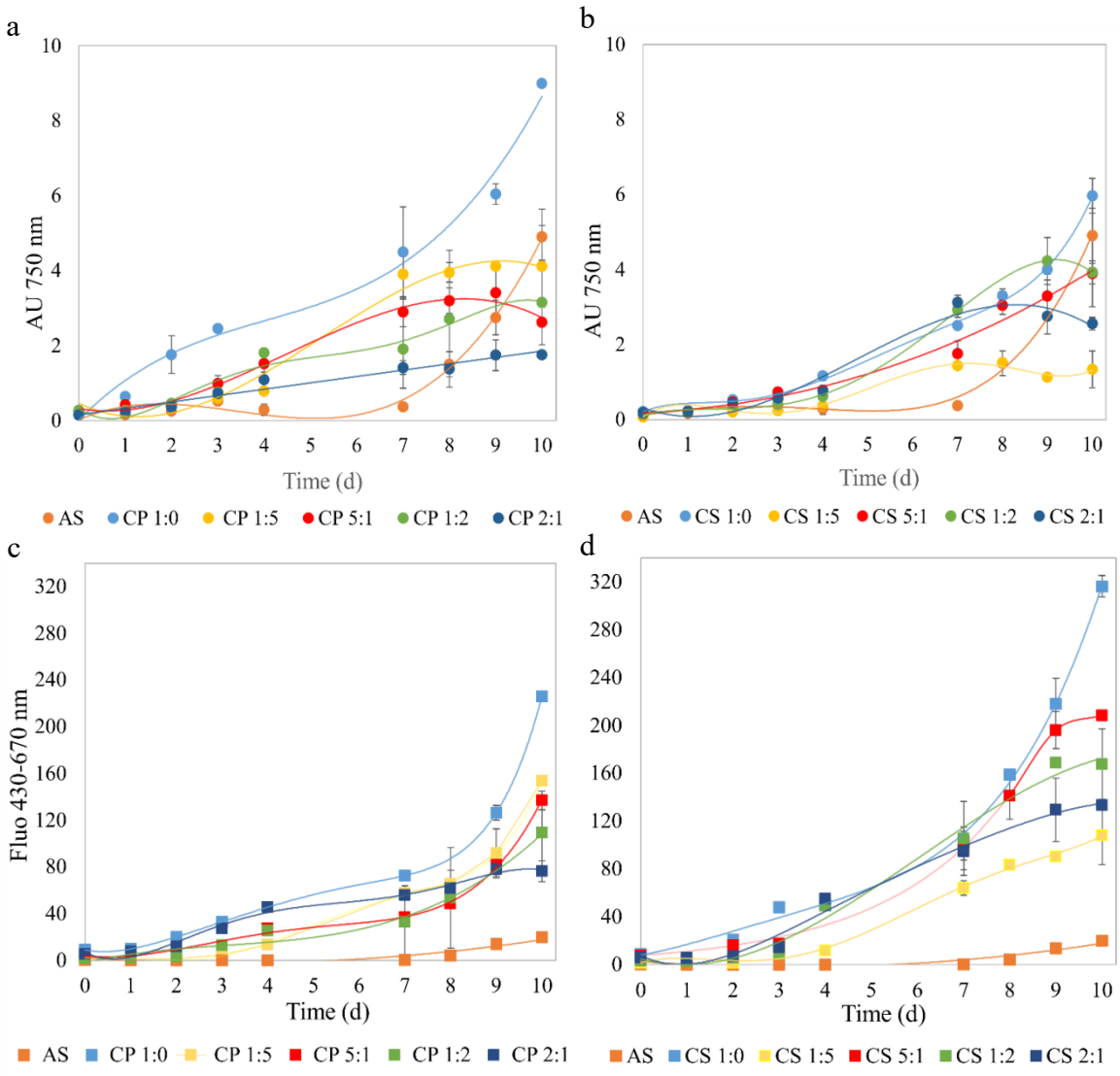

Figure 4: Growth curves of the two microalgal strains (*Chlorella sp* Pozzillo and *Chlorella sp* CW2) inoculated in different ratios (w/w) with activated sludge in municipal sewage. a) and c) show the values of absorbance (750 nm) and the fluorescence (430-670 nm), respectively, of the cultures containing *Chlorella sp* Pozzillo. b) and d), instead, show the same of the cultures containing *Chlorella sp* CW2. Values are reported as means (n = 3) and error bars report the standard deviations.

By analysing the fluorescence curves in Figures 5 b and d, in the first case (Figure 4 b) the fluorescence is not related to the initial ratio of inoculated microalgae, while in Figure 4 d there is a correspondence between the intensity of the fluorescence and the relevant quantity of algae inoculated into the initial sample, indicating that the inoculated microalgae well grew in the sewage. This may be connected to the adaption of *Chlorella sp* CW2 at the sewage and also at the AS itself, which, therefore, did not surmount it.

3.2. Abatement of COD and nutrients by consortia of microalgae and other microorganisms

After the growth, the biomass was harvested and the remaining treated sewage was tested for COD, total nitrogen and total phosphorous and data compared with that of the same analysis before the growth. The biomass was separated from the sewage by filtering the suspension before the analysis; this was necessary to avoid the biomass leading to an increase of the COD because of its carbon content. Figures 5 and 6 and Table 3 report the results of chemical analysis of the sewage before and after the growth of microalgae and microorganisms from AS. In general, COD (Figures 5 a and b and Table 3) is already well abated by the action of the AS (- 76,67±0), while the first microalgae, *Chlorella sp* Pozzillo, leads to a slight increase of the COD (+1,94±0). This increase was already observed in previous work [8] and is probably due to a release of organic compounds by the microalga. The other microalga, *Chlorella sp* CW2 is instead able to decrease the COD (-71,53±0,98), even though less efficiently than AS alone, but anyway below the loyal requirement for municipal wastewaters delivering in Italy (D.Lgs 152/06). A reason behind the failure of decreasing the COD of *Chlorella sp* Pozzillo may be that it cannot shift its metabolism to heterotrophy/mixotrophy and is forced to exploit the autotrophic one. To test this hypothesis, we performed a respirometric test providing glucose as the carbon source in the darkness and observed that *Chlorella sp* Pozzillo and *Chlorella sp* CW2 were both able to produce oxygen, meaning that them both can shift from autotrophic to mixotrophic/heterotrophic metabolism (data not shown). Therefore, a possible explanation to the failure of decreasing COD may be that *Chlorella sp* Pozzillo is not adapted to the carbon sources present in the sewage. In support of this hypothesis, other authors showed that *Chlorella sp* may differently respond to diverse organic carbon sources [24,25] and that the complex nature of the organic compounds present in the sewage may alter the microalgal behavior [26]. *Chlorella sp* CW2, instead, was able to perform a mixotrophic metabolism and therefore to decrease the COD of the sewage. This is probably due to its adaption to the sewage carbon source.

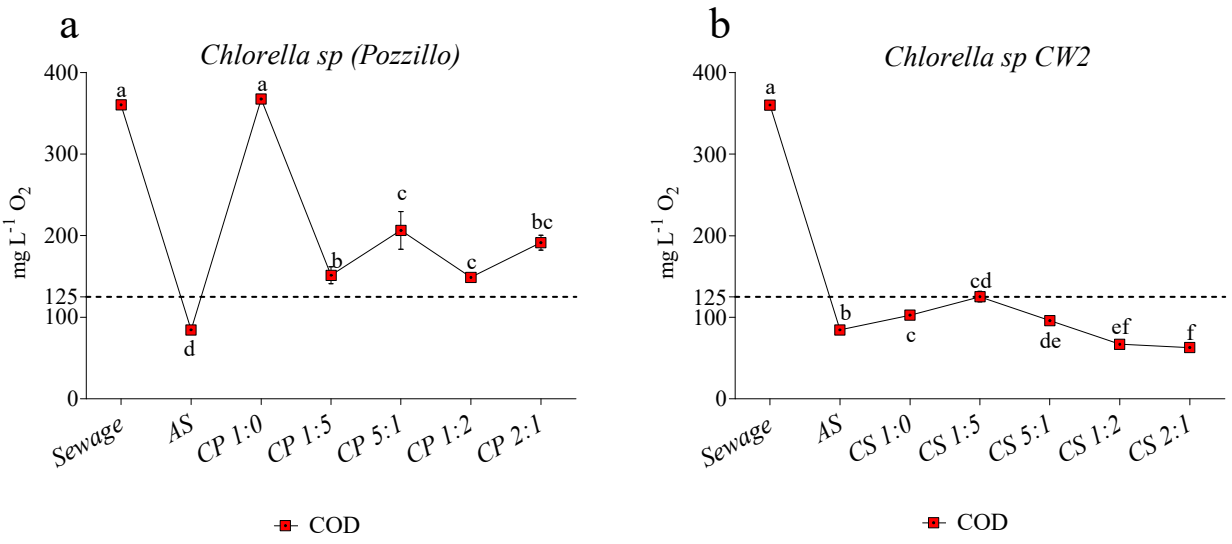

Figure 5: Chemical Oxygen Demand (COD) of the sewage before and after the growth of microorganisms. Values are reported as means (n = 3) and error bars report the standard deviations. Samples with the same letter are statistically identical. Dotted lines indicate the loyal requirements for delivering in sensitive locations of the Sicilian littoral.

The addition of microorganisms coming from the AS led to a better abatement of COD compared to the microalgal action alone, for both the algae as reported in Figures 5 a and b. When *Chlorella sp* Pozzillo was grown into the sewage, the COD levels were still beyond to the loyal requirement for urban wastewaters delivering in Italy (Figure 5 a) (D.Lgs 152/06). Instead, *Chlorella sp* CW2 added to AS resulted very efficient in removing COD, increasing the removal potential of the AS alone. In particular, the two most effective combinations for COD abatement were those of samples CS 1:2 and CS 2:1 (-81.39%±0.56 and – 82.5%±0.83, Table 3 and Figure 5 b).

Nitrogen removal is of relevance because in some areas of Sicily the concentrations of nitrite and nitrate in wastewater are particularly high probably as a consequence of the abuse of nitrogenous fertilizers [13]. On the other hand, low concentrations of nitrogen (below 10 mg L^{-1}) are needed for delivering in sensitive areas of the coastline, as reported in Figure 6 a and b. As it can be seen, the action of the activated sludge alone is not sufficient to decrease nitrogen concentration below the loyal requirement (the abatement is of the 42.69%±2.43). The addition of both the microalgae strains, instead, increased the percentages of abatement (see also Table 3), reaching nitrogen concentrations below the loyal limit. The best performance is provided by *Chlorella sp* CW2 without the help of AS (CS 1:0, - 93.79%±0.08) and after it by *Chlorella sp* CW2 with AS (CS 5:1, - 90.73%±0.46). As regards the microalgae *Chlorella sp* Pozzillo with activated sludge, the best percentages of removal are provided by the sample CP 5:1 (- 82.61%±2.4).

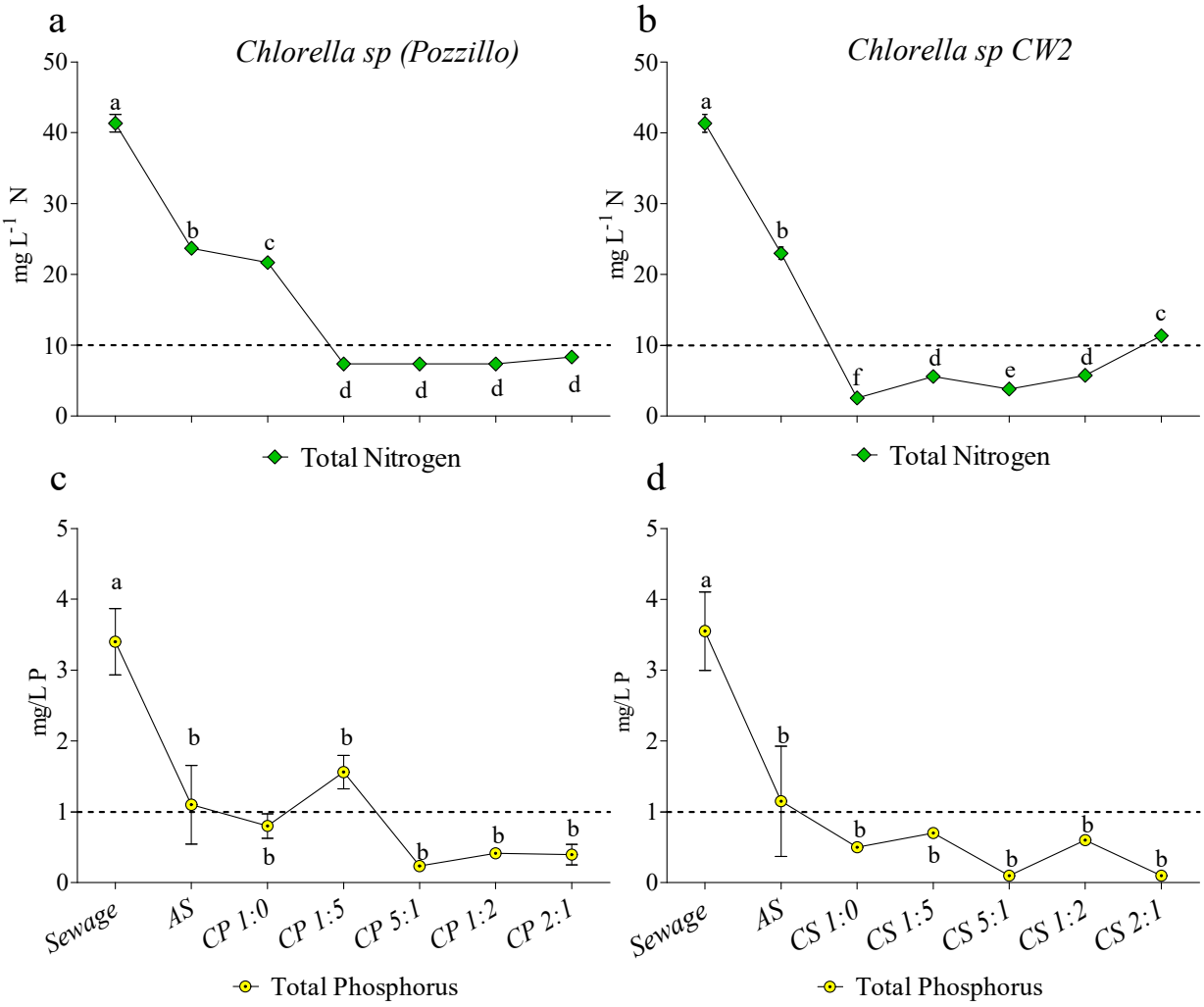

Figure 6: Total Nitrogen and Total Phosphorous of the sewage before and after the growth of microorganisms. Values are reported as means (n = 3) and error bars report the standard deviations. Samples with the same letter are statistically identical. Dotted lines indicate the loyal requirements for delivering in sensitive locations of the Sicilian littoral.

Phosphorous removal, shown in Figures 6 c and d, is efficient in all the experimental conditions. This is because phosphorous, although being a limiting nutrient, is needed in lower concentration compared to nitrogen, and is efficiently removed already by the microorganisms of the activated sludge. The best performances of phosphorous removal are realised by samples CS 5:1 and 2:1 (- 97.15%±0.44 Table 3) and for what concerns the microalgae *Chlorella sp* Pozzillo by sample CP 5:1 (- 94.3%±0.89). We may conclude that the inoculum ratio algae:activated sludge 5:1 is the best one to decrease the nutrient concentrations in our experiments.

Table 3: Percentages of COD, Total Nitrogen and Total Phosphorous removal in the sewage treated by Activated Sludge (AS), *Chlorella sp* Pozzillo (CP 1:0) and *Chlorella sp* CW2 (CS 1:0) alone and combined in different ratios (5:1, 1:5, 2:1, 1:2). Values are reported as means (n = 3) and standard deviations is reported.

Sample ID	% of removal of COD	% of removal of Total Nitrogen	% of removal of Total Phosphorus
AS	-76.57±0.16	-42.69±2.43	-65.49±27.27
CP 1:0	+2.04±0.16	-48.36±1.95	-80.04±3.1
CP 1:5	-57.96±2.85	-82.46±2.2	-53.94±16.32
CP 5:1	-42.69±6.43	-82.61±2.4	-94.3±0.89
CP 1:2	-58.7±0.16	-82.22±1.93	-87.45±1.95
CP 2:1	-46.85±2.52	-79.72±1.93	-88.46±3.78
CS 1:0	-71.48±0.7	-93.79±0.08	-85.74±2.21
CS 1:5	-65.19±1.79	-86.53±0.28	-80.04±3.1
CS 5:1	-73.33±1.47	-90.73±0.46	-97.15±0.44
CS 1:2	-81.39±0.56	-86.12±0.43	-82.89±2.66
CS 2:1	-82.5±0.83	-72.66±0.46	-97.15±0.44

The idea of inoculating algae and activated sludge in different ratios was firstly reported by Su et al. [27]. The researchers found that COD is better removed when algae and other microorganisms worked synergistically, in a similar manner to the present work. Furthermore, they also found that the best ratio microalgae/bacteria for N and P removal was 5:1. Also in that case the employed algae were isolated from the wastewaters, and this explains the similar results we obtained. Other authors, conversely, found different optimal ratios: for *Chlorella vulgaris*/AS=10/1 COD and N−NH4 removal efficiencies were of 85 and 86.3 % respectively [28]; for *Chlorella vulgaris*/AS=0.75/1 nitrogen removal was of 97–98% [29]; for *Chlorella sp*/AS 3:1 and 1:1 COD reduction were 37,5%-45.7% respectively [30].

Samer, 2015, indicates an ideal C:N:P ratio for optimal biological treatment of wastewaters 100:20:1 to 100:5:1. In our case, in the starting sample, the ratio between C:N:P was 100:10:1. Nevertheless, the activated sludge alone was not useful in removing the nitrogen concentration under the loyal requirement. This goal may be easily reached by adding known concentrations of alive microalgae to the wild microorganisms naturally occurred in the oxidation tanks. Moreover, the presence of microalgae within the activated sludge may be useful in the separation process of the treated water, as microalgae may be involved in the autoflocculation that occurs in activated sludge [32,33].

3.3. Biochemical analysis of the biomass

The obtained biomass was analysed to assess which kind of application may be advantageous for its valorisation. For this reason, the total carbohydrate and total lipid content were assessed, and results are shown in Figure 7. As it can be seen, the content of lipids is higher in the sample CP 1:0 constituted by the only microalga *Chlorella sp* Pozzillo grown in sewage (31,77%±2,5), while it is lower in all the other samples. This result is coherent with Gao et al. results [34], while Feng et al. found that *Chlorella vulgaris* grown continuously in wastewater has a lipid content higher than that reported in the present work, e.g. 42% in average [35]. For what concerns samples containing *Chlorella sp* CW2, the lipid content does not significantly vary when varying the ratio with activated sludge, ranging from 24,86%±1,14 of CS 1:0 to 14,07%±1 of CS 5:1. Anyway, Peng et al. showed that the concentration of lipids in *Chlorella vulgaris* grown in wastewaters may significantly vary in presence of different organic carbon sources [36]. This may explain the differences that can be found across different researches.

As regards the carbohydrate content in the samples containing *Chlorella sp* Pozzillo, it is highest in the sample CP 2:1 (21.13%±1.47); for what concerns *Chlorella sp* CW2, the carbohydrate concentration ranged from 34.04%±8.46 of CS 1:0 to 4.43%±0.84 of CS 2:1. Wang et al. found higher values of carbohydrate content in *Chlorella vulgaris* cultivated in swine wastewater [37], while Peng et al. showed that the concentration of carbohydrates in *Chlorella vulgaris* grown in wastewaters with different organic carbon sources was around 30% [36], similarly to this work.

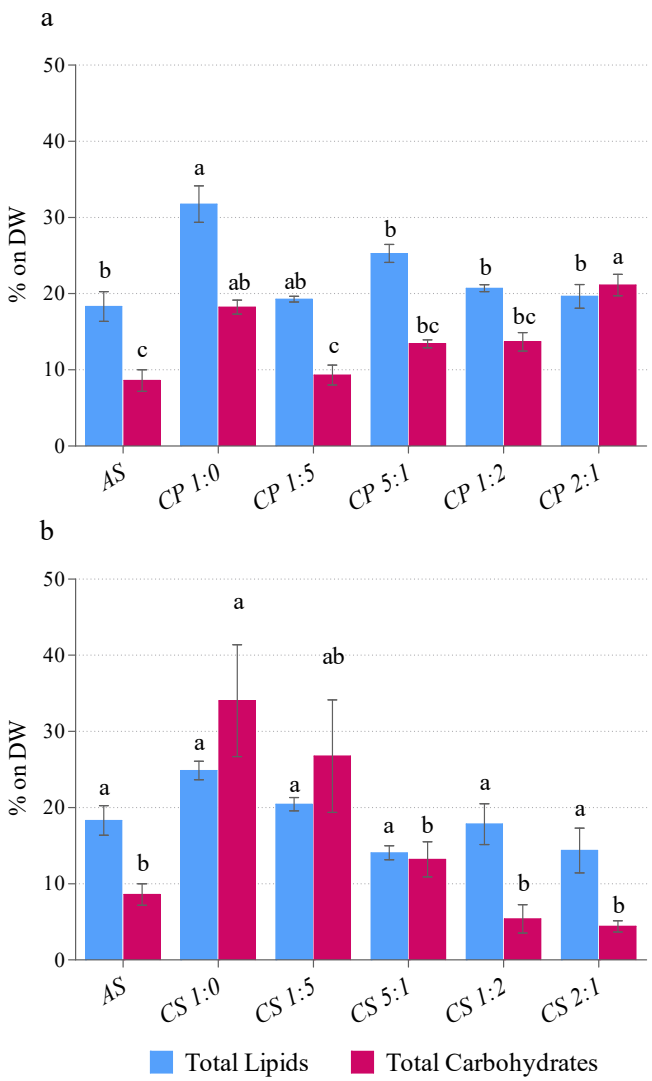

Figure 7: Biochemical analysis on the obtained biomass. All the results are reported for Activated sludge (AS), *Chlorella sp* Pozzillo (CP 1:0) and *Chlorella sp* CW2 (CS 1:0) alone and combined in different ratios (1:5, 5:1, 1:2, 2:1). Values are reported as means (n = 3) and error bars report the standard deviations. Samples with the same letter are statistically identical.

In general, all the analysed samples have a discrete content both of carbohydrates than lipids that may be exploited in several applications. Considering both the remediation potential and the biomass composition, the most promising sample, combining a high remediation performance and a good content of valuable products may be identified as CS 1:0. The biomass produced by treating wastewaters do not find application in the nutraceuticals or pharmaceuticals market but may be employed in other fields such as bioenergy, biofertilizers and production of high-value compounds [38,39]. By exploiting the recent view of microalgal cells as biorefinery, we propose that the carbohydrate fraction may be recovered by its transformation in glucose and consequently in fructose by isomerization. This feedstock may be then employed for dehydration in 5-Hydroxy methyl furfural, a platform compound with many applications in the industry [40]. At the same

time, the lipid fraction may be employed to produce biofuels and in particular biodiesel. It has been stated that the production of microalgae addressed to biodiesel production is not economically sustainable [41]; this is due to the high costs of microalgal cultivation systems and of separation and harvesting of microalgal biomass and oil, in particular, if the biomass is cultivated with this goal. When, instead, the biomass is derived from other processes (such as the remediation of wastewaters), it may be convenient to exploit the lipid fraction in a multi-product biorefinery view [42]. For this reason, the lipid content was analysed and the results are reported in Figure 8 as fatty acids class composition and in Table 4 and Table 5 where the detailed composition of fatty acids is reported for Activated Sludge (AS) and its combination with *Chlorella sp* Pozzillo and *Chlorella sp* CW2 respectively.

As it can be seen in Figure 8, the fatty acid composition varies among the different samples. In general, it can be stated that the amount of polyunsaturated fatty acids (PUFAs) decreases when microalgae are grown in addition to AS. The fatty acid class that increases is that of saturated fatty acids (SFAs). In Table 4 it can be seen that the SFAs fraction is minimum in CP 1:0 sample (8.61±0.89) and it progressively increases by adding activated sludge. Something similar happens also in the samples containing *Chlorella sp* CW2 (Table 5), in which there was a starting concentration of SFA (40.36±0.81) higher than that found in *Chlorella sp* Pozzillo. The fact that adding activated sludge to microalgae increases the SFAs content may be explained in two ways: (i) some of them come from the AS itself or (ii) the composition of microalgal oil is shifted because of co-cultivation with the microorganisms of the AS. A high degree of saturated fatty acids in lipid composition generally improves the biodiesel stability and therefore may be of interest for its production [43].

By looking at the single fatty acids in the samples containing microalgae, the most abundant are those of the classes C16 and C18, as observed also by other authors [44,45], for *Chlorella sp* cultivated in a highly concentrated municipal wastewater, and for *Chlorella vulgaris* cultivated in the pretreatment of dairy wastewater by UV and NaClO, respectively. Indigenous activated sludge fatty acids have a predominant composition of C16 and C18 as well, as observed by Fernández-Linares et al. in an indigenous consortium cultivated in a treated wastewater [46].

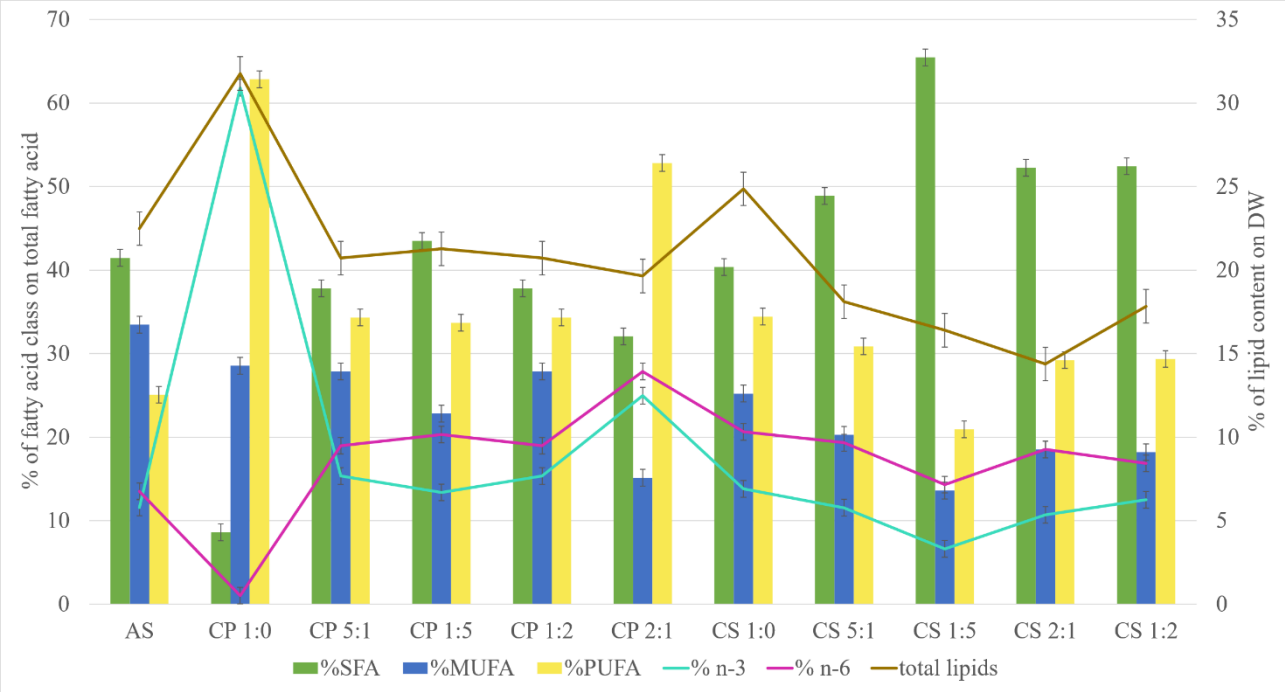

Figure 8: Fatty acids class composition (%,w/w) and total lipids of Activated sludge (AS), *Chlorella sp* Pozzillo (CP 1:0) and *Chlorella sp*. CW2 (CS 1:0) alone and in addition to microorganisms from activated sludge in different ratios (5:1, 1:5, 2:1, 1:2). Values are reported as means (n = 3) and error bars report the standard deviations. SFA: Saturated fatty acid; MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid.

The fatty acid composition of *Chlorella sp* Pozzillo was found significantly different with respect to a previous study in which it was cultivated in a growth medium [6]. As regards the presence of smaller chain-length fatty acid, in some cases they may be detected because of residuals coming from the sewage itself. Short-length fatty acid were detected also by Huo et al. in *Chlorella zofingiensis* grown in dairy wastewater [47]. Overall, considering the remediation potential and the content in carbohydrate, lipid and fatty acid, the most promising samples are CS 1:0 and CS 5:1 for its enrichment in SFA. Future perspective may be to assess the treatment of urban wastewaters in a continuous mode under laboratory conditions by inoculating *Chlorella sp* CW2 alone and in combination with activated sludge.

Table 4: Fatty acids Composition (%,w/w) Activated sludge (AS), *Chlorella sp* Pozzillo (CP 1:0) alone and combined with activated sludge in different ratios (5:1, 1:5, 2:1, 1:2). Data are reported as means (n=3) with standard deviation. Different letters indicate significant differences among various treatment in each species.

	AS	CP 1:0	CP 5:1	CP 1:5	CP 1:2	CP 2:1
Butyric Acid C4:0	-	-	0.12±0.05	-	0.12±0.05	-
Caproic Acid C6:0	-	-	0.07±0.03	-	0.07±0.03	-
Caprylic Acid C8:00	-	0.2±0^{a}	0.59±0.34	-	0.59±0.34^{a}	-
Capric Acid C10:00	-	0.17±0.01^{a}	1.11±0.62^{a}	2.3±0.84	1.11±0.62^{a}	-
Undecanoic Acid C11:0	1.02±0.92^{a}	0.36±0.11^{a}	1±0.55^{a}	2.25±1.05^{a}	1±0.55^{a}	-
Lauric Acid C12:0	0.13±0.22^{a}	0.39±0.09^{a}	0.61±0.21^{a}	1.77±0.96^{a}	0.61±0.21^{a}	-
Tridecanoic Acid C13:0	-	-	0.34±0.2^{a}	1.22±0.55^{a}	0.34±0.2^{a}	-
Myristic Acid C14:0	1.67±0.37^{a}	2.35±0.22 ^{a}	0.92±0.27^{a}	2.68±1.58^{a}	0.92±0.27^{a}	0.36±0.06^{a}
Myristoteleic Acid C14:1	0.65±1.12	-	0.33±0.19^{a}	1.47±0.9^{a}	0.33±0.19^{a}	-
Pentadecanoic Acid C15:0	0.37±0.35^{a}	0.73±0.03^{a}	0.56±0.26^{a}	2.28±1.37^{a}	0.56±0.26^{a}	-
cis-10-Pentadecenoic Acid c15:1	-	0.21±0.2 ^{a}	-	1.49±1.03^{a}	-	-
Palmitic Acid C16:0	28.19±6.67^{a}	-	25.14±0.81^{a}	23.8±3.28^{b}	25.14±0.81^{a}	24.52±1.62^{a}
Palmitoleic Acid c16:1	11.47±3.97^{a}	4.55±0.19^{a}	1.01±0.37^{a}	1.23±0.41^{a}	1.01±0.37^{a}	0.83±0.14^{a}
Heptadecanoic Acid c17:0	0.87±0.1^{ab}	1.54±0.01^{a}	0.25±0.09^{ab}	0.65±0.01^{ab}	0.25±0.09^{b}	0.33±0.42^{ab}
cis-10-Heptadecenoic Acid c17:1	12.67±12.75 ^{a}	-	9.47±4.97^{a}	8.39±0.61^{a}	9.47±4.97^{a}	11.22±0.21^{a}
Stearic Acid c18:0	4.89±0.65^{a}	2.86±0.5^{a}	3.94±1.73^{a}	3.37±0.98^{a}	3.94±1.73^{a}	1.62±0.02^{a}
Oleic Acid c18:1n9c + Elaidic Acid c18:1n9t	8.68±4.56^{a}	-	17.06±11.28 ^{a}	10.23±3.11^{a}	17.06±11.28 ^{a}	3.07±0.15^{a}
Linoleic Acid c18:2n6c + Linolelaidic Acid c18:2n6t	11.23±8.73^{a}	-	18.48±1.23^{a}	20.32±0.6^{a}	18.48±1.23^{a}	25.73±0.81^{a}
g-Linolenic Acid c18:3n6	2.28±1.29^{a}	1.02±0.17^{a}	0.49±0.31^{a}	-	0.49±0.31^{a}	2.13±1.29^{a}
a-Linolenic acid c18:3n3	11.56±3.17^{b}	61.84±0.89^{a}	15.35±5.73^{b}	13.37±0.97^{b}	15.35±5.73^{b}	24.95±0.37^{b}
arachidic acid c20:0	4.32±2.97^{a}	-	3.17±0.48^{b}	3.17±0.33^{ab}	3.17±0.48^{b}	5.22±0.46^{ab}
cis-11-eicosenoic acid c20:1n9	-	23.78±1.75	-	-	-	-
%SFA	41.46±3.41^{a}	8.61±0.89^{c}	37.81±1.17^{a}	43.49±1.96^{b}	37.81±1.17^{a}	32.06±1.01^{ab}
%MUFA	33.47±8.19^{a}	28.54±1.75^{a}	27.86±5.99^{a}	22.82±0.39^{a}	27.86±5.99^{a}	15.13±0.4^{a}
%PUFA	25.07±10.9^{c}	62.85±0.86^{a}	34.32±4.93^{c}	33.69±1.57^{bc}	34.32±4.93^{c}	52.82±1.02^{ab}
% n-3	11.56±3.17^{b}	61.84±0.89^{a}	15.35±5.73^{b}	13.37±0.97^{b}	15.35±5.73^{b}	24.95±0.37^{b}
% n-6	13.5±8.74^{a}	1.02±0.17^{b}	18.98±0.94^{a}	20.32±0.6^{a}	18.98±0.94^{a}	27.86±0.72^{a}

Table 5: Fatty acids Composition (%,w/w) Activated sludge (AS) and *Chlorella sp* CW2 (CS 1:0) alone and in addition to microorganisms from activated sludge in different ratios (5:1, 1:5, 2:1, 1:2). Data are reported as means (n=3) with standard deviation. Different letters indicate significant differences among various treatment in each species.

	AS	CS 1:0	CS 5:1	CS 1:5	CS 2:1	CS 1:2
Undecanoic Acid C11:0	1.02±0.92^{a}	1.66±0.01^{a}	1.95±0.49^{a}	3.57±0.99^{a}	-	-
Lauric Acid C12:0	0.13±0.22^{a}	-	-	-	-	-
Myristic Acid C14:0	-	-	0.72±0.05^{a}	-	-	-
Myristoteleic Acid C14:1	1.67±0.37^{a}	-	1.37±0.38	-	-	-
Pentadecanoic Acid C15:0	0.65±1.12	-	-	-	-	-
Palmitic Acid C16:0	0.37±0.35^{a}	34.16±0.89^{a}	25.75±0.64^{a}	24.91±6.21^{a}	26.42±1.11^{a}	27.62±0.34^{a}
Palmitoleic Acid c16:1	-	3.57±0.29^{b}	2.55±0.51^{b}	1.53±0.33^{b}	3.15±0.31^{b}	2.57±0.06^{b}
Heptadecanoic Acid c17:0	28.19±6.67^{a}	-	-	0.72±0.68	-	-
cis-10-Heptadecenoic Acid c17:1	11.47±3.97^{a}	9.09±0.16^{a}	6.64±1.68^{a}	3.71±0.52^{a}	6.05±0.47^{a}	6.46±1.06^{a}
Stearic Acid c18:0	0.87±0.1^{ab}	4.54±0.09^{a}	3.87±0.4^{ab}	3.66±0.32^{ab}	2.65±0.3^{b}	2.07±0.12^{b}
Oleic Acid c18:1n9c + Elaidic Acid c18:1n9t	12.67±12.75^{a}	12.55±0.35^{a}	9.69±0.61^{a}	8.37±1.7^{a}	9.32±0.64^{a}	9.17±0.32^{a}
Linoleic Acid c18:2n6c + Linolelaidic Acid c18:2n6t	4.89±0.65^{a}	20.63±1.06^{a}	15.75±2.53^{a}	11.13±2.3^{a}	17.47±0.78^{a}	16.87±0.14^{a}
g-Linolenic Acid c18:3n6	8.68±4.56^{a}	-	3.58±2.46^{a}	3.18±2.86^{a}	1.05±0.15^{a}	-
a-Linolenic acid c18:3n3	11.23±8.73^{a}	13.8±0.28^{a}	11.54±2.8^{ab}	6.62±0.56^{b}	10.7±0.61^{ab}	12.49±0.3^{ab}
arachidic acid c20:0	2.28±1.29^{a}	-	16.6±4.62^{ab}	32.6±8.7^{a}	23.19±2.69^{ab}	22.76±0.79^{ab}
%SFA	11.56±3.17^{b}	40.36±0.81^{bc}	48.88±4.74^{bc}	65.46±2.22^{a}	52.25±1.38^{b}	52.45±1.04^{b}
%MUFA	4.32±2.97^{a}	25.21±0.6^{ab}	20.26±2.03^{ab}	13.6±2.54^{b}	18.52±0.2^{ab}	18.2±0.93^{ab}
%PUFA	-	34.43±1.3^{a}	30.87±3.18^{ab}	20.94±0.32^{b}	29.22±1.2^{ab}	29.35±0.26^{ab}
% n-3	41.46±3.41^{a}	13.8±0.28^{a}	11.54±2.8^{ab}	6.62±0.56^{b}	10.7±0.61^{ab}	12.49±0.3^{ab}
% n-6	33.47±8.19^{a}	20.63±1.06^{a}	19.33±0.59^{a}	14.32±0.76^{a}	18.53±0.93^{a}	16.87±0.14^{a}

4. Conclusions

In this work, we analyzed the effect of inoculating two autochthonous microalgal isolates and with activated sludge at different ratios in municipal wastewaters. One of the strains, *Chlorella sp* CW2, was isolated from the activated sludge itself and molecularly characterized. Microalgae/activated sludge consortia were able to grow in the sewage. The contribute of the photosynthetic portion of each sample was highlighted by reading the fluorescence of the suspensions. The abatement of COD, total phosphorous and total nitrogen for each sample was assessed at the end of the cultivation. The best COD abatement was achieved by the samples CS 1:2 and CS 2:1, with a decrease of -81.39%±0.56, -and -82.5%±0.83, respectively, while the best nutrients abatement was obtained by the samples CS 1:0 and CS 5:1. The residual biomass was analysed for the content in carbohydrate, lipids and fatty acid and considering the remediation potential and the obtained biomass composition, the most promising sample may be the combination of *Chlorella sp* CW2 and activated sludge in the ratio 5:1 (sample ID CS 5:1). An increase of SFAs was found in samples containing microalgae and AS compared to that containing only microalgae. This suggests a possible application of the lipid

fraction as source of biodiesel (that is more stable with high concentration of SFAs). The remaining fraction containing carbohydrates may be applied for sugars fermentation or chemical conversion in chemical building blocks such as furfural compounds.

AUTHOR CONTRIBUTIONS

Serena Lima (SL) (serena.lima@unipa.it) concepted and designed the study, collected and assembled data, drafted the article. Alberto Brucato (AB) (alberto.brucato@unipa.it) , Giuseppe Caputo (GC) (giuseppe.caputo01@unipa.it) , Franco Grisafi (FG) (franco.grisafi@unipa.it) participated to the interpretation of data and to the final approval of the article. Francesca Scargiali (FS) (francesca.scargiali@unipa.it) participated to the obtaining of funding, helped to design the experiments, participated to the interpretation of data, revised the article critically for important intellectual content and participated to the final approval of the article.

ACKNOWLEDGEMENT

Authors wish to thank Amap SpA and Ing. A. Siragusa, Dr R. Arcuri, Dr Ferrara, for helping in experimental analysis and providing the sample of sewage and activated sludge and Dr D'Addelfio for performing some of the experimental analysis.

FUNDING SOURCE

This work was realized with the cofounding of European Union FESR or FSE, PON Ricerca e Innovazione 2014-2020 - DM 1062/2021

COMPETING INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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