Autochthonous microalgae grown in municipal wastewaters as a tool for effectively removing nitrogen and phosphorous

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Abstract

Microalgae have promising applications in wastewater treatment because of their ability to use inorganic compounds such as nitrates and phosphates as nutrients for their growth. Microalgae are applied to the secondary and tertiary bio-treatment with two benefits: *i)* pollutants removal from wastewater; *ii)* production of microalgal biomass, that can be exploited as a source of biomass and biomolecules. In the present work, four different microalgal strains (two from culture collections and two isolated from Sicilian littoral) were tested in municipal sewage bioremediation. The sewage of a municipal plant, already processed with primary treatment, was used for the cultivation of microalgal strains in order to test their potential on performing the secondary treatment. Microalgal cells were cultivated in growth medium and in sewage with the aim to compare their growth and biomass composition in different conditions. The efficiency of wastewater treatment was established through assessment of biochemical oxygen demand (BOD), chemical oxygen demand (COD), total nitrogen (TN) and total phosphorous (TP) of sewage before and after algal growth. Results showed that microalgal treatment alone is not effective in reducing COD and BOD, while all the tested strains were able to significantly reduce wastewater TN (up to 77%) and TP (up to 61%) concentrations. Amongst the tested strains, *Chlorella* genus can be considered the best candidate for wastewater treatment.

Keywords

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1. Introduction

The exponential growth of human population over the past few centuries, in conjunction with the contemporaneous industrial development, has strongly impacted the environment, due to the large increase of waste products. In this realm, the remediation of wastewaters has become one of the global priorities.

The traditional process for sewage treatment, summarized in several reviews (*e.g.* [1]), typically consists of three treatments (Figure 1). The *primary treatment* involves sand and grit removal. The removal of biodegradable organic matters is obtained in the *secondary treatment* via aerobic biological depuration and chemical precipitation. The removal of nitrogen and phosphorous typically occurs in the *tertiary treatment* [2]. In order to monitor the process of sewage depuration, several chemical analyses are performed, *e.g.* Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) [3]. These are indirect indicators of organic matter concentration and representative parameters for wastewater quality.

The biological depuration is achieved through consortia of microorganisms, usually heterotrophic bacteria, that, in the presence of oxygen, employ the carbon sources in the sewage for their growth and energy requirements. Because of their role in the biological degradation of organic matter, in this step an aeration system is needed for heterotrophic bacteria growth.
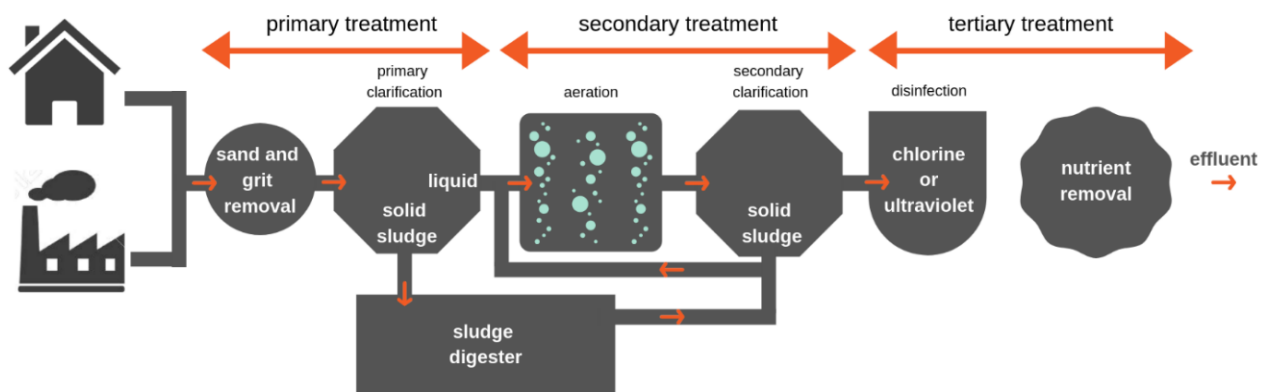

Figure 1: Schematic process flow diagram of a typical wastewater treatment plant. The process starts with a mechanical separation of debris and continues with primary, secondary and tertiary treatments.

Microalgae, instead, are phototrophic organisms able to convert light energy and inorganic compounds into oxygen and organic compounds. As a matter of fact, it has been shown that the use of microalgae in wastewater treatment has several advantages: *i)* the removal of pollutants such as ammonia (NH_4^+), nitrates (NO_3^-) and phosphates (PO_4^{3-}) [4, 5] and, in some cases, also of heavy metals [6]; *ii)* oxygen production that can be used by the heterotrophic bacteria to degrade compounds present in the matrix [7]; *iii)* CO_2 removal from the atmosphere [8].

An advantage of using microalgae for wastewater remediation is that the microalgal biomass so produced typically contains high-value molecules, that may be conveniently recovered. As a matter of fact, microalgae derived chemicals are currently employed in the production of cosmetics, nutraceuticals and pharmaceuticals [9, 10]. There are also some potential applications in the production of bulk chemicals, commodities for aquaculture, biofertilizers, biofuels as well as for CO_2 capture [11, 12]. Several biomolecules are extracted from algae, including carotenoids and chlorophylls (which are commercially highly valued [13]) as well as proteins, carbohydrates and lipids [14], among which the PUFAs deserve a special mention due to the proprieties and functions they carry out in animal bodies [15]. In particular, the omega-3 series, mainly made of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), has a large commercial interest. For this reason, the biotechnological accumulation of these lipids in microalgae has attracted significant attention in recent years [16] and strategies to trigger their production were proposed [17].

Moving to the topic of microalgae involvement in water remediation technologies, this has already received significant attention over the years, and several exhaustive reviews may be found in the literature [6, 8, 18]. In some cases, strategies were proposed to trigger the production of biomolecules coupled with wastewater remediation [19]. Although microalgae are photosynthetic microorganisms, some of them can act heterotrophically and are able to consume organic substrates via a fermentation process by producing CO_2. Microalgal fermentation is especially employed for the production of high-value algal biomass or bio-compounds [20], with a carbon substrate deliberately dissolved in the culture broth. Heterotrophic microalgae metabolism may also be applied to wastewaters treatment, with the aim of reducing both BOD and COD in the sewage.

When microalgae exploit the photosynthetic metabolism, instead, they employ CO_2 and (HCO_3^-) as the (inorganic) carbon source. After carbon, the second most important nutrient for microalgae is nitrogen, that is usually supplied as nitrate (NO_3^-), or ammonia (NH_4^+), or urea. In photosynthetic microalgae, nitrate assimilation starts with a transport step into the cell [21]. A subsequent reduction step to nitrite is catalyzed by a cytosolic Nitrate Reductase (NR). Nitrite is then transferred into the chloroplast where enzyme Nitrite Reductase (NiR) catalyzes its reduction to ammonium. Then, ammonium is incorporated as organic N into amino acids via glutamate by the action of synthetase/glutamine oxoglutarate amino transferase or glutamate synthase (GS/GOGAT). Phosphorous is another important nutrient for microalgae. As regards its assimilation, the preferred source is orthophosphate (PO_4^{3-}) and its uptake is energy-dependent. Algae can store more phosphorous than they need, especially when external conditions are harsh, in polyphosphate bodies during so-called luxury uptake [22].

Although microalgae have already been employed in wastewaters treatment, there are only a few examples of environmental isolates of microalgae employed for this application. Amongst them, *Scenedesmus* sp. and *Chlorella* sp. isolated from Kallar Kahar Lake, Pakistan, were grown in wastewaters with promising results [23]. In some cases, microalgae are isolated directly from wastewaters in order to increase their nutrients removal potential [24–26]. Isolating microalgae from the environment in which they will be employed should be beneficial as they are already adapted to the biotic and abiotic stresses in it and for this reason, in this work, microalgal strains from Sicilian littoral were harvested and isolated. Microalgae were then molecularly identified. This is the first time that isolates from Sicily are employed in a proof-of-concept work aimed at industrial applications. Furthermore, there are only a few other examples of isolation of microalgae (as well as macroalgae) from Sicily [27, 28].

In addition, the potential in remediating urban wastewaters of two microalgae strains coming from collections (*Nannochloropsis gaditana* and *Chlorella sorokiniana*) was compared with that of the two strains isolated from Sicilian coastal waters (*Chlorella* sp. and a consortium of *Chlorella* sp. and *Dunaliella tertiolecta*). The isolated microalgae were harvested from polluted areas and the objective of this work was to test whether they resulted effective in removing organic matter and nutrients from a primary treated sewage was tested. BOD, COD, nitrogen and phosphorous removal were assessed in order to evaluate microalgae effectiveness in wastewater treatment. In addition, microalgal biomass was also analysed in order to evaluate whether the contact with wastewaters caused a shift in their composition. The presence of high value molecules such as omega-3 fatty acids and their application potential in the process industry was also investigated.

2. Materials and Methods

2.1. Isolation of microalgae from marine samples

The isolation of microalgal species occurred by a combination of filtration methods using various pore-size sieves (from 5 to 200 μm) and serial dilutions in microplates. The medium employed for serial dilutions was f/2 medium [29]. Filtration method was employed in order to physically separate microalgae from other microorganisms and from macro-algae by exploiting their different cell-size. Furthermore, cell-suspensions were diluted in order to obtain a single-cell in each well. After obtaining a single strain in each well, cultures were transferred in solid medium (f/2 supplemented with agar 1.5%) in order to obtain single colonies. Monoalgal isolates were then characterized by microscopic morphological analysis followed by molecular analysis of the rDNA 18S aimed at assessing the isolated microalgae species. Molecular characterization was performed by colony polymerase chain reaction (PCR) using Q5 ® high-fidelity DNA Polymerase (NEB), the forward

primer A (5′-ACC CTG GTT GAT CCT GCC AG-3') and primerSSU-inR1 (5′-CAC CAG ACT TGC CCT CCA-3') and the following program: 95°C (5 min), 32 cycles of 95°C (30 s), 55°C (30 s) and 68°C (60 s) and a final 7 min extension step of 68°C. To identify the *Dunaliella* strains, PCR was performed in order to amplify the ITS region by using the genus-specific primers DITS_F 5′-AATCTATCAATAACCACA and DITS_R 5′-TTTCATTCGCCATTACTA [30] and the following program: 95°C (5 min), followed by 30 cycles of 95°C (30 s), 52°C (30 s) and 68°C (60 s) and by a final 5 min extension step at 68°C. The gene sequences were deposited in GenBank database (MT378213 and MT378213).

2.2 Microalgal growth

Four different strains were used in this work in order to compare their performance for sewage treatment. The first two strains were *Nannochloropsis gaditana* (CCAP 849/5) from the Scottish Association for Marine Science, and *Chlorella sorokiniana* (CCAP 211/11k) from the algal collection of Università di Napoli "Federico II". The other two microalgae employed in this work were harvested along sicilian coasts, isolated and identified with rDNA 18S sequencing. *Chlorella* sp. (*Pozzillo*) was harvested from the GPS coordinates 38,18372; 13,144250 while the consortium of *Chlorella* sp. and *Dunaliella tertiolecta* (Vergine Maria) was harvested from the location 38,167149;13,368573. All strains were kept in liquid medium. A modified version of f/2 medium [29] supplemented with 4 times the original nitrate and phosphate concentrations was used for *N. gaditana*, *Chlorella* sp. (Pozzillo) and the consortium of *Chlorella* sp. *and Dunaliella tertiolecta.* (Vergine Maria), while a commercial medium, the Cell-Hi JW (Varicon Aqua Solutions Ltd, UK) was used for culturing the freshwater microalga *C. sorokiniana*. A pre-culture of the microalgae was set up by inoculating 10 mL of sample from a culture flask in 100 mL of fresh medium. When cells were in late exponential phase (after about 10 cultivation days), 10 mL of the cell suspension were used to inoculate the medium containing cultivation flask (growth medium cultivations) or sewage (first and second cultivations). The algae were cultivated for 15 days. The concentration of the microalgal suspension was checked by counting the cells using a Burker Chamber and applying a suitable dilution factor in order to have 100-200 cells in a square. Measurements were done in triplicate (*n=3*) and the average value was retained and shown together with observed standard deviation (sd).

2.3 Set up of the experiment

Microalgal cells from the preculture in growth medium were inoculated in the original growth medium mixed in equal quantity with the sewage, in order to accustom them (data not shown). A sample from this cultivation was then inoculated into pure sewage, so obtaining the "first cultivation"

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series. In order to assess whether the cells were affected by a previous cultivation in wastewater, a second cultivation was performed by inoculating the cells from the first cultivation into fresh sewage obtaining the "second cultivation" series.

2.4 Sample preparation

After the growth in all cultivation conditions (growth medium, first and second sewage cultivation), the cell suspension was microfiltered and the biomass frozen in liquid nitrogen and freeze-dried for 48 h in a bench lyophilizator (FreeZone 2.5L, LABCONCO, US). The biomass was then stored at room temperature for further analysis. The filtered sewage was stored at -20°C for further analysis.

2.5 Sewage analysis

A pre-treated sewage coming from the municipal treatment plant AMAP "Acqua dei Corsari", located in Via Messina Marine, 592, 90121 Palermo PA, was used. This batch was analyzed for COD, BOD, total phosphorous (TP) and total nitrogen (TN) and then stored at -20°C until it was used for microalgae inoculation. After microalgal growth, the sewage was microfiltered (0.45 μm) and the above analyses were repeated. A batch of sewage was treated in the same way (frozen and microfiltered) and used as reference control. The COD analysis was performed following the APAT/CNR Method, IRSA Manuals 117/2014 – Method 513; BOD5 followed OXITOP method, compliant to UNI EN ISO 1899-1:2001; TN was analyzed according to disintegration ISO 11905-1; ISO 7890-1:1986 while TP EPA method 365.2.

2.6 Extraction and analysis of Fatty Acids

About 20 mg of lyophilized microalgae biomass was powdered with mortar and pestle using 5 mL of chloroform/methanol (2:1, v/v) and 1 mL of NaCl 1%. The mixture was vigorously mixed until the formation of two phases. The lower phase (chloroform phase) was transferred in a pre-weighted tube and the solvent was evaporated by heating below 50°C under nitrogen stream. After complete solvent evaporation, total lipids were trans-esterified by adding 1 mL of sodium methoxide (1 g NaOH in 100 ml MeOH) and 1 mL of hexane. The upper phase was then transferred in GC vials and analyzed by gas-chromatography using a GC 7890B System (Sigma-Aldrich, US) equipped with a FID detector and a capillary column Omegawax 250 (Sigma-Aldrich, US). Initial temperature was 50 °C, increased to 220°C as working temperature. Total analytic time was 79.5 minutes and argon was used as 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.

2.7 FTIR analysis

Samples of biomass were analyzed by Fourier Transform Infrared Spectroscopy (FTIR) in order to assess the general biochemical composition. The method was adapted from Stehfest et al. [31]. About 2 mg of freeze-dried biomass were weighted and transferred in a mortar together with 100 mg of Potassium bromide (KBr) to prepare glassy sample discs. Then the mixture was vigorously crushed, and a pellet was made by a hydraulic press (CrushIR, PIKE Technologies, US). The pellet was then scanned in a Cary 630 Spectrometer (Agilent Technologies, US). This technique links one or more peaks to the corresponding biochemical macromolecule, thanks to the vibrational frequencies of the relevant functional groups. By integrating the area under the curve, a semi-quantitative analysis of the macromolecule is obtained. Ratios between different areas were examined in order to make different samples comparable. In Table 1 the employed wavelengths to integrate different peaks are shown. Measurements were done in triplicate ($n=3$) and the average value was retained.

Table 1: Reference wavelengths for peaks integration connected to macromolecules by the vibrational frequency of the related functional group.

Wavelength (cm^{-1})	Assignment	Macromolecule
2799-300	CH of saturated CH	Lipids
1584-1725	Amide I C=O of amides from proteins	Protein
950-1200	C–O–C of saccharides	Carbohydrates

2.8 Statistical data analysis

Data in triplicate were tested for statistical significance of the variations in different strains and treatments. Two-way ANOVA analysis was performed to detect statistical differences among the parameters *treatments* and *strains*. The output F-values together with p-values were used to describe the impact of treatment on the variables.

Bonferroni´s correlation (p value) was used to quantify the variability between *control* and *treatments*. Data were considered significant for p-values smaller than 0.1.

Results are shown as means and standard deviations are reported as error bars.

3. Results and discussion

3.1 Molecular characterization of isolated strains

Two beaches in the littoral of Palermo, Sicily were selected as sampling site for the isolation of interesting strains. Both beaches, located in polluted areas, could host interesting microalgae strains to be employed for wastewater nutrient removal. A *Chlorella* strain was isolated from Pozzillo beach (38°11'05.6"N 13°08'31.5"E). A molecular characterization was obtained using universal primers for the amplification of 18S. A mixed culture of *Chlorella* and *Dunaliella* was isolated from the Vergine Maria beach (38°10'04.9"N 13°22'08.1"E). The PCR using the universal primers for the amplification of 18S gave two different products (Figure 2). For species identification of *Dunaliella* strain ITS region was amplified by using genus-specific primers. The obtained sequences were deposited in Genbank database: *Chlorella* sp. (Pozzillo) with the code MT378213 and *Dunaliella tertiolecta* (Vergine Maria) with the code MT378213.
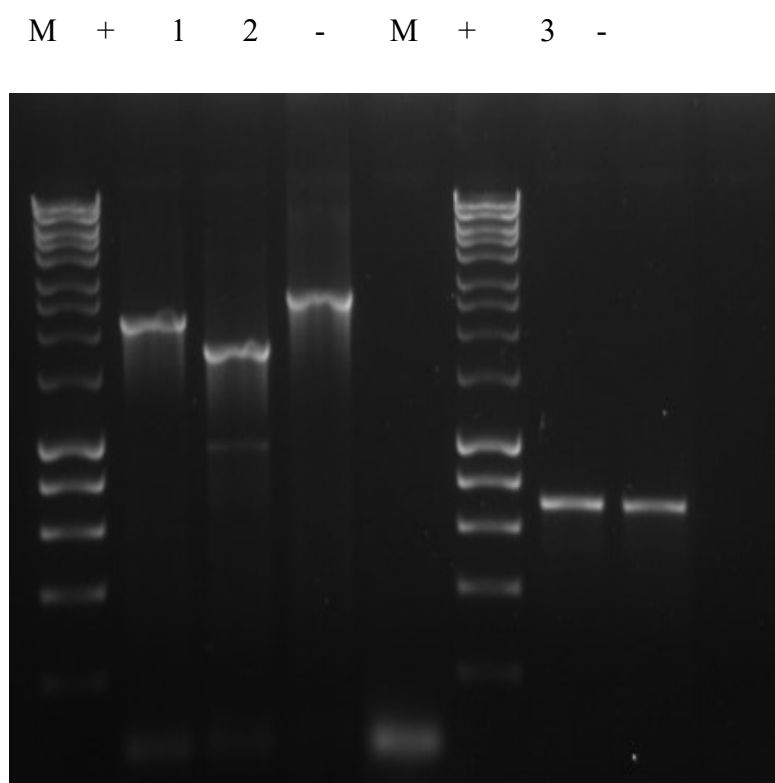

Figure 2: Gel electrophoresis of PCR product on 1% agarose gel; M: molecular marker, +: positive control, 1: 18S rDNA product of the mixed culture *Dunaliella tertiolecta/Chlorella sp. (Vergine Maria)* 2: 18S rDNA PCR product of *Chlorella sp (Pozzillo)*; 3: ITS1 product of the mixed culture *Dunaliella tertiolecta/Chlorella sp. (Vergine Maria)*.

3.2 Microalgal growth curves

The four microalgae strains were cultivated in their growth medium and in urban wastewaters during two cultivation series. The second cultivation series was carried out in order to assess the effect of acclimation, as cells coming from the first cultivation were employed for the inoculum of the second cultivation. In Figure 3, growth curves obtained in control medium and during first and second cultivations are reported. As it can be seen, both environmental strains, *Chlorella* sp. (Pozzillo) and *Chlorella* sp.*/Dunaliella tertiolecta* (Vergine Maria), reached a higher cell density than the culture collection strains *N. gaditana* and *C. sorokiniana* in all the tested conditions (Figure 3 A-D). Interestingly, both the *Chlorella* isolates reached higher cells concentrations than other algae belonging to the same genus reported in literature [5,23]. Almost all the employed species reached higher concentrations in the growth medium (red line) than in the sewage (green and blue lines). However, the consortium of *Chlorella* sp.*/Dunaliella tertiolecta* (Vergine Maria) reached comparable cell densities in both control and first cultivation series (Figure 3 D). These strains, hence, showed the best growth performance on sewage in the first cultivation series compared to the other tested strains.

During the first cultivation days *N. gaditana* showed a faster growth in the first cultivation series as compared with cultivation in the growth medium, but with a lower final cell density (Figure 3 A). *C. sorokiniana* showed the same trend (Figure 3 B). Slightly different considerations apply to the isolate *Chlorella* sp. (Pozzillo), in which a marked increase in growth during the first cultivation series is observed in comparison with the second one (Figure 3 C). The anticipated growth observed in the first cultivation may be due to a best adaptation to sewage nutrient composition as compared to the growth medium. Future analysis may assess the elemental composition of the sewage in order to investigate this aspect.

A different situation is shown in Figure 3 D where the consortium of *D. tertiolecta/Chlorella* sp. showed a better growth performance in the first cultivation series than in the growth medium. The reason may rely on the ability of the consortium to grow better and enhance nutrients removal from wastewaters, as reported by other authors [32–34].

It is worth noting that in this work both freshwater and salt compliant species were employed. In particular, *N. gaditana* is a salt species (the medium employed for its cultivation has a conductivity of 42,03 mS/cm), *C. sorokiniana* prefers low salt concentrations (in fact, the growth medium for its cultivation has a conductivity of 132,3 µS/cm) and the environmental isolates *Chlorella* sp. (Pozzillo), *Dunaliella tertiolecta/Chlorella* sp. (Vergine Maria) can grow in a wide range of salt concentrations (data not shown). Since sewage conductivity is low (5,9 mS/cm) and growth was not inhibited in it, we can conclude that salinity did not influence growth during these experiments.

A *N. gaditana*

B *C. sorokiniana*

C *Chlorella sp. (Pozzillo)*

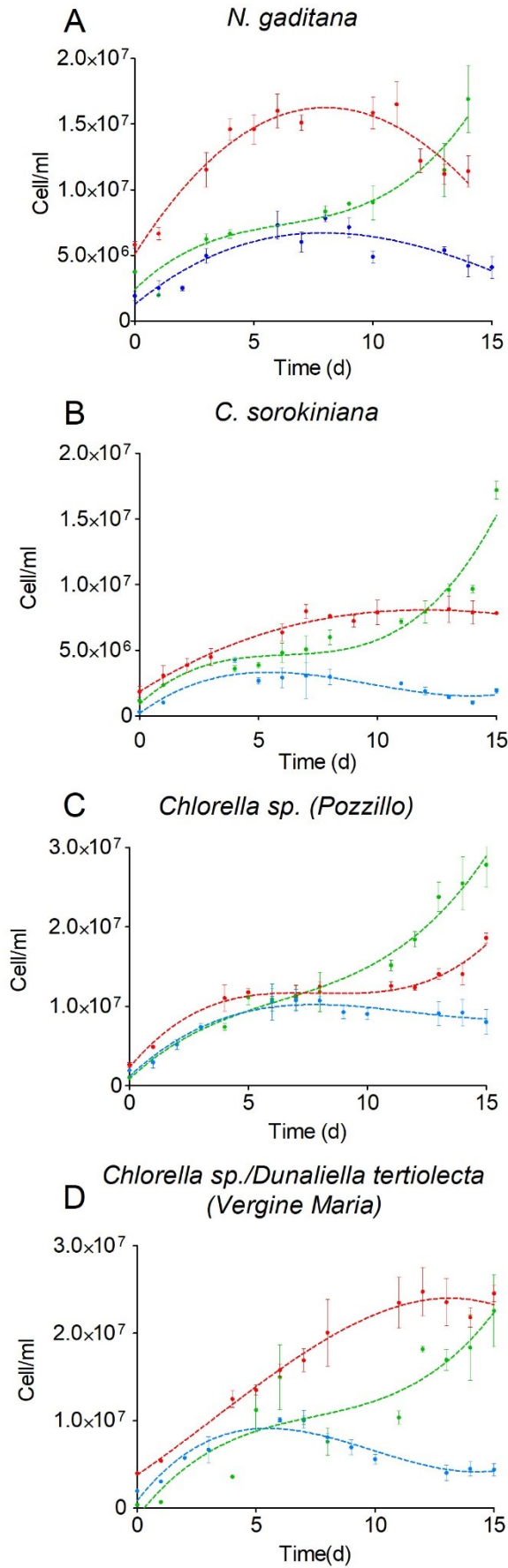**D** *Chlorella sp./Dunaliella tertiolecta (Vergine Maria)*

Figure 3: Growing curves of the four microalgal strains grown in: growth medium (green), first cultivation series (red) and second cultivation series (blue). A *N. gaditana*; B *C. sorokiniana*; C *Chlorella* sp. (Pozzillo); D *Dunaliella tertiolecta/Chlorella* sp. (Vergine Maria). Measurements were done in triplicate (n=3), obtained average and standard deviation values were shown in the graphs.

- Growth medium
- 1st Cultivation Series
- 2nd Cultivation Series

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Although some authors [5,32] reported that wastewater treatment is improved by acclimation, the results of the present work show that all employed microalgae had a poorer growth performance in the second cultivation series in comparison with the first one, even if cells were expected to be more acclimated. The reason behind this behavior may relay on the lack of macronutrients in the second cultivation, that were instead still present in the first cultivation series because this cultivation was inoculated from the growth medium, very rich in nitrates and phosphates. Future work may therefore assess the concentration of these nutrients during the cultivation. As reported also by others [35,36], and supported by chemical analysis of the sewage (see next paragraphs), the limiting nutrient may be phosphorous, that is a well-known essential nutrient for plants and microalgae growth in wastewater remediation application [37,38]. It is worth observing that the environmental isolated microalgae, *Chlorella* sp. (Pozzillo) and *Chlorella* sp./*Dunaliella tertiolecta* (Vergine Maria), grown in the sewage reached higher cell concentrations than the algae from collections and had a better growth performance. This is in accordance with the locations of algae's sampling sites, which were selected for their high pollution level. Therefore, acclimation of the strains to their natural habitat plays a role in their abilities also after the isolation process, as observed also by other authors in different conditions [25, 39].

3.3 Analysis on the biomass

After growth, the biomass was harvested, freeze-dried and analyzed in order to assess its biochemical features. FTIR spectroscopy was employed to identify the presence of vibrationally active functional groups (including O–H, N–H, C=O, C–H, CH_2, C–O–C) and peak areas related to the corresponding macromolecules were estimated. These are reported in Figure 4 as ratios between different areas. By using two ratios, Lipid/Amide I (L/A), Carbohydrates/Amide I (C/A), the accumulation of lipid and carbohydrates was approximately estimated.

Under a statistical viewpoint, L/A ratio was influenced both by the treatment ($F=41,82$, $p<0.001$) and by the strains ($F=44,35$, $p<0.001$). C/A ratio was affected only by the treatment ($F=75,99$, $p<0.001$) and not by the strain ($F=0,74$, $p=0,5424$).

In the case of *N. gaditana* both the L/A and the C/A ratios increased from the growth medium to the first cultivation (by 38 and 145% respectively) while L/A ratio was similar (- 4%) from the control and the second cultivation. It can be hypothesized that both lipid and carbohydrate contents increased in the first cultivation series, but carbohydrate levels increased more than lipid ones. The C/A ratios slightly increased by 45% also in the second cultivation series as compared to control, probably due to carbohydrates increase (Figure 4 A).

A

N.gaditana

B

C. sorokiniana

C

Chlorella sp. (Pozzillo)

D

Chlorella sp. /Dunaliella tertiolecta (Vergine Maria)

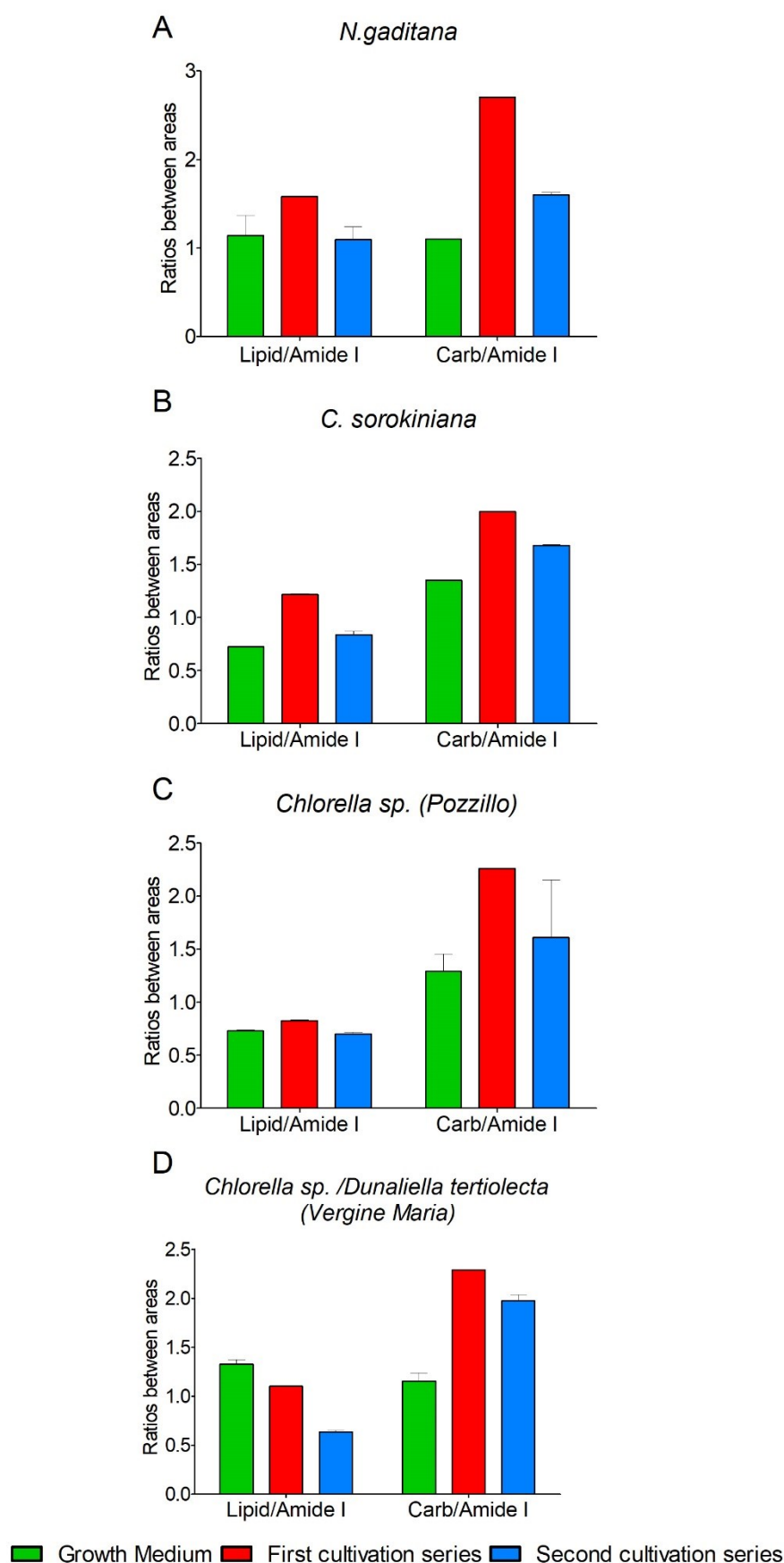■ Growth Medium ■ First cultivation series ■ Second cultivation series

Figure 4: FTIR analysis on the microalgal biomass. Ratios between areas under peaks related to macromolecules are reported. Following the tendency of different ratio, the total composition of the biomass can be estimated. Values are reported as means (n=2) and error bars report the standard deviations.

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The first and second cultivation series of *C. sorokiniana* showed similar results to *N. gaditana* (Figure 4 B): L/A ratio increased of 68 and 15 % from control to first and second cultivation and C/A ratio of 48 and 24%, respectively.

Chlorella sp. (Pozzillo) did not show an increase of L/A ratio in any of the two cultivations (+ 13% and -4% respectively in the first and second cultivations), therefore the lipid content is probably the same between them. By contrast, the C/A ratio increased more in the first cultivation series (75%), and less in the second cultivation series (24%), compared to the control, with a consequent probable rise of carbohydrate content (Figure 4 C).

A slightly different situation is displayed in the consortium of *Dunaliella tertiolecta* and *Chlorella* sp. (Vergine Maria). In fact, in this sample L/A ratio slightly decreased in both first and second cultivation series (-51 and -71%), as compared to control, as shown by the L/A ratio. The only increasing compounds are the carbohydrates, as shown with the rise of C/A ratios in both first and second cultivation series (+ 98 and 71%, respectively) (Figure 4 D).

In general, sewage treatment brought in an increase of both carbohydrates and lipids in microalgal biomass. This may be connected to the nitrogen limitation at the end of the cultivation, a well-known trigger for lipids accumulation in the biomass, as reported by Liang and collaborators [40] and Metsoviti et al. [41]. Results show that the differential increase of carbohydrates or lipids depends on the species and on the acclimation state of the cultivation (first or second one). The increase of lipids content in the algae biomass grown in wastewaters, as compared to the growth medium control, is supported by literature, *e.g.* in *Nannochloropsis oceanica* grown in industrial wastewaters [42]. The observed increase in carbohydrates levels was also previously observed in *Nannochloropsis oculata* and *Tetraselmis suecica* grown in municipal wastewaters [43], as well as in *Desmodesmus* spp and *Scenedesmus obliquus* grown in a mixture of raw wastewater with different leachate ratios [44].

3.4 Characterization of lipid content

Biomass samples were also analyzed by Gas Chromatography to assess fatty acids composition.

Under a statistical viewpoint, saturated fatty acids (SFAs) were influenced both by the treatment ($F=222.7$, $p<0.001$) and by the strains ($F=306.9$, $p<0.001$). Mono-unsaturated fatty acids (MUFAs) were affected more by the treatment ($F=362.6$, $p<0.001$) than by the strain ($F=138.6$, $p<0.001$). By contrast, poly-unsaturated fatty acids (PUFAs) were influenced more by the strain ($F=494.1$, $p<0.001$) than by the treatment ($F=92.08$, $p<0.001$).

As shown in Figure 5, the shift in composition of fatty acids is species-specific. *N. gaditana* did not show a marked shift in composition: there is a slight decrease in SFAs and an increase in PUFAs in the first and second cultivation series, as compared to control, while no significant effects were observed on eicosapentaenoic acid (EPA) content (Figure 5 A).

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Otherwise, the major effect in *C. sorokiniana* in the first cultivation series is a decrease of SFAs in favor of MUFAs and of PUFAs, as shown in Figure 5 B. Opposite results were obtained in second cultivation series where the same strain showed an increase in SFAs and a decrease in MUFAs and PUFAs. Furthermore, EPA could not be detected in the two cultivations in the sewage. *Chlorella* sp. (Pozzillo) showed an increase in SFAs in both first and second cultivation series, while an increase in MUFAs was observed in the first cultivation series and in PUFAs in the second one (Figure 5 C). EPA could not be detected in the first cultivation series. The consortium of *Chlorella* sp*./Dunaliella tertiolecta* (Vergine Maria) showed instead a decrease in MUFAs and an increase in PUFAs and EPA both in first and second cultivation series (Figure 5 D). Both PUFA and EPA were not found in the control.

The change in fatty acid composition is not always of easy interpretation because of different factors, such as nutritional conditions, physicochemical conditions as well as growth phases can act on the composition of fatty acid [45,46]. Also, other environmental factors, such as salinity, could play a role in assessing, the fatty acid profile [47]. However, this work showed similar results to other works in the same field. For example, *Nannochloropsis oceanica* grown in municipal sewage wastewater, showed a decrease in SFAs and MUFAs together with an increase in PUFAs on dry weight [42], similarly to present work findings with algae from the same genus. Furthermore, De Bhowmick et al. [48], reported a decrease in SFAs together with an increase in PUFAs in *Chlorella minutissima* grown in a mix of kitchen waste, poultry litter waste and 5% flue gas in comparison with growth medium culture, matching present work findings with *C. sorokiniana* and *Chlorella* sp*./D. tertiolecta* (Vergine Maria).

Regarding EPAs content, even though *Chlorella* genus is not known as an EPA producer, in many cases good concentrations of this fatty acid were found in this genus [49,50]. Some authors found that both PUFAs and EPA content decreased under nutritional (nitrogen) limitation [32,44] and this might explain the change in fatty acid composition observed in *C. sorokiniana*. A similar observation about PUFAs content was also made by Breuer et al. [51], when studying triacylglycerol (TAG) accumulation under nitrogen-deficient cultivation conditions. Another interesting aspect regards the fatty acids composition differences observed between the first and the second cultivation series in *C. sorokiniana* and *Chlorella* sp. (Pozzillo). This is not the first time that in two subsequent cultivations a shift in fatty acid composition is observed: similar findings were also reported by Daneshvar et al. [52].
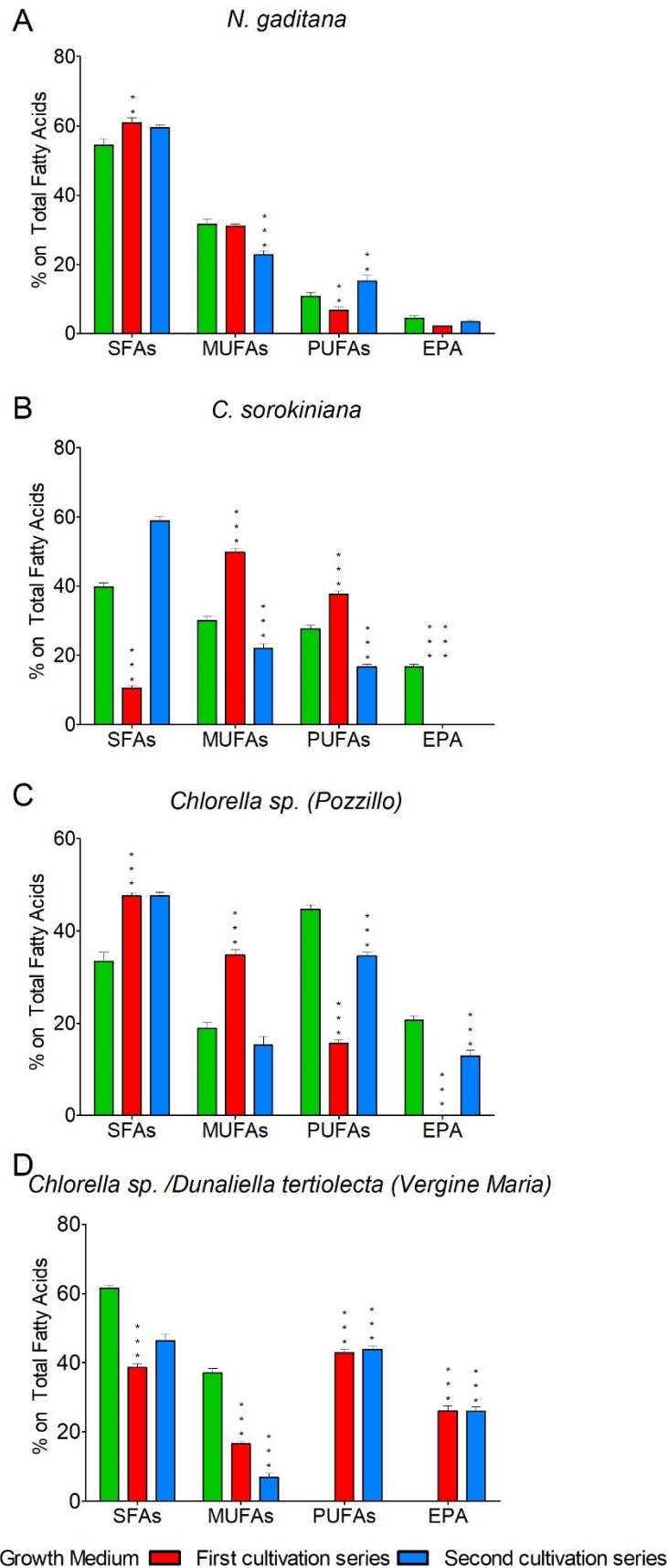
Figure 5: Composition in percentage of fatty acid on total fatty acids of the microalgal biomass grown in urban wastewaters in the analyzed microalgae: A) *N. gaditana*, B) *C. sorokiniana*, C) *Chlorella* sp. (Pozzillo), D) *Chlorella* sp.*/Dunaliella tertiolecta* (Vergine Maria). Fatty acids are shown as saturated fatty acids (SFAs), mono-unsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs). Eicosapentaenoic acid (EPA) is shown individually. Values are reported as means (n=2) and error bars report the standard deviations. Asterisks indicate if the treatment is statistically different to the control growth medium. Two asterisks indicates a P value <0.01 and three asterisks < 0.001.

A

N. gaditana

B

C. sorokiniana

C

Chlorella sp. (Pozzillo)

D

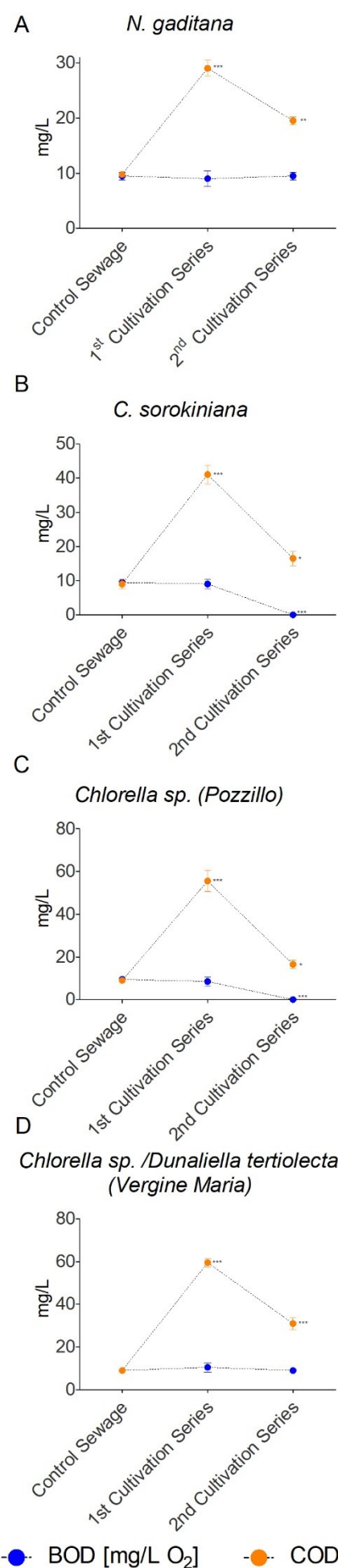### Chlorella sp. /Dunaliella tertiolecta (Vergine Maria)

Figure 6: Chemical analysis for the characterization of the sewage before and after the microalgae treatment: Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) are reported for the untreated sewage (control), first Cultivation Series and second Cultivation Series. Values are reported as means (n=2) and error bars report the standard deviations. Asterisks indicate if the treatment is statistically different to the control sewage. One asterisk indicates a P value <0.1, two asterisks <0.01 and three asterisks < 0.001.

BOD [mg/L O_2]   COD [mg/L O_2]

Interestingly, in the case of *C. sorokiniana* and *Chlorella* sp.*/Dunaliella tertiolecta* (Vergine Maria) EPA was found to be induced by the growth in wastewaters, while it was not detected in the control. In conclusion, the reported results confirm that the fatty acid profile depends both on the analyzed microalgae and on the employed matrix for its cultivation (growth medium, first or second sewage cultivation series).

3.5 Sewage chemical analysis

The sewage treated with microalgae was filtered and analyzed for the determination of COD, BOD, total nitrogen and total phosphorous. Results are compared with the control sewage, which underwent the same analytical procedures than the treated samples. Results obtained in the present work are summarized in Figures 6 and 7. BOD concentration (Figure 6 A) was stable in all samples except *Chlorella* sp. (Pozzillo) second cultivation series and *C. sorokiniana* second cultivation series, where it went down to zero. Otherwise, COD level increased in all the samples (Figure 6 B). Notably, COD level was higher in the first cultivation series than in the second one in all the analyzed microalgae, and this correlates with the higher cell concentrations obtained in the first cultivation series compared to the second one. By contrast, several other authors found an opposite trend in the COD values that was decreased by microalgae treatment. For example, *Chlorella vulgaris* decreased COD values in several kind of wastewaters [53]; in another case, a consortium of *Chlorella prototheicodes* and *Brevundimonas diminuta* decreased COD values of wastewaters in a continuous system [33].

Results of the present work showing increased COD values may be due to the release of compounds from microalgae not degradable with the BOD test, yet oxidizable with the COD test. It may be that these compounds are constituted by cellulose and hemicellulose, the main carbohydrates of microalgae together with starch [54]. Microalgae are, in fact, protected by a lipid-rich plasma membrane and a rigid cell-wall with a complex composition rich in different carbohydrates [55]. Cellulose was detected in *Nannochloropsis* genus [56], in *Chlorella sorokiniana* [57] and in *Chlorella vulgaris* [58] between others. COD analysis can detect the polysaccharide content of a matrix, including cellulose levels [59]. On the other hand, in the past BOD presented criticisms for the monitoring of wastes with significant ligno-cellulosic content [60]. In fact, in the normal time of the BOD analysis (5 days), only the easily degraded carbohydrates are oxidized, while longer times would be needed by cellulose and hemicellulose [61].

A

N. gaditana

B

C. sorokiniana

C

Chlorella sp. (Pozzillo)

D

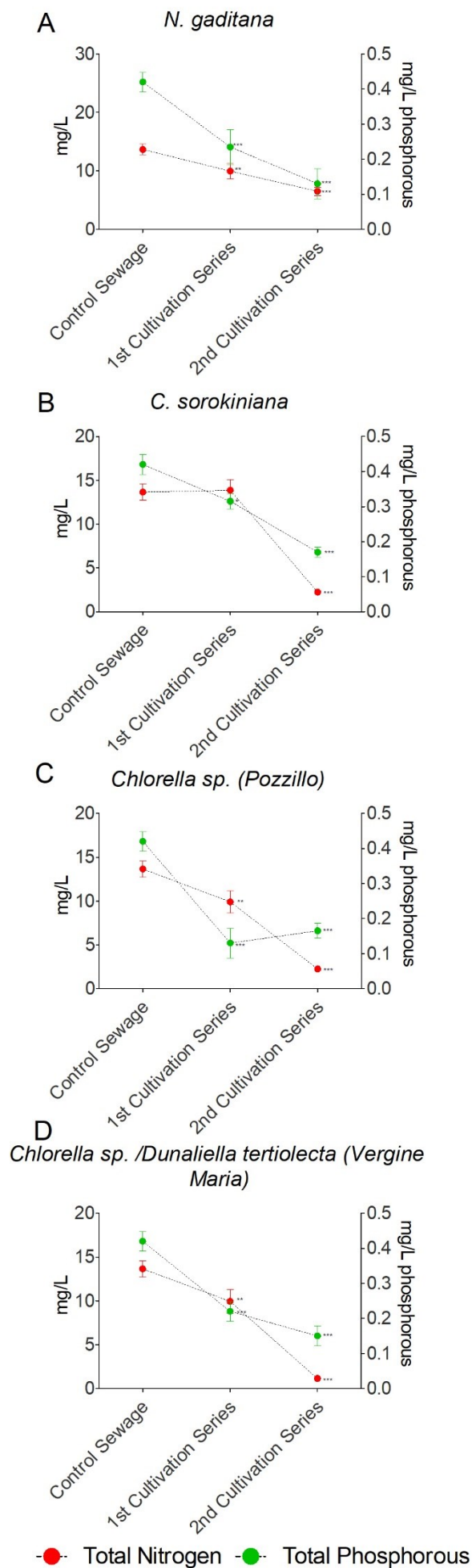### *Chlorella sp. /Dunaliella tertiolecta (Vergine Maria)*

Figure 7: Chemical analysis for the characterization of the sewage before and after the microalgae treatment: Total nitrogen (TN) and total phosphorous (TP) are reported for the untreated sewage (control), first cultivation series and second cultivation series. Values are reported as means (n=2) and error bars report the standard deviations. Asterisks indicate if the treatment is statistically different to the control sewage. One asterisk indicates a P value <0.1, two asterisks <0.01 and three asterisks < 0.001.

● Total Nitrogen ● Total Phosphorous

Another possible explanation for the increase of COD levels in microalgae treated wastewaters was found by Wang et al. [62] in their work with *Chlorella* sp. According to these authors, when organic substrates are not available, microalgae grow in phototrophic conditions, using CO_2 as carbon source. In such conditions they excrete small molecular organic substances, such as glycolic acid as a by-product of photosynthetic carbon reduction cycle, which may explain the increase of COD in the effluent after algal cultivation.

Total nitrogen and total phosphorous analysis are shown in Figure 6. As it can be seen, their trend is similar in all the analyzed microalgae and consists of a progressive concentration reduction between first and second cultivation series. During the first cultivation series TN was removed by 0,9% by *N. gaditana*, while an increase of 33% was detected when *C. sorokiniana* was grown in wastewaters. TN was furthermore decreased by 1,8 and 0,9% respectively by *Chlorella* sp. and by the consortium of *Chlorella* sp. and *D. tertiolecta*. During the second cultivation a larger removal of TN was observed: 36,4%, 77,3%, 76,4% and 88,2% of removal by *N. gaditana, C. sorokiniana, Chlorella* sp. and the consortium of *Chlorella* sp. and *D. tertiolecta,* respectively. As regards TP, during the first cultivation it was removed by 34,1%, 19,5%, 61,0% and 41,5% and during the second cultivation by 61,0%, 56,1%, 17,1% and 58,5% in cultures of *N. gaditana, C. sorokiniana, Chlorella* sp. and the consortium of *Chlorella* sp. and *D. tertiolecta,* respectively,. The above results seem to be coherent with literature data. For example, Chamberlin et al. [4] showed a decrease of both nitrogen and phosphorous values in wastewaters after the cultivation of *C. vulgaris*. The same strain was found to decrease nitrogen and phosphorous values also by other authors, that showed the differences between the acclimated strain and the not acclimated one [5]. Moreover, the present work results show an example of acclimation, as the reduction of both nitrogen and phosphorous is increased in the second cultivation series.

3.6 Effectiveness of microalgae compared to other treatments

Microalgal treatment of urban sewage was compared with other state-of-the-art solutions, as shown in Table 2. Microalgal treatment appears to be competitive as regards nitrogen and phosphorous removal. It is not   for organic matter removal, as already observed in previous paragraphs.

Table 2: comparison amongst microalgal and other treatments

Treatment	Kind of wastewater	Efficiency in COD reduction	Efficiency in BOD reduction	Efficiency in TN reduction	Efficiency in TP reduction	Reference
Autochthonous microalgae treatment	pre-treated municipal sewage	No effective	No effective	77%	61%	This paper
Bio-derived MgO nanopowders	tannery wastewaters	95,4%	99,9%	-	-	[63]
Nano Fe(0)-induced denitrification by means of a spinning disk reactor	aqueous solution	-	-	90 % of nitrate	-	[64]
Photocatalytic degradation using a green nanoparticle TiO₂ catalyst	petroleum refinery effluent	-	-	67%	-	[65]
Porous ceramsites	synthetic organic wastewaters	91%	-	85% of ammonia	80%	[66]
Electrocoagulation	secondary treated waste- water	82,4%	-	-	88,3%	[67]
Multiple anoxic and aerobic treatment	synthetic wastewater	95,3%	-	74,1%	98,2%	[68]
Pyrite-based bioretention system	stormwater	-	-	89.3 %	81.6 %	[69]
Zeolite-based bioretention system	stormwater	-	-	47.1 %	47.5 %	[69]
Modified biosand filters	synthetic wastewater	45,2%	-	-	-	[70]
Bioelectrochemically-based system	groundwater	-	-	90%	-	[71]
Reverse osmosis	urban wastewater	76,5%	91,8%	79,1%	49,4%	[72]
Carbide derived carbon	synthetic wastewater and treated sewage	-	-	-	90% of phosphate	[73]

In particular, TN and TP removal results successfully compare with photocatalytic degradation [65], zeolite-based bioretention systems [69], and are comparable with respect to the use of porous

ceramsites [66], electrocoagulation [67], pyrite-based bioretention system [69]. Quite surprisingly, also the reverse osmosis treatment plant in Medina Sidonia [72] showed poorer performance than microalgal treatment. It is worth noting that in many cases literature results are hardly comparable, because of the different wastewaters employed in the experimentation and treatment diversities. Some treatments include subsequent steps (e.g. [68]) and the overall performance is therefore positively affected. The microalgal treatment may be included in a multi-steps treatment and this may bring about further advantages, such as the removal of organic matter together with that of nitrogen and phosphorous. As an example, a denitrification step involving microalgae may be included before the oxidation of the secondary treatment of municipal sewage. As single step, the treatment with autochthonous microalgae may be instead an ideal solution for treating wastewaters with little or no organic matter concentration and high phosphate and/or nitrate content, such as stormwater or groundwater. Overall, microalgae wastewater remediation competes with other state-of-the-art treatment for what concerns nitrogen and phosphorous removal. Furthermore, it does not require expensive and complex systems: microalgae can be grown in quite simple raceway pond reactors which, if well designed, ensure low capital and operational costs and high efficiencies for wastewater treatment [74].

3.7 Scale-up, harvesting and cleaner production

The problem of eutrophication of groundwaters and stormwaters with fertilizers from agriculture is well-known. This, together with the management of urban wastewaters points out some critical issues since the traditional wastewater treatment plants may be ineffective in removing nutrients such as nitrates and phosphorus and their management (especially aeration) is often expansive, especially in old wastewater treatment plants [75]. From our results, microalgae are not able to decrease wastewater organic carbon content, but they may be conveniently employed in a novel concept of wastewaters-treatment plant that takes advantage from the presence of both photosynthetic organisms and bacteria. In comparison with traditional treatment plants, this process results in a reduced production of CO_2 with no need for aeration [76] and reduced sludge formation [77].

From a practical viewpoint, the cheapest option in an industrial perspective is to grow microalgae and bacteria in raceway ponds containing primary-treated urban sewage in a continuous system. There are some examples in literature [78, 79] and the technology is mature for a commercial step. Moreover, being a cheap and simple technology it is applicable in developing countries.

After growth, the sludge made of microalgae and bacteria needs to be harvested. It is worth pointing out that harvesting is a critical step in microalgal production. As a matter of fact, it amounts to 20-30% of the total production costs: it is therefore mandatory to choose efficient harvesting methods. Several processes may be employed to this end. Mechanical methods include centrifugation, efficient

but expensive, and sedimentation, easy and inexpensive, by employing sedimentation tanks. Another mechanical treatment is froth-flotation, consisting in bubbling a gas from the bottom [80]. This carries the biomass to the top of the tank, where it is separated by skimming or filtration. In order to make the last two processes effective, a primary step of flocculation and coagulation may anyway be necessary. Flocculation consists in aggregating the suspended cells/algal material in agglomerates which possess a faster settling rate. It may employ inorganic, organic or chemical flocculants. A promising and effective option for separating microalgae by reducing costs is the immobilization technique [81]: microalgae grow attached to a solid matrix and the separation may be obtained by a simple filtration. Innovative technology for microalgae harvesting are the electrocoagulation [82], magnetic separation [83] and the ultrasound technique [84].

Once the biomass is separated from the remediated water, it may be involved in a circular economy cycle, as it may enter in the formulation of fertilizers and in particular biostimulants [85], as well as constitute a feedstock for biofuels [23], bioplastics [86], or it can be employed to produce syngas in a supercritical water gasification plant (SCWO) [87].

4. Conclusions

In this work four different strains of microalgae were tested for their ability in bioremediating pretreated municipal wastewaters. Biomass was characterized and an increase in lipid and carbohydrate fractions was observed in almost all the tested strains, together with a specie-specific shift in fatty acid composition, leading in one case to EPA accumulation. Chemical analyses were performed, and COD value was found to increase in all samples, while nitrogen and phosphorous levels always decreased (by 77 and 61%, respectively). *Chlorella* strains, especially the Sicilian one (the one denominated "Pozzillo" and the one in consortium with *Dunaliella* "Vergine Maria"), were the most effective in the general bioremediation of the samples. In conclusion, this work is a proof-of concept research aimed at demonstrating that the autochthone strains analyzed may be considered as suitable candidates for a commercial bioremediation process, with the advantage of accumulating high-value compounds (*e.g.* PUFAs).

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