

Microalgae cultivation by uncoupled nutrient supply in sequencing batch reactor (SBR) integrated with olive mill wastewater treatment

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Abstract

The growth of bacteria contaminants can be controlled in heterotrophic microalgae cultures by using an uncoupled supply of glucose and nitrate. However, till now this strategy was only described for fed-batch cultivation. The cultivation in a sequencing batch reactor (SBR) could be more promising for the industrial scale.

Here in this work, we tested the uncoupled supply of substrates on microalgae cultivated in SBR (feast/famine ratio = 1.33), with an optimized culture medium (based on the microalgae elemental formula), and the integration of this strategy with olive mill wastewater (OMW) treatment. SBR allowed to attain biomass productivities (P_X) proportional to the initial biomass concentration ($P_X = 0.13 \cdot X_0$), showing the possibility to reach the same productivities as conventional axenic cultures, by maintaining bacteria contamination at negligible values ($< 5 \%$, as CFU/algae). The SBR system showed a stable biomass production (1.54 folds X_0) throughout 8 consecutive cycles (53 days), uncoupling biomass production and cell duplication. However, relevant grazer contamination reduced the growth of microalgae cells between the 4th and 7th cycle and the biomass yield on glucose (from 0.31 to 0.17 g g⁻¹). The integration with OMW treatment proved the possibility to remove 52 % of phenols, but the loss of fermentable substrates during OMW storage and preliminary processing (by membrane filtration) hindered the exploitation of OMW as a relevant alternative source for organic substrates.

Keywords: wastewaters, contaminant control, grazers, bacteria, pollutant removal, heterotrophic growth

Nomenclature

OMW = olive mill wastewater.

SBR = sequencing batch reactor.

PBR: photobioreactor

C_{MF} = concentrate (retentate) from microfiltration.

C_{UF} = concentrate (retentate) from ultrafiltration.

C_{NF} = concentrate (retentate) from nanofiltration.

P_{MF} = permeate from microfiltration.

P_{UF} = permeate from ultrafiltration.

P_{NF} = permeate from nanofiltration.

N^+G^- = phase with N and without glucose (organic/C source) in the culture medium (famine phase).

N^-G^+ = phase with glucose (organic/C source) and without N in the culture medium (feast phase).

Q_{max} = maximum N quota ($g_N g_x^{-1}$).

Q_0 = minimum N quota ($g_N g_x^{-1}$).

q = N quota at the beginning of the cycle ($g_N g_x^{-1}$).

f_c = Amount of biomass consumed for mass of N assimilated during G^-N^+ ($g_x g_N^{-1}$).

$Y_{X/S}$ = biomass to glucose yield ($g g^{-1}$).

P_X = biomass productivity ($g L^{-1} d^{-1}$)

X_N = biomass concentration at the end of the whole cycle ($g L^{-1}$).

X_G = biomass concentration at the end of the G^+N^- phase ($g L^{-1}$).

X_0 = biomass concentration at the beginning of the cycle ($g L^{-1}$).

$X(t)$ = biomass concentration measured at time t ($g L^{-1}$).

S = substrate concentration ($g L^{-1}$).

S_0 = initial substrate (nutrient) concentration ($g L^{-1}$).

$S(t)$ = substrate concentration measured at time t ($g L^{-1}$).

S_G = glucose concentration measured at the end of the G^+N^- phase ($g\ L^{-1}$).

ΔN = N uptake during the G^-N^+ phase ($g\ L^{-1}$).

$Cell(t)$ = microalgae cell concentration measured at time t ($10^6\ mL^{-1}$)

$Cell_0$ = microalgae cell concentration at the beginning of the cycle ($10^6\ mL^{-1}$)

$[n]_i$ = concentration of the n-compound in the i-stream ($g\ L^{-1}$).

V_i = volume of the i-stream (L).

M_n = initial mass of the n-compound (g).

BOD_5 = biochemical oxygen demand measured after 5 d ($g\ O_2\ L^{-1}$).

1. Introduction

In recent years microalgae have emerged as a promising sustainable feedstock for different industrial applications as a source of food, feed and chemicals [1]. Microalgae are photosynthetic microorganisms able to synthesize several compounds of industrial interest, such as proteins, pigments, antioxidants, polymers and fatty acids [2]. They can grow by using different metabolisms, the main are photoautotrophic, heterotrophic and mixotrophic [3,4]. Industrial cultivations are carried out in two main processes: fully photoautotrophic and fully heterotrophic. Photoautotrophic processes have the advantage to use only light, CO_2 and minerals salts as nutrients, however, there is a relevant consumption of energy for culture mixing, light exposition, temperature control and gas supply [5,6]. Weighting the costs of such energy consumption as compared to the typical low biomass productivities ($0.01\text{--}1\ g\ L^{-1}\ d^{-1}$) [5], the minimum reported production costs are about $3\text{--}10\ \text{€}\ kg^{-1}$, too high for a large part of the envisaged applications [2,7]. As a result of the energy consumption, GHG gas emissions ($CO_2\ kg^{-1}$) reported are typically higher than conventional crops [6]. In contrast, heterotrophic processes can attain higher biomass productivities (more than $20\ g\ L^{-1}\ d^{-1}$) [8], but about half of the production cost is given by the organic substrate, and, also, aseptic reactors and axenic cultures are required to avoid bacteria contamination, increasing remarkably the capital costs [8]. Limited data are available about microalgae biomass production cost and environmental sustainability from heterotrophic cultures, but some preliminary assessments indicate that

environmental sustainability higher than photoautotrophic culture may be attained [9]. The integration of microalgae cultivation with wastewater treatment is a promising way to enhance sustainability, by replacing synthetic substrates with organics from wastes and by integrating biomass production with pollutant removal. Many wastewaters contain high concentrations of organic substrates (up to 100 g L^{-1} COD), thus they can be used to increase the productivity of microalgae cultivation processes, by exploiting heterotrophic metabolism [2,10–12]. However, contamination by heterotrophic bacteria is one of the main issues hindering wastewater exploitation in microalgae cultivations [13–15]. Wastewater sterilization is difficult, because of the high volume involved, complex chemical composition and for the presence of suspended materials, that make difficult thermal and physical treatments. In addition, sterilization treatment invariably increases the cost of substrates furnished by wastewaters, affecting their potential advantages. In a recent study, we described a process by which microalgae can be cultivated in heterotrophic fed-batch conditions in non-axenic environment by uncoupling organic substrate and N substrate feeding [14]. With this strategy, bacteria contamination could be efficiently controlled. The aim of this work is to integrate this cultivation strategy with the treatment of real wastewater, namely Olive Mill Wastewater (OMW).

OMW is produced from olive oil production, mainly in the Mediterranean area, for an estimated worldwide production of $7\text{--}30 \cdot 10^6 \text{ m}^3 \text{ year}^{-1}$ [16]. Currently, there is not a common policy about OMW treatment, for instance, in Italy OMW is mainly displaced by spreading on agricultural lands, with negative antimicrobial and phytotoxic effects [17] and at $10\text{--}40 \text{ € m}^{-3}$. Several studies have investigated the use of microalgae to treat OMW [18–22], however, OMW usually reduces microalgae growth due to antimicrobial effects by phenols [23,24] and due to its dark color [21], that strongly reduces light penetration. Because of the dark color, and because of the high content of sugars and other organic substrates, previous works indicated heterotrophy as the best way to maximize microalgae growth and pollutant removal, by exploiting a two-stage process (phototrophic + heterotrophic) or by a fully heterotrophic process [18,19,23,25]. However, in these latter works, the

management of bacterial contamination still remains a challenge. Therefore, the aim of this study is to couple the insights found in previous works about bacteria contamination control in microalgae cultivations and those about cultivation integrated with OMW treatment, by investigating an integrated heterotrophic process to achieve microalgae biomass production, pollutant removal and negligible bacteria contamination.

Another relevant issue affecting the application of OMW treatment processes is the seasonality of OMW production, which affects the economical sustainability of an industrial process. To overcome such an issue, we tested the integration with a membrane filtration pre-treatment, by which OMW volume can be reduced approximately ten folds, allowing easier storage. OMW treatment by membrane filtration can be considered a ready technology, but the main limit for its wide application is the cost (5–10 € m³) [26]. This cost could be overcome by a valorization of the side concentrate streams produced, giving an economic gain. In this context, microalgae can be integrated for the treatment of the concentrate side streams produced by OMW membrane filtration [25].

This study aimed at testing, at lab scale, the integration of the heterotrophic cultivation of microalgae with uncoupled nutrient feeding strategy (to control bacterial contamination) with the treatment of organic concentrate obtained from OMW membrane filtration (Fig. 1.). To uncouple nutrient feeding, a sequencing batch reactor (SBR) was tested in this study in place of the previously tested fed-batch [14], because: SBR is expected to be more easily implementable at larger scale, SBR should attain higher biomass productivity and it was largely applied in similar feast and famine processes for the selection of bacteria producing PHA integrated with wastewater treatment [27].

2. Materials and methods

2.1 Microalgae strain

A strain of *Tetradismus obliquus* (generally known as *Scenedesmus obliquus*) was selected and identified as previously described [18]. The strain was maintained in solid and liquid BG11 medium, in phototrophic and non-axenic conditions, as previously described [19].

The microalgae biomass used as starting inoculum for the heterotrophic experiments was grown under phototrophic conditions in closed 4 L column photobioreactors (PBR) with BG11 culture medium (table S1) renewed every 10-15 days by 1 to 10 dilution. PBRs were maintained under magnetic stirring, 2.5 L min⁻¹ air feeding and 100 μmol s⁻¹ m⁻² light supply (24 h/24) by using cool white fluorescent lamps. After growth in PBRs, microalgae were harvested and inoculated for heterotrophic cultivations.

2.2 Experimental-based optimization of the chemical composition of the culture medium

In this experiment, microalgae were cultivated in heterotrophic conditions by following the protocol described in a previous work [14], but in a sequencing batch reactor (SBR) instead of fed-batch. This cultivation protocol was proved to be effective in avoiding contamination by bacteria competitors. Microalgae grown in PBRs were harvested by centrifugation at 4,500 rpm for 5 min and washed twice with distilled water. This biomass was used as inoculum for three cultivation conditions, with different initial biomass concentrations $X_0 = 1 \text{ g L}^{-1}$, 5 g L^{-1} and 10 g L^{-1} . Microalgae were suspended in 100 mL medium and cultivated in 200 mL glass bottles ($h = 10 \text{ cm}$, $d = 5 \text{ cm}$), fed with air at 150 mL min⁻¹ ($1.5 \text{ L L}^{-1} \text{ min}^{-1}$) and maintained at a constant temperature of $30 \pm 1 \text{ }^{\circ}\text{C}$. The pH was daily measured and adjusted at 7 with HCl 1 M or NaOH 1 M. The bottles were completely covered with aluminum foil to ensure heterotrophic growth. For $X_0 = 1 \text{ g L}^{-1}$, BG11 medium without NaNO₃ was used, supplemented with 2 g L⁻¹ glucose, for $X_0 = 5 \text{ g L}^{-1}$, the medium was BG11 5x (without NaNO₃) supplemented with 10 g L⁻¹ glucose, while for $X_0 = 10 \text{ g L}^{-1}$ the medium was BG11 10x (without NaNO₃) supplemented with 20 g L⁻¹ glucose (the composition is reported in table S1). After 2 days of cultivation (corresponding to the exhaustion of glucose), NaNO₃ was added at 0.18 g L⁻¹ (for $X_0 = 1 \text{ g L}^{-1}$), at 0.6 g L⁻¹ (for $X_0 = 5 \text{ g L}^{-1}$) and at 1.4 g L⁻¹ (for $X_0 = 10 \text{ g L}^{-1}$). After 2 days of growth with NaNO₃, the biomass was harvested by centrifugation and a fraction of it was used as inoculum for another subsequent new cycle of growth in the same condition (with the same X_0).

The biomass productivity (P_X) was measured as:

$$P_X = \frac{(X(t)-X_0)}{(t-t_0)} \quad (1)$$

with $x(t)$ indicating the biomass measured at a time = t of the cultivation cycle, while t_0 and x_0 are the time and the biomass concentration at the beginning of each cycle.

A fraction of the biomass used as starting inoculum was dried at 105 °C until constant weight and then analyzed for elemental composition determination (CHNS analyzer). A fraction of the culture medium was collected at the beginning of the experiment, at the end of the phase with glucose (before NaNO_3 addition) and at the end of the whole cycle. These samples were separated from biomass using centrifugation at 10,000 rpm for 5 min and stored at – 18 °C until analysis.

For the sake of clarity, it is emphasized that the word “cycle” is used in this work to indicate a phase of cultivation with glucose (in N-starvation), followed by a phase of cultivation with N (in glucose starvation).

The percent biomass content of P, K, Mg, Fe, Ca and Na were determined by means of the measured consumption of nutrients during cultivation, as follows:

$$\% = \frac{(S_0-S(t))}{(X(t)-X_0)} 100 \quad (2)$$

Where S_0 and $S(t)$ are the concentrations of the nutrients at the beginning of the cultivation and at the end of the cycle.

2.3 Heterotrophic cultivation in SBR with the optimized culture medium.

Two culture mediums were used for each SBR cycle, the first one with glucose but without N ($\text{N}^- \text{G}^+$) and a second one with N but without glucose ($\text{N}^+ \text{G}^-$) (table 1). Both the mediums were prepared using tap water (composition reported in a previous work [28]), with nutrients added based on the results of the previous experiment. NaNO_3 was added in excess in the $\text{N}^+ \text{G}^-$ medium, with respect to the expected consumption calculated as described in a previous study [13], to assure the restoring of maximum N quota ($Q_{\max}$).

[Table 1]

Microalgae were harvested from PBRs by centrifugation at 4,500 rpm for 5 min and washed twice with distilled water. After that, this biomass was used as inoculum to start the cultivation in the SBR (Fig. 1A). This biomass was suspended in 250 mL of N^-G^+ medium in 800 mL conical flask, at $X_0 = 4.5 \pm 0.7 \text{ g L}^{-1}$ and maintained in the dark under orbital shaking at 150 rpm at $27 \pm 3 \text{ }^\circ\text{C}$. The pH was daily monitored and adjusted at 8 by adding NaOH 5 M or HCl 1M. The speed of the shaking and the ratio between medium volume and flask volume was chosen to ensure enough O_2 supply, based on a previous work [29]. After 4 days of cultivation, the whole biomass was harvested by centrifugation, washed twice with distilled water and suspended in the N^+G^- medium, maintained in the same conditions for other 3 days. At the end of each cycle (7 days) a new consecutive cycle with N^-G^+ medium was started. For the new cycle, the biomass in excess was removed to have the same X_0 . The cultivation cycle in the SBR took 7 d (Fig. 1B) and can be summarized with the following sequencing operations: a carbon/energy substrate (glucose) feeding phase (10 min, 0.25 L), a first reaction/aeration phase of 4 d, a withdrawal phase of the exhausted medium (5 min), a N substrate feeding phase (NO_3^-) (5 min, 0.25 L), a second aeration/reaction phase of 3 d, and a harvesting and biomass separation phase of 2 h. This cultivation system can be also viewed as a SBR with feast/famine ratio = 1.33 and a feast phase length that is 60 % of the whole cycle [27,30].

This cultivation was carried out for 8 consecutive cycles.

For each cycle, the f_C factor has been measured as:

$$f_C = \frac{X_N - X_G - \Delta N}{\Delta N} \quad (3)$$

With X_N and X_G indicating the biomass concentration at the end of the whole cycle and at the end of the G^+N^- phase, respectively, and ΔN the amount of N consumed in the G^-N^+ phase.

Minimum (Q_0) and maximum (Q_{max}) N quota were obtained as follow:

$$Q_0 = \frac{X_0 q}{X_G} \quad (4)$$

$$Q_{max} = \frac{X_0 q + \Delta N}{X_N} \quad (5)$$

with X_0 the biomass concentration at the beginning of each cycle and q the N quota at the beginning of each cycle. For the first cycle, $q = 0.07$ was assumed, based on previous measurements [14].

Glucose to biomass yield has been calculated as follows:

$$Y_{X/S} = \frac{(X_i - X_0)}{(S_0 - S_G)} \quad (6)$$

With X_i the biomass concentration measured at the end of the G^+N^- phase (X_G) or at the end of the whole cycle (X_N), while S_0 and S_G the glucose concentration at the beginning and at the end of the G^+N^- respectively.

2.4 Olive Mill Wastewater (OMW) pre-treatment

OMW was collected from a continuous three-phase olive oil production plant located in Italy (Rocca Massima, LT). It was stored for about one month in closed tanks at 15-20 °C and then filtered by means of subsequent membrane filtration processes, that is microfiltration (MF), ultrafiltration (UF) and nanofiltration (NF) in series. Thin-film composite membranes in 2.51 m² spiral wounded modules supplied by GE Water were used for all the filtrations. The model JX4040 with 300.0 nm pore size was used for MF, the model GM4040 with 2.0 nm pore size was used for UF, and the model DK4040 with 0.5 nm pore size was used for NF. The membrane plant consisted of a 100 L feedstock tank, two pumps (centrifugal booster and volumetric piston) that could be selected to drive the feed-stream to the membrane module inserted in housing; two regulation valves with a precision of 0.5 bar and 10 L h⁻¹, respectively (that served to set the desired operating pressure and crossflow over the membrane independently), measured and displayed by analogue manometers and a turbine flow meter respectively; two plate heat exchangers to maintain the temperature of the streams stable at ambient temperature. More details about the filtration system can be found in Fig. S1 and in a previous work [31]. The membrane performances concerning productivity (fluxes) and selectivity (rejection values) are in line with those observed previously, reported in a previous work [31]. Some research pointed out that pretreatment processes for this type of wastewater stream are mandatory to avoid severe membrane fouling, such as the use of photocatalysis [32,33]. This process is well studied and appears

to enhance efficiencies with the use of nanoparticles as a catalyst [34]. However, because in this study we aimed at minimizing the degradation of organic compounds (to use them to feed microalga), the photocatalysis pre-treatment was replaced by MF.

The concentrate and permeates obtained have been analyzed within 2 days from the membrane treatment, and then stored in the dark in closed PET bottles at environmental temperature (15-20 °C). The recovered mass (%) for each n-compound in the concentrate and permeate streams produced throughout the membrane treatment was calculate as follows:

$$\%_n = \frac{[n]_i V_i}{M_n} 100 \quad (7)$$

where the percent mass for each n-compound in each i-stream was calculated considering its concentration in the stream ($[n]_i$), stream volume (V_i) and the initial mass of the n-compound (M_n). To minimize mistakes due to degradation during OMW storage, the initial mass of each n-compound was calculated as follows:

$$M_n = V_{CMF} [n]_{CMF} + V_{PMF} [n]_{PMF} \quad (8)$$

as the sum of the mass in the permeate and concentrate MF streams. For the volumes, a separation ratio of 7/3 L L⁻¹ (2.3) was assumed for all the membrane treatments.

A fraction of the raw OMW was stored in three 50 mL glass bottles, closed with rubber caps, and stored for more than 6 months in a dark environment at 25 ± 3 °C. These bottles were periodically analyzed for BOD₅, sugars and phenols.

2.5 Cultivation integrated with treatment of OMW concentrates

A fraction of the biomass harvested from the sequencing batch cultivation at the end of the cycles n° 3 to 7 was suspended ($X_0 = 5.0 \pm 1.2$ g L⁻¹) in 300 mL conical flasks with 100 mL culture medium. The culture medium was obtained by adding the nutrients of the G⁺N⁻ medium (table 1), but at a diluted amount at 9 g L⁻¹ glucose, and with 12 mL (0.12 v v⁻¹) of OMW concentrate solution (60 % concentrate from UF, C_{UF}, and 40 % concentrate from NF, C_{NF}). The flasks were maintained for 4 days in the dark under 150 rpm orbital shaking. The pH was daily monitored and adjusted to 8 by

adding NaOH 5 M or HCl 1M. The OMW concentrate solution (60 % C_{UF}/40 % C_{NF}) were analyzed at the beginning of each batch to assess the initial phenol concentration.

2.6 Chemical and biological analyses

Elemental composition was measured by CHNS elemental analyzer (EA 1110 CHNS/O), while Ca, Fe, K, Mg and Na were measured by Atomic Adsorption Spectroscopy (AAS) with the instrument contrAA[®] 300 Analytik Jena AG. Oxygen content was obtained as a difference between 100 and the percentage of the other analyzed elements. Sugars concentration in the culture medium was determined by the phenol-sulfuric acid (Dubois) method [19]. Total phenols were determined by means of the colorimetric Folin-Ciocalteu method and reported as tyrosol equivalents [23].

Total N (TN) and total phosphorous (TP) have been analyzed by the official method for water analysis by IRSA-CNR 4060, as follows: sample was diluted to 25 mL with distilled water and then transferred in a glass bottle together with 3.5 mL of oxidant reagent (5 g of K₂S₂O₈, 3 g of H₃BO₃ and 1.4 g of NaOH in 100 mL of water) and 0.15 g of K₂S₂O₈ (VWR CA1.05092.0250). The sample was then digested for 30 min at 120 °C in autoclave. After that, for TN, 10 mL of sample were acidified with 0.15 mL of H₂SO₄ (96%), diluted to 25 mL and the absorbance read at 220 nm. The concentration was determined by using a calibration line obtained with glycine. For TP, 15 mL of the sample obtained from autoclave digestion were mixed with 0.9 mL of reducing solution (7 g of ascorbic acid, 0.03 g of Na₂EDTA and 0.6 mL of formic acid in 100 mL water solution) and, after 2 min, with 0.9 mL of reagent solution (10 mL of potassium antimony tartrate 20.06 mM mixed with 20 mL of ammonium heptamolybdate 68.5 mM and 60 mL of H₂SO₄ 6 M). The absorbance was read after 5 min at 882 nm. Chemical oxygen demand (COD) was measured by means of the colorimetric kit Lovibond[®] Vario vials (2420721). Biochemical oxygen demand (BOD₅) was measured using the official method IRSA 5120 B1. Samples aliquots were diluted to 130 mL in closed bottles filled with a water solution containing 0.25 mg L⁻¹ FeCl₃ 6H₂O; 55 mg L⁻¹ CaCl₂; 45 mg L⁻¹ MgSO₄ 7H₂O and 2 mL L⁻¹ of a buffer solution at pH 7.2 (0.16 g L⁻¹ of K₂HPO₄; 0.065 g L⁻¹ of KH₂PO₄; 0.26 g L⁻¹ of

Na₂HPO₄ 7H₂O and 0.013 g L⁻¹ of NH₄Cl). The bottles were left in incubation 5 d in the dark at room temperature. O₂ before and after incubation was measured by using the InLab OptiOX sensor and a Mettler Toledo SevenExcellence™ multiparametric station. The BOD₅ was calculated as follows:

$$BOD_5 = [(C_{S,5} - C_{S,0}) - (C_{B,5} - C_{B,0})]D \quad (9)$$

where C is oxygen concentration, S indicates sample, B indicates blank, D indicates the dilution factor, while 5 and 0 indicate the incubation time.

Total solids were measured by drying 10 mL of sample in glass vials for 7 days (constant weight) at 105 °C. Microalgae cell concentration was measured by optical counting in a Thoma chamber by using an optical microscope (Leitz Laborlux 12). To measure biomass dry weigh a sample aliquot was filtered through 0.7 µm fiberglass filter (VWR), washed with distilled water and then dried at 105 °C till constant weight. Bacteria contamination was measured by measuring colony forming units (CFU) on solid (15 % agar) Lysogeny broth (LB) medium after 4 to 5 d of incubation at 25-27°C.

2.7 Statistical treatment of data

All the growth experiments were carried out by testing two biological replicates. Differences among groups were analyzed by the Welch one-way analysis of variance (ANOVA) by using the software R (3.6.1). For comparison between two groups, the t-test was used. Differences were considered significant when $p < 0.05$.

[Figure 1]

3. Results and discussion

3.1 Experimental-based optimization of the chemical composition of the medium

Heterotrophic cultivation of microalgae has the advantage to allow reaching biomass concentrations significantly higher than photoautotrophic cultivations. However, the utilization of conventional culture mediums, developed for conventional photoautotrophic cultures, can limit biomass production

due to the limited amount of nutrients. Therefore, to avoid nutrient limitation, the heterotrophic cultures should be fed with concentrations of nutrients proportional to the expected biomass production and its elemental composition. However, the information in the literature about the elemental composition of microalgae is poor, and mainly focused on those few species (e.g. *Chlorella* sp.) most widely cultivated in heterotrophic systems [2,8]. Despite *T. obliquus* is one of the most promising and employed species for wastewater treatments (because of its adaptability to several stressing conditions and for its good growth rate) only one previous work was found that reported its elemental composition (Table 2)[35]. For this reason, the first part of this work was aimed at the measurement of the elemental composition of *T. obliquus*, to develop a tailored culture medium for heterotrophic cultivation.

To this end, *T. obliquus* was cultivated in heterotrophic conditions by following a substrate feeding strategy based on the principles of the uncoupled addition of glucose and N source. Such strategy was described in detail in a previous work [14]. It has the advantage to avoid relevant contamination by competitor bacteria. All the other nutrients were considered in excess, based on the assumption that the nutrient concentration in BG11 synthetic medium is enough to produce 1 g/L *T. obliquus* biomass in balanced growth. Because the growth in N-starvation is limited by the accumulation ability of cells [14] and by the amount of glucose added, no more than 1 g L⁻¹, 5 g L⁻¹ and 10 g L⁻¹ of new biomass were expected to be produced for the tests with $X_0 = 1, 5$ and 10 g L⁻¹, respectively [14]. For this reason, BG11, BG11 5x and BG11 10x were used for these three conditions, respectively.

As expected, biomass concentration increased ~2 folds ($1.9 \pm 0.1 X/X_0$) during the cultivation phase with glucose ($G^+ N^-$), irrespective of the X_0 (Fig. 2A). The biomass then decreased by $21 \pm 6 \%$ during the phase with NO_3^- ($G^- N^+$). This behavior was maintained throughout the subsequent cycles. Cell duplication was uncoupled with respect to biomass growth, and it happened, as expected, only during the phases with NO_3^- (Fig. S2). Bacteria contamination was monitored to check the effectiveness in controlling the growth of heterotrophic bacteria. The strategy was confirmed effective, because the

CFU did not increase during the cultivation (Fig. 2C), remaining a negligible fraction of the microalgae population (Fig. 2D).

[Figure 2]

As expected, biomass productivity was linearly related to X_0 (Fig. 2B). However, the angular coefficient (d^{-1}) of the regression lines changed by changing the cultivation end-time considered to calculate P_X ; it was maximum by considering only 1 d of the G^+N^- phase ($0.72 \cdot X_0$) and minimum at the end of the whole cycle ($0.13 \cdot X_0$). This difference is a result of the logistic behavior in the G^+N^- phase and of the biomass consumption in the G^-N^+ phase.

By assuming X_0 between 10 - 100 g L⁻¹ in a realistic operative industrial plant [8], the described SBR process could allow attaining P_X between 1.3 and 13 g L⁻¹ d⁻¹, comparable to conventional microalgae heterotrophic cultivations carried out in axenic environment [8].

The biomass to glucose yield ($Y_{X/S}$) was higher ($p = 7 \cdot 10^{-8}$) at the end of the G^+N^- phase (0.54 ± 0.14) as compared to the end of the whole cycle (0.28 ± 0.04). This latter value, in agreement with a previous study [14], also included the consumption of biomass (stored carbohydrates) as the energy source in the G^-N^+ phase. Total carbohydrates inside biomass increased to 44 ± 9 % in the G^+N^- phase and decreased to 31 ± 5 % in the G^-N^+ phase ($p = 0.007$). A little difference ($p = 0.02$) was also found between $Y_{X/S}$ attained at $X_0 = 1$ g L⁻¹ (0.22 ± 0.03 for the whole cycle; 0.43 ± 0.08 for G^+N^- phase), with respect to those at 5 and 10 g L⁻¹ (0.30 ± 0.04 for the whole cycle; 0.59 ± 0.13 for G^+N^- phase). To have a more general term of comparison of the metabolic yield on substrate, the yields were also calculated in term of C-mol_X C-mol_S⁻¹, finding values equal to 0.35 ± 0.05 C-mol_X C-mol_S⁻¹ for the whole cycle and 0.68 ± 0.18 C-mol_X C-mol_S⁻¹ for the G^+N^- phase, in agreement with previous data from *Chlorella* [36].

The analysis of nutrients indicated that every nutrient was consumed both in the G^+N^- phase and G^-N^+ phase (Fig. S3). The percentage content of the different chemical elements was determined by eq.

(2) and compared to the previous literature data (Table 2). The only significant difference was found for S ($p = 0.03$), which was lower in our study [35]. All the other elements were within the ranges usually reported for *T. obliquus* [35] and other microalgae [8].

[Table 2]

The elemental formula $\text{CH}_{1.8}\text{O}_{0.4}\text{N}_{0.12}\text{P}_{0.015}\text{S}_{0.001}\text{Mg}_{0.01}\text{K}_{0.014}\text{Ca}_{0.002}\text{Na}_{0.008}\text{Fe}_{0.0008}$ was calculated in this study for *T. obliquus*. It is comparable to those determined for microalgae in two previous studies [35,37]. Finally, the elemental composition of the biomass was used to design a tailored growth medium for heterotrophic cultivation. For each phase of growth, the calculated demand of inorganic ions ($\text{mg}_S \text{g}_x^{-1}$) has been multiplied by the $Y_{X/S}$ ($\text{g}_x \text{g}_{\text{Glu}}^{-1}$) to obtain the stoichiometric amount of ions required for each gram of supplied glucose ($\text{mg}_S \text{g}_{\text{Glu}}^{-1}$) (Table S1 and S2). By rounding up the so obtained values, the amounts of FeSO_4 , K_2HPO_4 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in the medium (Table 1) were calculated. Ca^{2+} was not added because already available in the used tap water [19]. NaNO_3 was added in slight excess with respect to the calculated amount required to re-establish the maximum N quota in the expected produced biomass upon the $\text{G}\cdot\text{N}^+$ phase [14]. All the other micro nutrients were added based on the initial assumption made about BG11 medium.

3.2 OMW pre-treatment by membrane filtration

OMW was collected from the production plant immediately after its production and quickly analyzed within the same day of collection. The measured values, reported in Table 3, are in line with previous data [21,38]. The samples were then stored up to 6 months, because this is approximately the time passing in Italy between an olive oil production season and the following one. As the storage was performed at environmental temperature (to be representative of real conditions), a relevant degradation of organic compounds was registered (Fig. 3A), mainly due to anaerobic fermentation. The degradation was quicker for sugars, which were reduced up to 72 % in 1 month, and up to 90 %

in 6 months. Phenols and BOD₅ showed a slower decrease, attaining no more than 40-45 % degradation. This behavior was likely given by the easier biodegradation of sugars, as compared to other organic compounds, and by the anaerobic environment which impaired complete substrate oxidation. After one month of storage, the OMW was treated by membrane processes. As expected, the concentrate from microfiltration (C_{MF}) was mainly composed of high viscous suspended solids, for this reason it was not considered as a substrate for microalgae feeding (microalgae have usually scarce ability in biodegrading solid matter). Comparing to other possible pretreatment processes [33], MF appears to separate a high fraction for all compounds, including phenols. Apart from C_{MF}, for every analyzed compound the higher concentrations were found in the C_{UF} and C_{NF} (Table 3). The same trend was found in terms of percent mass recovery (Fig. 3B), which was calculated by assuming a 2.3 (70/30) separation factor (L L⁻¹) between permeate and concentrate volumes, for all the membranes. For these reasons, C_{UF} and C_{NF} have been chosen as streams to feed microalgae in the following experiments. The mass balances also showed a relevant loss for every nutrient, which was 70 % (for N), 60 % for sugars and about 50 % for phenols, P and BOD₅. These losses were likely ascribable to the biological activities of endogenous microorganisms and at phenomena as adsorption and volatilization upon the filtration.

[Table 3]

[Figure 3]

The BOD₅ to phenol ratio and the phenol concentration are fundamental parameters to take into consideration to determine the minimum feasible dilution for feeding microalgae culture. It should be considered that the phenol concentration in the reactor should be no more than 0.1 g L⁻¹, which is the threshold value previously determined to avoid negative effects on microalgae growth [23]. Ideally, we would like to obtain a concentrated solution with high BOD₅ (to replace pure glucose as

substrate for microalgae) and low phenol concentration (to minimize antimicrobial effects). However, the membrane treatment did not significantly change the BOD₅/phenols ratio, which was 2.7 in raw OMW, 2.4 in C_{UF} and 4.4 in C_{NF} (table 3). Considering that each liter of OMW treated produced 0.21 L of C_{UF} and 0.15 L of C_{NF}, the microalgae cultures should be fed with a solution containing 60 % v v⁻¹ C_{UF} and 40 % v v⁻¹ C_{NF}, corresponding to 2.8 g L⁻¹ BOD₅ and 1.08 g L⁻¹ phenols. Therefore, to avoid attaining too high phenol concentration, the OMW concentrate should be added to no more than 10 % of the culture medium, corresponding to no more than 280 mg L⁻¹ of BOD₅. This latter concentration is too low to exploit it as a significant source of substrate for heterotrophic cultivation.

3.3 SBR cultivation

The SBR cultivation aimed at assessing the stability of heterotrophic microalgae growth throughout repeated SBR cycles with uncoupled nutrients feeding, by using the culture medium optimized in the previous tests. The results reported in Fig. 4 indicate that, as expected, the biomass was only produced in the G⁺N⁻ phases, up to a maximum value ($X(t) X_0^{-1}$) corresponding to 2.65 ± 0.37 (Fig. 4B), without variations throughout the cycles ($p = 0.09$). This maximum value is higher than that one reported in a previous study for this strain [14], maybe because of the different chemical composition of the medium. Instead, the biomass was reduced during the G⁻N⁺ phases ($p = 1 \cdot 10^{-7}$), because of the utilization of the internally stored biomass as the only carbon and energy source. The biomass produced at the end of the whole cycle was 1.54 ± 0.23 folds X_0 , without statistically relevant variations throughout the cycles ($p = 0.16$). The P_X varied during the batches depending on the phase and on the considered time. In the G⁺N⁻ phase, the average P_X was 1.80 ± 0.39 g L⁻¹ d⁻¹, while the average value for the whole cycle was 0.43 ± 0.12 g L⁻¹ d⁻¹ lower than the expected value of 0.58 g L⁻¹ d⁻¹ for the used X_0 , based on the equation previously found (Fig. 2B). This was likely because in the SBR, the G⁺N⁻ phase lasted 3 days (instead of 2). In fact, by considering only 2 days for the G⁺N⁻ phase, the average calculated P_X was 0.66 ± 0.11 g L⁻¹ d⁻¹, in agreement with the expected value. Further, during the G⁺N⁻ phases, there was not a relevant variation in biomass concentration upon the

last day of the phase, indicating that the length of this phase could be reduced to 3 days, further increasing the expected P_X to $0.79 \pm 0.13 \text{ g L}^{-1} \text{ d}^{-1}$.

As expected, the cell concentration increased during the G^-N^+ phases (Fig. 5), due to cell cycle reactivation, as a result of N availability and the utilization of the internally stored energy (previously stored in G^+N^- phases). For the G^-N^+ phases in the first 4 cycles, cell duplication produced on the average 2.66 ± 0.20 folds the initial cell concentration ($cell_0$) (Fig. 5B). In contrast, cell concentration remained the same during the G^+N^- phase, due to cell exit from the cell cycle and entrance in G_0 phase.

[Figure 4]

Microalgae cell duplication showed a relevant reduction since the 5th cycle on ($p = 0.003$), in concurrence with a relevant contamination by grazers (Fig. 5), likely due to the utilization of untreated tap water for preparing the culture medium. The lower cell duplication also gave a lower cell concentration at the beginning of the subsequent G^-N^+ phases ($p = 4 \cdot 10^{-4}$), which was reduced from $355 \pm 57 \cdot 10^6 \text{ cell mL}^{-1}$ (1th cycle) to $184 \pm 7 \cdot 10^6 \text{ cell mL}^{-1}$ (8th cycle). About this aspect, it should be underlined that the uncoupled addition of nutrients is a strategy developed to control competitors, by selecting microalgae for their substrate accumulation capacity. However, this strategy is not expected to be effective against grazers, which can instead get all the nutrients by eating microalgae. Grazer contamination looked reduced after the 7th cycle (qualitative observation at microscope), with a microalgae cell duplication that became comparable to the previous cycles (Fig. 5B). Further experiments are required to elucidate if there was an effective grazer control due to the cultivation strategy or if this behavior was just a result of the predator-prey oscillation described by the Lotka-Volterra model [39]. We expect that grazers can be controlled at the industrial scale by applying the same procedures followed for photobioreactors, such as preliminary filtration of the culture medium, CO_2 asphyxiation, or surfactants [40,41].

[Figure 5]

The glucose was completely consumed in almost all the G^+N^- phases, for an average consumption of $14.0 \pm 1.5 \text{ g L}^{-1}$ for each cycle (Fig. 6A), without relevant variation among the different cycles. The $Y_{X/S}$ was significantly lower ($p = 4 \cdot 10^{-11}$) for the whole cycle, as compared to the sole G^+N^- phase, decreasing from $0.52 \pm 0.08 \text{ g g}^{-1}$ to an average value of $0.27 \pm 0.07 \text{ g g}^{-1}$. The values agree with those obtained in the previous test on a few cycles with the different culture medium (section 3.1). The $Y_{X/S}$ of the whole cycle decreased during the cultivation ($p = 1 \cdot 10^{-4}$) from an initial $0.31 \pm 0.03 \text{ g g}^{-1}$ to a minimum value of $0.17 \pm 0.04 \text{ g g}^{-1}$ attained at the 6th cycle, likely due to the described grazer contamination. The average N consumption (supplied as NO_3^-) was $280 \pm 70 \text{ mg L}^{-1}$ for each G^+N^- phase (Fig. 6B). However, this value decreased almost 50 % during the cultivation ($p = 9 \cdot 10^{-3}$), from an initial value of $315 \pm 14 \text{ mg L}^{-1}$ to a minimum value of $190 \pm 40 \text{ mg L}^{-1}$ at the 7th cycle. This decrease was likely due to the reduced cell duplication attained in the last cycles, as a result of grazer contaminations. The average N consumption per unit cell was $0.76 \pm 0.22 \text{ pg N cell}^{-1}$. In this experiment, the variation in N consumption was managed by suspending the biomass in a fresh culture medium (table 1) after the end of each cycle, avoiding in this way the presence of residual accumulated N at the beginning of the following G^+N^- phase, which could induce relevant contamination by competitor bacteria [14]. However, the replacement of the entire culture medium should be avoided in real industrial plants. To overcome the issue of the oscillation in N consumption, and thus N accumulation, three possible strategies could be implemented: i) an accurate filtration of every stream fed to the reactor to minimize the entrance of grazers. ii) the implementation of control systems to kill grazers [40], coupled with a monitoring system able to detect early any grazer contamination [42]. iii) a control system to feed N proportionally to its consumption.

The f_C factor has been calculated (eq. 3), to quantify the cost paid by the cells for N uptake and cell duplication, measured as biomass consumed in each G^+N^- cycle for each gram of consumed N. An

average $f_C = 17.2 \pm 5.7 \text{ g g}^{-1}$ was found (Fig. 6D), without relevant variations among the different cycles ($p = 0.23$). This number is in agreement with 17 g g^{-1} , the value previously found with uncoupled fed-batch cultivation [14].

[Figure 6]

The N quota was determined based on mass balances for biomass and N (eq. 4 and eq. 5). The quotas oscillated throughout the cycles as a result of biomass accumulation and N uptake, attaining Q_0 and $Q_{\max}$ at the end of the G^+N^- phase and at the end of the whole cycle, respectively. For both Q_0 and $Q_{\max}$ there was not a significant variation among the different cycles, with average values of $Q_0 = 0.035 \pm 0.005 \text{ g}_N \text{ g}_X^{-1}$ and $Q_{\max} = 0.095 \pm 0.014 \text{ g}_N \text{ g}_X^{-1}$. These values agree with those usually reported for different eukaryotic green microalgae [14,43,44].

[Figure 7]

3.4 Batch cultivation with OMW concentrates

Based on the considerations described in section 3.2, a solution containing 60 % C_{UF} and 40 % C_{NF} was fed at the beginning of each batch, diluting it at 10 % to the final volume, to avoid too high phenol concentration. Because at this dilution only 0.28 g L^{-1} of BOD_5 equivalents (table 3) were furnished from OMW, no relevant advantage by this surplus in substrate on biomass production was expected. In agreement, the average $Y_{X/S}$ obtained (calculated based on the sole pure glucose added) from this test was $0.49 \pm 0.14 \text{ g g}^{-1}$, like the one found for the SBR cultivation with only glucose (Fig. 6C), indicating that the BOD_5 furnished was, as expected, too low to furnish any detectable positive effect on biomass growth. Despite this expected value, a lower glucose concentration was added in this test (9 g L^{-1} instead of 15 g L^{-1}). This lower glucose concentration was adopted to maximize the

removal of organic compounds from OMW concentrate, and in particular to stimulate the removal of phenols, which are usually removed only after the exhaustion of other substrates more easily metabolizable [19]. Because of this lower glucose concentration, the batch cultivation with OMW concentrate gave a lower biomass production as compared to the SBR cultivation with synthetic medium (G^+N^- phase), corresponding on the average at 1.85 ± 0.29 folds X_0 (Fig. 8A), without relevant variations among the different batches ($p = 0.81$). This strategy allowed to attain a relevant removal of phenols, which corresponded on the average at 52 ± 7 % reduction, without relevant variations among the different batches performed (Fig. 9A). This result gives an added value to the integration of microalgae growth with OMW concentrates. This remarkable yield in phenol removal is comparable with the highest values previously found in other studies about phenols removal from OMW by microalgae [19,24]. The metabolic pathways involved in phenol removal are still mostly unknown, requiring further specific studies to be elucidated [24].

[Figure 8]

An increase of 2.01 ± 0.62 folds in cell concentration was measured upon the batch cultivation with OMW concentrates (Fig. 8B). This result could be given by the utilization of some residual N in OMW concentrates. CFU analyses performed at the end of each batch indicated that culturable bacteria cells were a significant fraction of the total cells (CFU + microalgae). This fraction increased from 31 % in the first batch to 70 % in the last batch, however, this difference was not significant ($p = 0.25$). This bacteria concentration is still in line with the values found for many photoautotrophic cultures, where contamination by heterotrophic competitors is per definition negligible [19,45]. An estimation of the bacteria mass was made by assuming 1 pg for each CFU. Based on this assumption, we converted the values obtaining that the bacteria mass should have been between 3.5 and 21 % of total dry weight, thus indicating that microalgae biomass was the predominant biomass produced. It should be underlined that this is only a preliminary and conservative estimation because on one hand

CFU could underestimate total bacteria cells [46], while on the other hand we used a conservative high conversion factor for bacteria mass of 1 pg/cell, while many bacteria are known to have masses up to 2 order of magnitude lower [46,47].

[Figure 9]

Conclusions

The heterotrophic cultivation of non-axenic microalgae cultures by uncoupled addition of glucose and NO_3^- in a feast and famine sequencing batch reactor (SBR) allowed attaining biomass productivities comparable to those of conventional axenic cultures, without relevant contamination by competitor bacteria. Biomass productivity was proportional to the initial biomass concentration and varied based on the length of the SBR phases. The experiments conducted allowed us to determine the minimum formula for *T. obliquus* and to develop an optimized culture medium, which has been tested for 8 consecutive SBR cycles with feast-to-famine ratio = 1.33. Microalgae grew throughout the 8 cycles by uncoupling biomass production (performed in the G^+N^- feast phases) and cell duplication (performed in the G^-N^+ famine phases). However, relevant contamination by grazers was detected after four cycles, suggesting the necessity to couple this cultivation strategy (developed to control competitors) with adequate systems to control contamination by predators. The integration of this SBR system with a batch reactor for OMW treatment allowed attaining high yield in phenol removal (- 52 %), without attaining worrying bacteria contaminations. However, the preliminary OMW processing (storage + membrane treatments) strongly reduced the concentration of the fermentable substrates, hindering its utilization in replacing pure glucose as a substrate for microalgae growth. Although feast and famine SBR configuration was widely applied to select microorganisms producing PHA, little was made for cultivating other industrially relevant microorganisms. This work

showed how such a configuration can be applied to select microalgae against competitor contaminants.

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Figure Captions

Figure 1. A) Process scheme of the cultivation in sequencing batch reactor (SBR) integrated to the treatment of olive mill wastewater (OMW) concentrate obtained from ultrafiltration (C_{UF}) and nanofiltration (C_{NF}). The photobioreactors were used to produce the inoculum for the start-up of the first cycle of the SBR. Every SBR cycle (7 d) included two different phases (feeding + reaction), a first phase with glucose without N (G^+N^-) followed by a subsequent phase with N without glucose (G^-N^+). The biomass produced from the SBR was used as inoculum for the batch reactor used for wastewater (OMW concentrates) treatment. B) schematic description of the operations performed in each SBR cycle.

Figure 2. Biomass production (A) and productivity (B) during the SBR cultivation conducted to assess the elemental composition of *T. obliquus* for three different initial biomass concentration (X_0). In A), in white the phases with glucose without NO_3^- (G^+N^-) are evidenced, while in grey the phases with NO_3^- without glucose (G^-N^+) are evidenced. In B), the productivities calculated after 1 d of G^+N^- phase ($P_{X,1}$), after 2 d of G^+N^- phase ($P_{X,2}$) and at the end of the whole cycle ($P_{X,C}$) are reported, as function of X_0 . C) Heterotrophic bacteria measured as CFU at the beginning and end of the SBR cultivation. C) Percent amount of bacteria cells (as CFU) compared to microalgae, at the end of the SBR cultivation. Two biological replicates were tested for each X_0 . Data are reported as mean $\pm$ SD.

Figure 3. (A) Reduction in sugars, BOD_5 and phenols content in OMW during storage in closed and dark vessels. (B) Results of the mass balances performed to calculate the % mass repartition of the different compounds among the different streams. The values are reported as mean $\pm$ SD of three replicates.

Figure 4. Biomass concentration (A) and biomass production divided by the initial X_0 (B) as function of cultivation time. The G^+N^- phases are indicated in white, while the G^-N^+ phases are indicated in grey. The end of each cycle (1-8) is indicated with a dashed line. Data are reported as mean $\pm$ SD of two replicates.

Figure 5. Cell concentration (A) and cell duplication divided by the initial cell₀ (B) as function of cultivation time. The G^+N^- phases are indicated in white, while the G^-N^+ phases are indicated in grey. The end of each cycle (1-8) is indicated with a dashed line. Data are reported as mean $\pm$ SD of two replicates.

Figure 6. A) Glucose consumption, initial and final glucose concentration during the G^+N^- phases. B) N consumption, initial and final N concentration during the G^-N^+ phases. C) Biomass to glucose yield calculated in the G^+N^- phases and for the whole cycles. D) Amount of biomass consumed for every gram of N assimilated (f_c) during the G^-N^+ phases. For each figure, statistically significant differences are indicated with different letters. Data are reported as mean $\pm$ SD of two replicates. A significant difference ($p < 0.05$) among different cycles was found for the data reported in B and C. For C, a significant difference was found between G^+N^- and the whole cycle.

Figure 7. Internal biomass N quota measured during SBR growth. The G^+N^- phases are indicated in white, while the G^-N^+ phases are indicated in grey. The end of each cycle (1-8) is indicated with a dashed line. Data are reported as mean $\pm$ SD of two replicates.

Figure 8. Biomass production divided by the initial biomass X_0 (A) and biomass concentration (C) as function of cultivation time. Cell duplication divided by the initial cell₀ (B) and cell concentration (D) as function of cultivation time. The end of each separated batch (1-5) is indicated with a dashed line. Data are reported as mean $\pm$ SD of two replicates.

Figure 9. A) Phenol removal (%), initial and final phenol concentration for the different batch cultivation with C_{UF} and C_{NF} . B) % of CFU with respect to microalgae. D) % for heterotrophic bacteria (from CFU) and microalgae dry weight on total microbial biomass estimated by assuming 1 pg cell^{-1} for each CFU. Data are reported as mean $\pm$ SD of two replicates.

Table Captions

Table 1. Chemical composition of the two different culture mediums employed during heterotrophic cultivation of microalgae in SBR mode.

(*) salts already present in the tap water used to make the culture medium.

Table 2. Percentage content of the chemical elements in the biomass of *T. obliquus* as determined by this study and by a previous study for the same species. The values are reported as mean $\pm$ SD.

(*) statistically significant difference between the two studies ($p < 0.05$).

Table 3. Chemical parameters measured in raw OMW and in the concentrate (C) and permeate (P) fractions obtained after microfiltration (MF), ultrafiltration (UF) and nanofiltration (NF). Data for raw OMW have been measured after removal of suspended solids by centrifugation at 4500 rpm. The values are reported as mean $\pm$ SD.

Table 1

Salt (g L ⁻¹)	N ⁻ G ⁺	N ⁺ G ⁻
K ₂ HPO ₄	0.54	0.54
MgSO ₄ 7H ₂ O	0.48	0.48
FeSO ₄	0.024	0.024
Ca(HCO ₃) ₂ (*)	0.4	0.4
Mg(HCO ₃) ₂ (*)	0.11	0.11
EDTA	0.0075	0.0075
H ₃ BO ₃	0.00045	0.00045
MnSO ₄ H ₂ O	0.0012	0.0012
ZnSO ₄ 7H ₂ O	0.0021	0.0021
(NH ₄) ₆ Mo ₇ O ₂₄ 4H ₂ O	9·10 ⁻⁵	9·10 ⁻⁵
CuSO ₄ 5H ₂ O	1.8·10 ⁻⁵	1.8·10 ⁻⁵
Na ₂ SO ₄	0.27	0.7
Glucose	15	0
NaNO ₃	0	2.6

Table 2

C (%)	O (%)	H (%)	N (%)	S (%)	P (%)	Fe (%)	Mg (%)	K (%)	Ca (%)	Na (%)	Reference
51 $\pm$ 1	27 $\pm$ 3	7.6 $\pm$ 0.2	7.4 $\pm$ 0.1	0.13 $\pm$ 0.03*	2.0 $\pm$ 0.2	0.20 $\pm$ 0.06	1.1 $\pm$ 0.4	2.4 $\pm$ 1.0	0.4 $\pm$ 0.2	0.8 $\pm$ 0.6	This study
n.d.	n.d.	n.d.	6.8 $\pm$ 0.8	0.35 $\pm$ 0.07*	1.4 $\pm$ 0.6	n.d.	0.55 $\pm$ 0.2	1.4 $\pm$ 0.3	0.055 $\pm$ 0.04	n.d.	[35]

[Table 3]

	Raw OMW	C _{MF}	P _{MF}	C _{UF}	P _{UF}	C _{NF}	P _{NF}
COD (g L ⁻¹)	140 ± 20	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BOD ₅ (g L ⁻¹)	19 ± 6	6.11	5.72	3.79	0.66	1.36	2.02
TS (g L ⁻¹)	94 ± 2	92 ± 20	28.3 ± 0.3	19.3 ± 0.2	5.8 ± 2.4	8.1 ± 0.2	5.9 ± 0.5
Phenols (g L ⁻¹)	6.8 ± 0.1	2.82 ± 0.02	2.06 ± 0.05	1.59 ± 0.01	0.202 ± 0.01	0.31 ± 0.00	0.17 ± 0.00
Sugars (g L ⁻¹)	23.5 ± 1.8	2.26 ± 0.00	1.93 ± 0.09	2.41 ± 0.80	0.27 ± 0.00	0.399 ± 0.01	0.16 ± 0.01
pH	5.2 ± 0.1	4.67 ± 0.04	4.62 ± 0.00	5.13 ± 0.00	5.35 ± 0.03	5.48 ± 0.01	5.49 ± 0.01
TN (mg L ⁻¹)	580 ± 130	1039 ± 37	232 ± 5	246 ± 9	19.48 ± 0.01	22 ± 2	11 ± 1
TP (mg L ⁻¹)	369 ± 100	226 ± 24	329 ± 37	111 ± 27	52 ± 3	82.3 ± 0.6	51.2 ± 0.0