Annals of Microbiology (2018) 68:899–913
https://doi.org/10.1007/s13213-018-1400-9

ORIGINAL ARTICLE

Nutrient removal from high strength nitrate containing industrial wastewater using *Chlorella* sp. strain ACUF_802

Jairo Hernan Moreno Osorio[1] · Vincenzo Luongo[2,3] · Angelo Del Mondo[4] · Gabriele Pinto[4] · Antonino Pollio[4] · Luigi Frunzo[2] · Piet Nicolaas Luc Lens[5,6] · Giovanni Esposito[1]

Received: 2 August 2018 / Accepted: 1 November 2018 / Published online: 16 November 2018

Abstract

the research aim of this study was to characterize an isolated native strain of *Chlorella* sp. ACUF_802, well adapted to a high nitrate concentration environment and to investigate its potential to nitrate and phosphate removal from industrial wastewaters with the minimal addition of chemical reagents and energy. The isolated strain was identified and evaluated for its capability to support biomass growth and nutrient removal from synthetic wastewater in batch tests using different concentrations of carbon and nitrogen, different carbon sources and N:P ratios. The strain was isolated via the plating method from the settler of a pilot scale moving bed biofilm reactor performing a nitrification process. The strain was identified using molecular analysis with rDNA primers. Using sodium bicarbonate as carbon source, the batch productivity (71.43 mg L^{-1} day^{-1}) of the strain *Chlorella* sp. ACUF_802 was calculated with a logistic model and compared to the values reported in the literature. Assays on the effect of the N:P ratio indicated that the productivity was increased 36% when the N:P ratio was close to 1 (111.96 mg L^{-1} day^{-1}), but for a complete phosphorus removal a 5:1 N:P ratio with nitrate concentrations ≤125 mg·L^{-1} is recommended. The isolated microalgae strain *Chlorella* sp. ACUF_802 showed versatility to grow in the synthetic industrial wastewaters tested and can be considered as an appropriate organism for nitrogen removal from industrial wastewaters in the presence of an organic or inorganic carbon source.

Keywords Biomass production · Bioremediation · Freshwater microalgae · Isolation · Nitrogen and phosphorus removal

Introduction

It is widely recognized that water treatment and sanitation are essential to the achievement of many sustainable development goals (WWAP 2015). As a consequence, stricter government-issued regulatory wastewater effluent standards that give special attention to inorganic pollutants have been recently developed (Ibisch et al. 2016), especially nutrients such as nitrogen (N) and phosphorus (P), which contribute to eutrophication. Ammonium, nitrate, and phosphate are the primary forms of N and P in wastewater (Renuka et al. 2015). To date, biological and chemical systems for the removal of nutrients from wastewater are available. Chemical systems are expensive due to the addition of chemicals, while the main cost of biological systems is due to the required aeration and the sludge management and disposal (Mallick 2002). Under this scope, many efforts have been devoted to the research of alternative wastewater treatment approaches aiming to set sustainable and innovative solutions to remove N and P compounds from wastewater (Ruiz et al. 2011; Ge and Champagne 2016).

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13213-018-1400-9) contains supplementary material, which is available to authorized users.

✉ Jairo Hernan Moreno Osorio
jhernanm@gmail.com

[1] Department of Civil and Mechanical Engineering, University of Cassino and Southern Lazio, via Di Biasio, 43, 03043 Cassino, Italy

[2] Department of Mathematics and Applications Renato Caccioppoli, University of Naples "Federico II", via Cintia - Complex Monte S. Angelo, 26, 80126 Naples, Italy

[3] Department of Civil, Architectural and Environmental Engineering, University of Naples "Federico II", via Claudio 21, 80125 Naples, Italy

[4] Department of Biology, University of Naples "Federico II", via Cintia - Complex Monte S. Angelo, 26, 80126 Naples, Italy

[5] UNESCO-IHE institute for water education, Westvest 7, 2611 AX Delft, The Netherlands

[6] National University of Ireland Galway, University Road, Galway H91 TK33, Ireland

In wastewater treatment, biological systems for nutrient removal are the base of the secondary treatment process, which aims to reduce the biochemical oxygen demand (BOD) by reducing the organic matter content in advanced oxidation processes, aerated lagoons, aerobic granular reactors, and activated sludge plants. This is mediated, primarily, by a mixed population of heterotrophic bacteria and protozoa that utilize the organic compounds for energy and growth (Abdel-Raouf et al. 2012). However, in comparison to the entirely heterotrophic systems, the primary attraction of algal systems stands in the low-grade technology and in the saving of energy, since photosynthetic oxygen production can replace mechanical aeration (Mallick 2002). Furthermore, algal systems present other beneficial effects like valuable biomass and co-product generation and nutrient removal from wastewaters (Oswald et al. 1957; Gonzalez et al. 1997; Mallick 2002; Cai et al. 2013; Renuka et al. 2013; Arbib et al. 2014). More precisely, the *Chlorella* genus has shown promising properties for wastewater treatment (Mata et al. 2010; Abdel-Raouf et al. 2012). However, only a limited number of researchers have focused on the performance and cultivation of these microalgae associated to the treatment of nutrient-rich industrial wastewaters (Table 1).

In addition, industrial wastewaters can contain very high N and P concentrations, up to three orders of magnitude higher than the limits in water bodies (De la Noüe et al. 1992) and can vary highly depending on the industry source (Arbib et al. 2013; Zhang et al. 2014a). For example, anaerobic digestion effluents from different bioreactors treating poultry litter can contain 1570 mg L^{-1} of total nitrogen (TN) and 154 mg L^{-1} of total phosphorous (TP) (Singh et al. 2011), citric acid production effluent can contain 305 mg L^{-1} TN and 35 mg L^{-1} TP (Li et al. 2013), carpet mill industrial effluent can contain 0.1–28.1 mg L^{-1} $N\text{-}NO_3^-$ and 20–35 mg L^{-1} $P\text{-}PO_4^{3-}$ (Chinnasamy et al. 2010), and cattle manure can contain 2313 mg L^{-1} TN and 1119 mg L^{-1} TP (Kobayashi et al. 2013).

The ability of microalgae to adapt their metabolism to different culture conditions provides opportunities to several biotechnological applications (Bumbak et al. 2011; Mirzaie et al. 2016). Currently, natural photoautotrophic conditions for biomass production are implemented with CO_2 supplementation (Gupta et al. 2016). However, these cultivation conditions are limited due to low growth rates, low light penetration and photoinhibition (Liang et al. 2009). Under mixotrophic conditions, microalgae can grow using both light as energy source and CO_2 as carbon source (autotrophic growth) or using organic carbon as energy and carbon source (heterotrophic growth) (Zhang et al. 2014a). The higher growth rates normally achieved under mixotrophic or heterotrophic conditions are advantageous for industrial biomass production (Cerón García et al. 2006; Liang et al. 2009). Although mixotrophic cultivation of microalgae can provide higher biomass concentrations than under autotrophic conditions, the selection of the organic carbon source plays a key role in biomass production systems (Yeh and Chang 2012; Zhang et al. 2014a, b; Gupta et al. 2016). The effects of organic compounds on the growth of microalgae to produce biomass and simultaneously perform wastewater treatment by mixotrophic cultivation offer interesting opportunities. However, little is known about the effect of elevated nutrient concentrations on mixotrophic algal growth (Zhang et al. 2014a).

Each microalgal strain has its optimal growth conditions, making the strain selection process an important step in the establishment of a microalgal treatment system (Richmond 2004). To date, a considerable number of algae species have been screened and numerous microalgae species libraries have been established (Ji et al. 2015). However, microalgae species that tolerate elevated nutrient concentrations, as in the case of using industrial wastewaters as the culture medium, still need to be screened (Mata et al. 2010; Ji et al. 2015).

The aim of this study is, therefore, to characterize an isolated native strain of *Chlorella* sp. ACUF_802 (Electronic supplementary material Fig. S1), well adapted to a high nitrate concentration environment (the nitrification stage of an industrial wastewater treatment plant), for its potential application in nitrate and phosphate removal from industrial wastewater with minimal addition of chemical reagents and energy. The characterization was carried out by analyzing the biomass growth rate under specific operational conditions and different nutrient concentrations and by taking into account the biomass productivity (P_b) and the nutrient removal capability with two specific strategies: cultivate *Chlorella* sp. ACUF_802 growth in synthetic industrial wastewater under (1) autotrophic and mixotrophic conditions and (2) with only atmospheric air at different N:P ratios. Furthermore, in order to evaluate the biomass productivity, a microalgal growth curve has been described through a logistic regression model (Verhulst 1838).

Material and methods

Isolation and growth of the microalgae strain from wastewater

The strain *Chlorella* sp. ACUF_802 was isolated from the settler of a pilot scale Moving Bed Biofilm Reactor (MBBR). The MBBR was used for the treatment of industrial wastewaters generating a nitrate rich effluent, i.e., 2500 mg L^{-1} NO_3^- and 1–2 mg L^{-1} PO_4^{3-} (Vannecke et al. 2016). An initial sample from the algal biofilm attached on the wall of the settler was taken and cultivated in a 2-L bioreactor (Pyrex bottle). A modified Bold's basal medium (BBM) was used to stimulate the microalgae growth and activity (Nichols and Bold 1965). The bioreactor was continuously exposed to artificial light (Phillips TLA, 30W/55), and the modified BBM was refreshed every 2 weeks.

Table 1 Treatment of concentrated wastewater by different strains of the *Chlorella* genus reported in the literature

Algae strain	Strain origin	Culture medium	Carbon source*	Reactor	Operational condition	Culture time (h)	Max. biomass (gL^{-1})
C. pyrenoidosa	Isolated, high ammonia leachate	Dilutions landfill leachate	–	500 mL borosilicate flasks	Batch	288	–
C. pyrenoidosa	Chinese Academy of Sciences, Wuhan, China	Dilutions soybean processing wastewater (SPW)	–	500 mL conical flask	Batch and fed-batch	168	2.09–2.15
C. pyrenoidosa	National chemical laboratory of Pune, India.	Fogg's medium	–	1000 mL Erlenmeyer flasks	Batch	576	0.32
C. pyrenoidosa	Isolated, freshwater	Primary piggery wastewater	Glucose	500 mL conical flasks	Batch	240	0.3
C. pyrenoidosa	Chinese Academy of Sciences, Wuhan, China	Anaerobic digested SPW	–	890 L airlift	Batch	336	2.05 ± 0.03
C. pyrenoidosa	Chinese Academy of Sciences, Wuhan, China	Anaerobic digested starch wastewater (ADSW) and alcohol wastewater (AW)	Volatile fatty acids	2000 mL glass conical flasks	Batch	216	3.01 ± 0.15
C. zofingiensis	Institute of Energy Conversion of China Academy	Piggery wastewater	–	Tubular bubble column	Batch	240	2.4
Chlorella sp.	Isolated, Freshwater lake	Autoclaved and raw centrate WWTP	CO_2	Coil reactor (25 and 9 L)	Batch continuous	336	1.2
Chlorella sp.	Isolated, freshwater lake	Raw centrate WWTP	CO_2	Coil reactor (25 and 9 L)	Batch continuous	336	1
C. vulgaris	Lab of Industrial Biotech., Jiangnan University	Citric acid fermentation effluent	Citric acid 10%	5 L HRPB	Batch	240	0.765
C. vulgaris	UTEX collection	Growth medium	Glucose 1%	I L polycarbonate bottles	Batch	288	0.722 ± 0.012
C. sorokiniana	UTEX collection	10% Anaer dig, effl. and BBM	–	Hanging bag	Batch	504	0.80 in ADE 0.360 in BBM
Chlorella sp.	Isolated. MBBR settler	SWAA	CO_2 + ammonium acetate	100 mL Erlenmeyer flasks	Batch	336	0.48
Chlorella sp	Isolated. MBBR settler	SWSB	CO_2 + sodium bicarbonate	100 mL Erlenmeyer flasks	Batch	336	0.35

Algae strain	Initial concentration (mg L^{-1})					Removal %					Reference
	TN	NO_3^-	NH_4^+	TP	PO_4^{3-}	TN	NO_3^-	NH_4^+	TP	PO_4^{3-}	
C. pyrenoidosa	–	–	135	–	0.5	–	–	80–90	–	60–75	Lin et al. 2007
C. pyrenoidosa	267.1	–	52.1	–	–	88 ± 1	–	89 ± 0.7	70.3 ± 11.4	–	Hongyang et al. 2011
C. pyrenoidosa		400	–	–	–	–	–	–	–	–	Nigam et al. 2011
C. pyrenoidosa	100	–	138.8	16	–	75	–	93.7	77.7	–	Wang et al. 2012
C. pyrenoidosa	300	–	–	33	32.5	84	–	–	90	90	Tan et al. 2014
C. pyrenoidosa	290	–	–	30	–	91.6	–	–	90.74	–	Yang et al. 2015
C. zofingiensis	148			156	–	83	–	–	98	–	Zhu et al. 2013

Table 1 (continued)

Algae strain	Initial concentration (mg L^{-1})					Removal %					Reference
	TN	NO_3^-	NH_4^+	TP	PO_4^{3-}	TN	NO_3^-	NH_4^+	TP	PO_4^{3-}	
Chlorella sp.	132.5 ± 5		85.9 ± 1	215 ± 7	–	90		93	79	–	Li et al. 2011
Chlorella sp.	116.1 ± 4		82.5 ± 2	212 ± 7	–	89		94	81	–	Li et al. 2011
C. vulgaris	300		60	30	–	96.3			94.7	–	Li et al. 2013
C. vulgaris		18.2			111.2	–	–	–	–	–	Liang et al. 2009
C. sorokiniana	23.,3	250	893	111.9	250		89–95	64–74	61–64 7–12	47–55 13–24	Kobayashi et al. 2013
Chlorella sp.	–	45	–	–	217	–	100	100	–	11.4	This study
Chlorella sp	–	45	–	–	217	–	100	–	–	17.8	This study

– data not supplied, *SWSB* synthetic wastewater with sodium bicarbonate, *SWAA* synthetic wastewater with ammonium acetate

To establish a pure culture of the microalga species, 10 mL of sample was collected from the bioreactor, poured in a closed 15 mL falcon tube, and exposed for 1 week to continuous artificial white light (95 μmol photons m^{-2} s^{-1}). Then, standard plating methods were used to separate the algal population. One hundred microliters from the sample were plated on Petri dishes containing 25 mL of agar BBM and 200 μg mL^{-1} of antifungal and antibacterial combos (amphotericin + rifampicin, 1:1). Petri dishes were incubated for 2 weeks at 22 °C under continuous illumination. Single green colonies were transferred to a new Petri dish with agar BBM without antibiotics. After an additional week, each single colony was inoculated in liquid BBM and algal growth was monitored daily by optical density measurements at 680 nm wavelength and by cell counting using a Bürker counter chamber.

Strain identification

The strain was identified by light microscopic examination as belonging to the *Chlorella* genus (Fig. 1) using a Nikon Eclipse E800 (Nikon Instruments Europe B.V. Düsseldorf, Germany). Further molecular analyses were conducted on *Chlorella* sp. ACUF_802 at the algal collection of the Department of Biology, University of Naples "Federico II" (www.acuf.net), where the strain was deposited and included in a special microalgae strain collection for industrial applications. DNA was extracted from liquid culture with the protocol by Doyle and Doyle (1990) and used for a polymerase chain reaction (PCR) with primers targeting the internal transcribed region of rDNA (ITS1_F 5′-TCCG TAGGTGAACCTGCGG-3 (White et al. 1990); ITS_rev_R 5′-TTCAAAGATTCGATGATTCAC-3′). PCR was carried out in a 25-μL aliquot containing approximately 100 ng DNA, a deoxynucleoside triphosphate mixture (0.2 mM each), buffer (1/10 volume of the supplied 10x buffer), supplemented to give a final concentration of 2.5 mM $MgCl_2$, 1. 25 U of Taq polymerase (EconoTaq, Lucigen), and 0.5 pmol of each primer. Amplification was run in an Applied Biosystem 2720 thermal cycler. The profile used was 5 min at 95 °C, 33 cycles of 95 °C for 30 s, 54 °C for 45 s, and 72 °C for 45 s and a final elongation step of 10 min at 72 °C. The amplification product (423 bp) was evaluated on 1.2% (*w*/*v*) agarose gel in a electrophoretic run and purified using the QIAquick® PCR Purification kit (Qiagen Inc., Valencia, CA, USA).

Sequence reaction was obtained with the BigDye Terminator Cycle Sequencing technology (Applied Biosystems, Foster City, CA), purified in automation using the Agencourt CleanSEQ Dye Terminator Removal Kit (Agencourt Bioscience Corporation, 500 Cummins Center, Suite 2450, Beverly, MA 01915, USA), and a robotic station Biomek FX (Beckman Coulter, Fullerton, CA). Product was

Fig. 1 Microscopic photograph of the *Chlorella* sp. ACUF_802 strain, isolated from a high nitrate concentration environment (the nitrification stage of an industrial wastewater treatment plant)

analyzed on an Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems). The amplification primers were used as the sequencing primers. Nucleotide sequence similarity was determined by using BLAST version 2.0 (National Center for Biotechnology Information databases) (Boratyn et al. 2013). To identify the isolate to the specie level, rDNA (ITS) sequences were aligned and compared with 87 sequences belonging to different strains of *Chlorella* and other genera (see Supplementary data) which showed a high identity percentage by BLAST analysis. The sequences were downloaded in the GenBank nucleotide database. The multiple nucleotide alignment was obtained by UGENE software (Okonechnikov et al. 2012) with the addition of the *Chlorella* sp. ACUF_802 strain sequence. The alignment, consisting of 326 sites, was manually inspected for quality and the end gaps trimmed. Manual inspection is a widely used technique for reducing the number of sequencing errors and improving quality (Alachiotis et al. 2013).

Bayesian inference was obtained with MrBayes 3.2.0, running two million generations and a sample frequency of 200, and using the general time reversible model (Tavaré 1986) with an invariable four gamma-distributed substitution rate category to correct for among site rate variation (GTR + G + I). The analysis was stopped at an average standard deviation of split frequencies of 0.00608. The first 25% of sampled trees were discarded as burn-in before calculating posterior probabilities (Electronic supplementary material Fig. S2).

Culture media and experimental procedure

Two different culture media were tested to simulate different types of wastewaters and to study the performance of the microalgae growth under different nutrient concentrations and different carbon sources (Electronic supplementary material Fig. S1). The composition of the synthetic industrial wastewater used as the basis of the cell culture consisted of a modified version of the BBM. BBM is commonly used for green algae (Nichols and Bold 1965). The modified version of BBM was prepared as described in Table 2. The medium simulates a wastewater from the phosphorus manufacturing process with a phosphorus concentration of 200 mg L^{-1} (Yapijakis and Wang 2006). Two different carbon sources were added to the synthetic wastewater (SW): either sodium bicarbonate ($NaHCO_3$, SB), to simulate an alkaline rich wastewater, denoted as SWSB, or ammonium acetate (CH_3COONH_4, AA), denoted as SWAA (Table 3). The latter simulated the condition of mixing diverse wastewaters resulting in the presence of two different inorganic nitrogen and carbon sources, similarly to the experiments carried out by Kobayashi et al. (2013) for the treatment of the anaerobic digestion effluents.

Table 2 Initial nitrate, phosphate, sodium bicarbonate and ammonium acetate concentrations used in tests to investigate: (I) performance with different carbon source, (II) effect of the N:P ratio in cultures with medium at different N and P concentrations with a N:P ratio close to 1, and (III) at different N and P concentrations with a 5:1 N:P ratio

	I. Effect of carbon and nitrogen source			II. Nitrogen limiting conditions (low N:P ratio)				III. Phosphorous limiting conditions (high N:P ratio)		
	Modified BBM	SWSB	SWAA	T 1.1.	T 1.2.	T 1.3.	T 1.4.	T 2.1.	T 2..2	T 2.3.
N:P ratio (molN:molP)	1.7	0.3	0.9	3.5	2.8	2.5	2	7.6	7.5	7.4
NO_3^- (mg L^{-1})	250	45	45	70.86	141.72	247.87	199.29	249.11	124.45	59.79
NH_4^+ (mg L^{-1})	–	–	23.28	–	–	–	–	–	–	–
PO_4^{3-} (mg L^{-1})	217	217	217	30.65	76.62	149.23	153.23	49.95	24.98	11.95
$NaHCO_3$ (mg L^{-1})	–	2000	–	–	–	–	–	–	–	–
CH_3COONH_4 (mg L^{-1})	–	–	76.62	–	–	–	–	–	–	–

– not supplied in the medium composition, *BBM* Bold's basal medium, *SWSB* synthetic wastewater with sodium bicarbonate, *SWAA* synthetic wastewater with ammonium acetate

Table 3 Biomass kinetic parameters of *Chlorella* sp. ACUF_802 determined by the Verhulst logistic model for the carbon sources and the different N:P ratios tested. X_0 (mg biomass L^{-1}): initial biomass concentration; X_m (mg biomass L^{-1}): maximum final biomass concentration; μ_m (day^{-1}): maximum specific growth rate; P_b (mg L^{-1} day^{-1}): batch productivity

Kinetic parameter	Carbon and nitrogen sources		Low N:P ratio				High N:P ratio		
	SWSB	SWAA	T 1.1	T 1.2	T 1.3	T 1.4	T 2.1	T 2.2	T 2.3
X_0	20.54	45.94	27.24	43.44	38.91	29.77	16.1	10.73	20.49
X_m	350.22	485.21	866.97	1234.19	1090.3	899.17	834	644.02	1488.09
μ_m	1.19	0.67	0.75	0.57	0.61	0.74	0.61	0.8	0.47
R^2	0.9825	0.9716	0.9843	0.9973	0.9934	0.9882	0.9941	0.9993	0.9984
P_{max}	416.51	325.63	654.25	703.16	660.85	664.61	505.5	513.87	697.39
P_b	71.43	59.59	102.45	111.96	105.44	104.87	73.69	73.33	96.85

SWSB synthetic wastewater with sodium bicarbonate, *SWAA* synthetic wastewater with ammonium acetate

The stock culture of the strain was maintained in modified BBM. Experiments were carried out in triplicate in 250-mL flasks closed with a cotton plug in an illuminated culture room at 25 (±2) °C with agitation (mixing) supplied by a table shaker at 100 rpm and constant illumination (95 μmol photons m^{-2} s^{-1}). Fluorescent lights are commonly used in algal bioreactors and have been shown to satisfactorily simulate photosynthetically active radiation (Zippel et al. 2007). In this study, the batch reactors were inoculated with a specific volume of microalgae in order to obtain a similar initial biomass concentration (X_0) in all the assays (initial optical density at 680 nm close to 0.10 corresponding to 2.5×10^6 cells mL^{-1}). The experiment lasted 2 weeks without additional pH adjustment along the experiments.

Effect of the different carbon and nitrogen sources and different nutrient availabilities

Three different sets of experiments were carried out to evaluate the capability of metabolic adaptation and the nutrient removal efficiency of *Chlorella* sp. ACUF_802 from different media composition. The first set was performed by using a different nitrogen and carbon source. The second one was executed by lowering the N:P ratio, below 5:1 (molN:molP), and by focusing on nitrogen limiting conditions (see Table 3). The third set of experiments was performed by keeping a higher N:P ratio, i.e., higher than 5:1, with phosphorous limiting conditions, as reported for aerobic treatment of industrial wastewater (Ammary 2004; Klausmeier et al. 2004; Li et al. 2010). Specifically, the initial N concentration of the medium was increased. The nutrient concentrations are detailed in Table 3.

Analytical methods and data analysis

The biomass concentration (X) was determined according to Moheimani et al. (2013). Samples were diluted by appropriate ratios to ensure that absorbance values were in the range of 0.1–1 (dimensionless). To convert the OD_{680} values to biomass as dry weight (mg L^{-1}), a calibration curve was determined. Biomass dry weight (DW) was measured by filtering an aliquot of an algal sample on pre-weighed glass-fiber filter paper with 0.45-μm pore size. The filters were then dried at 105 °C in an oven for 12 h. Algal biomass DW was determined by the difference of the two weights. Chlorophyll α (Chl α) was measured in vivo with an Aquafluor (Turner Designs) fluorimeter as described by Gargano et al. (2016), and pH values were measured with a pH meter Bench model AD-1030 (Adwa Instruments Inc., Szeged, Hungary).

The Verhulst logistic kinetic model (Verhulst 1838) was used to predict the evolution of the experimental biomass concentration. This model can accurately describe the growth of biomass in different culture conditions occurring in many batch bioreactors as a sinusoidal curve (Gong and Lun 1996, Arbib et al. 2013). The microbial growth could be expressed as described by Eq. (1), where μ_m is the maximum specific growth rate (day^{-1}), and X_0, X, and X_m are the concentration of biomass (mg L^{-1}) at an operation time equal to 0, t, and infinite (i.e., the maximum concentration) respectively.

$$X = \frac{X_0 X_m e^{\mu_m f}}{X_m - X_0 + X_0 e^{\mu_m f}} \quad (1)$$

To estimate the P_b with a standardized methodology, the lag phase should be minimized or even not be considered due to its high variability related to the experimental conditions and the experimental methodology (Arbib et al. 2013). The biomass productivity (mg L^{-1} day^{-1}) was then calculated as in Eq. (2).

$$P_b = \frac{\mu_m (0.9 \cdot X_m - 1.1 \cdot X_0)}{\ln\left(\frac{9 \cdot (X_m - 1.1 \cdot X_0)}{1.1 \cdot X_0}\right)} \quad (2)$$

Phosphorus was measured as orthophosphate ions (PO_4^{3-}) and nitrogen as nitrate (NO_3^-) and ammonium (NH_4^+, where

present). Analyses of nitrate and phosphate were performed with ion chromatography using a761 compacts IC analyzer (Metrohm, Herisau, Switzerland) according to APHA (2005). Amonium was measured by a titrimetric method as described by Wagner (1940). The nutrient removal rates were calculated considering the total nitrogen and phosphorus amounts removed during the incubation period (days). Kinetic modeling was performed using the SOLVER tool of Microsoft Excel 2011(Microsoft®), and the values were estimated by least squares. A confidence interval for $p \leq 0.05$ (C.I.) was used for the estimation of the determination coefficient.

Results

Isolation and identification of the microalgae strain

The strain *Chlorella* sp. ACUF_802 was isolated and purified from the effluent of a nitrification reactor. The strain demonstrated tolerance to high nitrate concentrations growing spontaneously in this environment. The cells were planktonic and spherical with a diameter range of 3–6 μm, single chromatophore, and a parietal mantle-shaped covering nearly the whole of the cell wall. A conspicuous round pyrenoid was present in each chromatophore (Fig. 1). The ITS phylogenetic tree suggests a close relation (0.99) of this strain with other *Chlorella* sp. isolated from wastewater (*Chlorella* sp. Iso4) (Electronic supplementary material Fig. S2).

Microalgae growth and nutrient removal from different wastewaters

Chlorella sp. ACUF_802 was able to grow in the synthetic industrial wastewaters tested. A typical evolution of growth in batch cultures was observed for all experiments. The Verhulst kinetic model showed a good alignment (lines in Fig. 2a) to the experimental data. In particular, SWSB showed the best alignment with R^2 of 0.98 (Table 3). SWSB showed the highest maximal growth rate (μ_m) (1.19 day^{-1}) and P_b (71.43 mg L^{-1} day^{-1}) (Fig. 2; Table 3) during the experiments. The maximum biomass concentration (X_m) of 485.21 mg L^{-1} was achieved by the cultures growing in the SWAA medium (Fig. 2a).

The cultures grown in SWSB and SWAA showed a similar growth trend during the first 4 days. Afterwards, the growth rate of SWAA reached the highest biomass concentration prior to achieving the stationary phase (after 11 days of incubation). The Chl α concentration of *Chlorella* sp. ACUF_802 cells growing in SWAA medium increased until the sixth day but declined at day 7. In contrast, cultures growing in SWSB globally showed a low Chl α concentration (Fig. 2b).

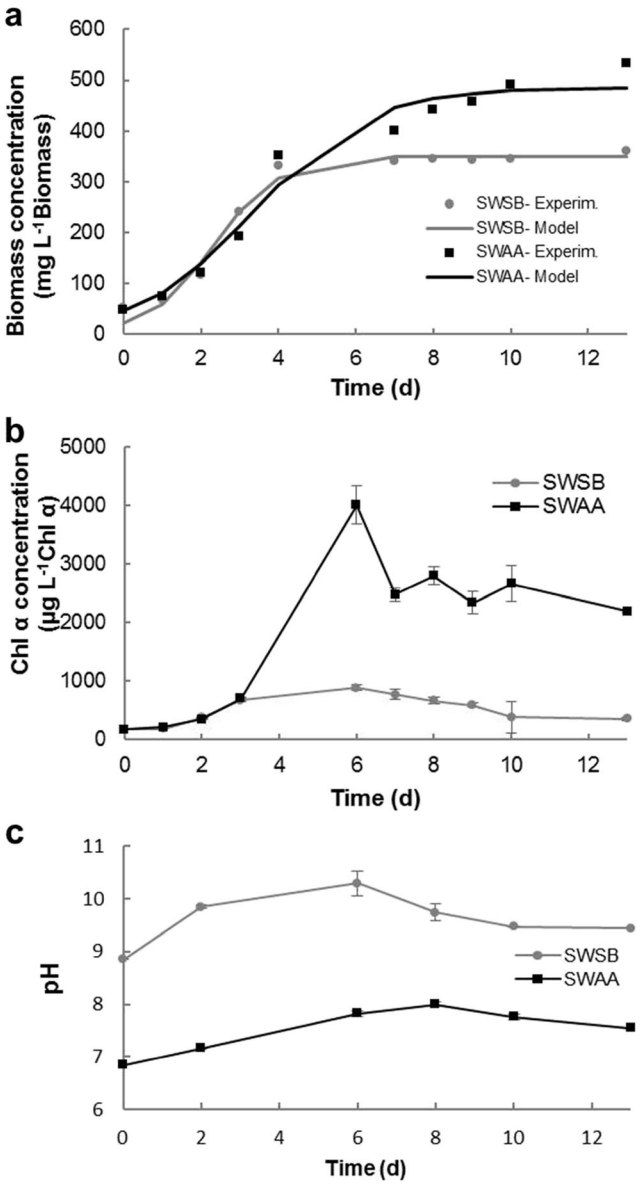

Fig. 2 Variation of biomass growth (**a**), chlorophyll α concentration (**b**), and pH (**c**) of *Chlorella* sp. ACUF_802 growing at different nitrogen concentrations with N:P ratio close to 1. SWSB (circles) and SWAA (squares). Error bars indicate standard deviation, where $n = 3$

The pH evolution over time showed a typical increase in both the SWSB and SWAA media reaching a maximum value of 10.2 and 8.0, respectively. This difference is partially due to the presence of different carbon sources in the modified BBM media tested, leading to the different initial pH values of 8.8 and 6.8, respectively. In both experiments, the pH slightly decreased during the last monitoring days (Fig. 2c).

The nitrogen removal rate by *Chlorella* sp. ACUF_802 was faster in SWSB medium that contains an inorganic carbon source (Fig. 3a). A concentration lower than 8 mg L^{-1} N was achieved at the second day of the experiment. On the other hand, for the SWAA medium, low NO_3^- concentrations were achieved only at the 6th day (Fig. 3b). Indeed, in this

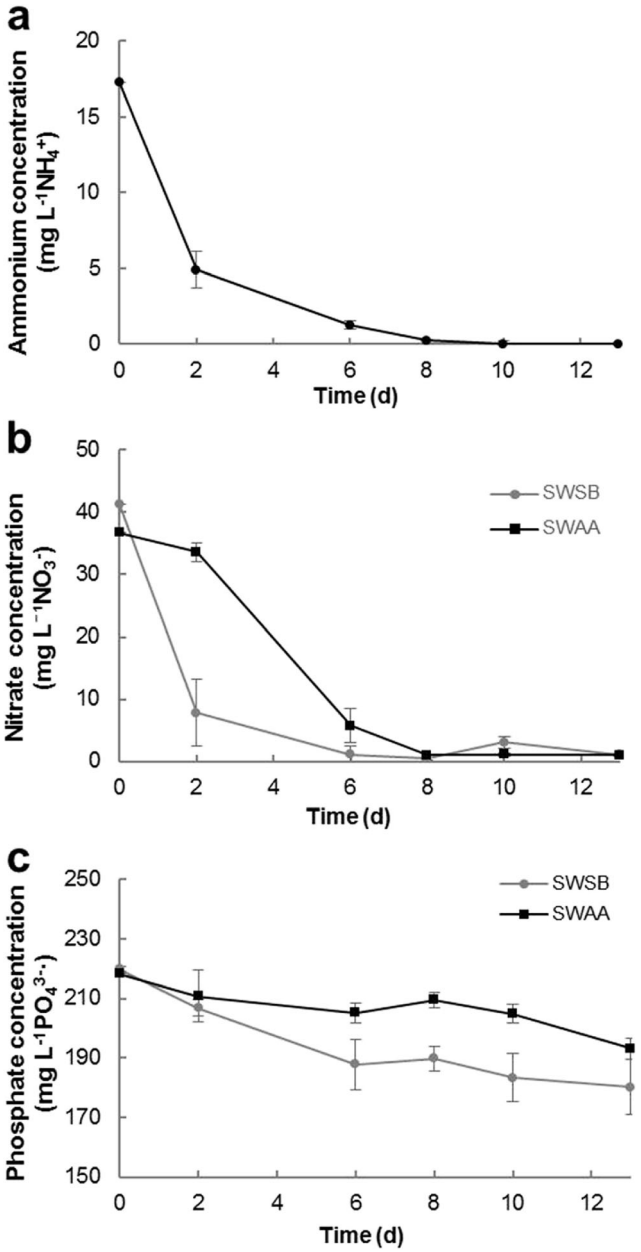

Fig. 3 Nutrient removal performance of *Chlorella* sp. ACUF_802. Ammonium removal from SWAA media (**a**), nitrate removal from SWAA (**b**), and phosphate removal (**c**) in SWSB and SWAA. Error bars indicate standard deviation, where $n = 3$

experiment, the nitrogen sources were both nitrate and ammonium, as ammonium acetate was added as the carbon source. The experiment showed that ammonium was the preferred N source, with 71.6% removal obtained in the first 2 days. During this incubation time, only 8.5% of the nitrate was removed, while during the next 6 days, 82% of the remaining nitrate was consumed (Fig. 3b). The complete uptake of phosphate was not achieved in both experiments (Fig. 3c). Only a modest decrease of the phosphate concentrations was observed: SWSB gave a PO_4^{3-} removal efficiency of 17.8% and SWAA only 11.4% from the high strength (220 mg L^{-1} PO_4^{3-}) synthetic industrial wastewater.

Nitrogen limiting conditions (low N:P ratio)

To simulate the composition of a phosphate-rich wastewater, the first N:P set of experiments was carried out by fixing the N:P ratio lower than 5:1 and by varying the initial nitrogen and phosphorous concentrations (Table 2). During the exponential phase, the four experiments exhibited a similar growth trend, and variations were observed after the 6th day when algal cultures with a lower nitrogen concentration (T 1.1) showed a slightly lower growth rate (Fig. 4a). The highest X_m was observed in the T 1.2 assay (1237.19 mg L^{-1}), and the

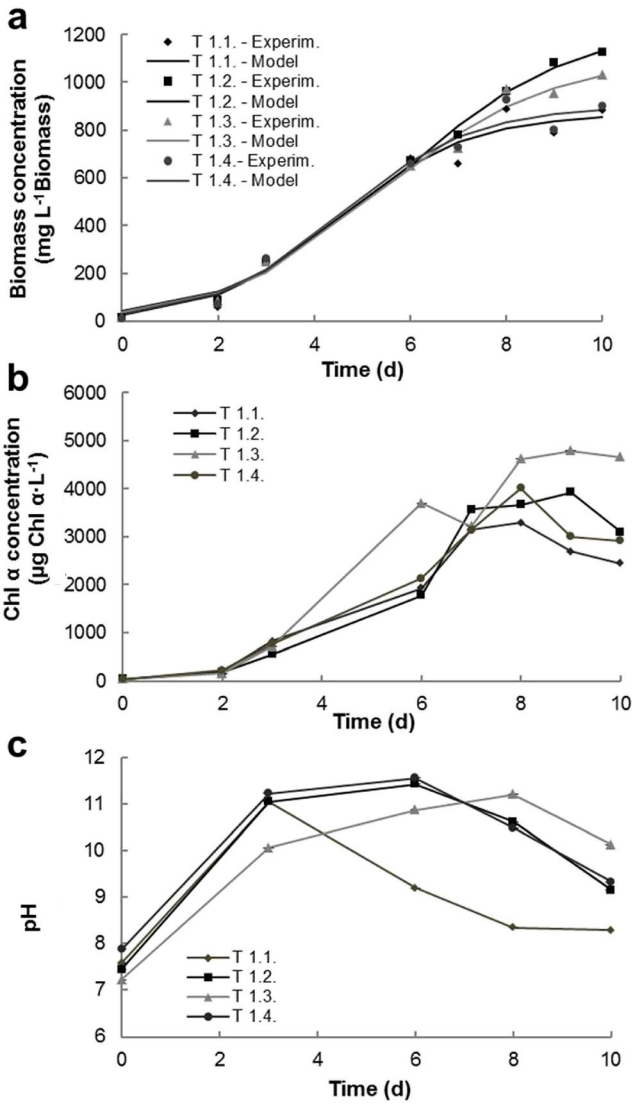

Fig. 4 Variation of biomass growth (**a**), chlorophyll α concentration (**b**), and pH (**c**) of *Chlorella* sp. ACUF_802 growing at different nitrogen concentrations with a N:P ratio close to 1. Treatment symbols: T 1.1 (diamonds), T 1.2 (squares), T 1.3 (triangle), and T 1.4 (circles). Error bars indicate standard deviation, where $n = 3$

maximum specific growth rate was observed during the experiment T 1.1 (0.75 day^{-1}) (Table 3).

The Chl α concentration had a strong relation with the initial nutrient concentrations. Experiments T 1:3 and T 1.4, with the highest N and P concentration yielded the highest Chl α concentration (4784.8 ± 213.6 and 4012.8 ± 21.6 μg L^{-1} Chl α, respectively), while the experiment T 1.1 gave the lowest Chl α concentration (3289.3 ± 87.9 μg L^{-1} Chl α) (Fig. 4b). A similar pH trend was observed in the T 1.2 and T 1.4 experiments: both reached the maximum pH value at the third day of incubation (pH > 11), which decreased after the 6th day (Fig. 4c). The nitrate removal rate was faster in experiment T 1.1 (depleted on day 2) than in the other experiments, which showed a complete removal around the 6th day of incubation (Fig. 5a). The highest phosphate removal efficiency was achieved in the experiments T 1.2 and T 1.1, 37.4 and 31.7%, respectively (Fig. 5b).

Phosphorous limiting conditions (high N:P ratio)

Chlorella sp. ACUF_802 was incubated in a medium characterized by a N:P ratio higher than 5:1 (initial P and N for the 3 conditions reported in Table 2). The exponential growth phase was similar for the different experiments: the medium with a higher nitrate concentration (T 2.1) exhibited a lower growth rate than the other experiments until day 8. The exponential growth phase lasted until day 10 (Fig. 6a).

The Chl α variation showed a significant relationship with nutrient availability: a higher nutrient concentration resulted in higher Chl α accumulation (T 2.1; Fig. 6b). Nevertheless, T 2.1 did not achieve the highest biomass concentrations in this experiment. T 2.3 biomass concentration was higher at the 10th day of incubation than in the T 2.1 experiment. In the case of X_m and P_b, the higher values were achieved in the

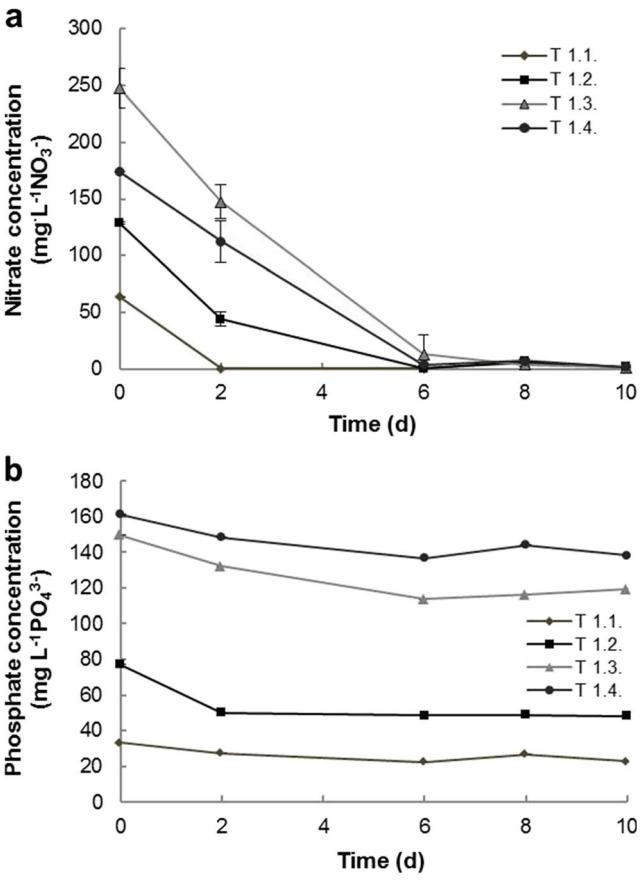

Fig. 5 Nitrate (**a**) and phosphate (**b**) removal with different nitrogen concentrations when incubating *Chlorella* sp. ACUF_802 in media with a N:P ratio close to 1. T 1.1 (diamonds), T 1.2 (squares), T 1.3 (triangle), and T 1.4 (circles). Error bars indicate standard deviation, where $n = 3$

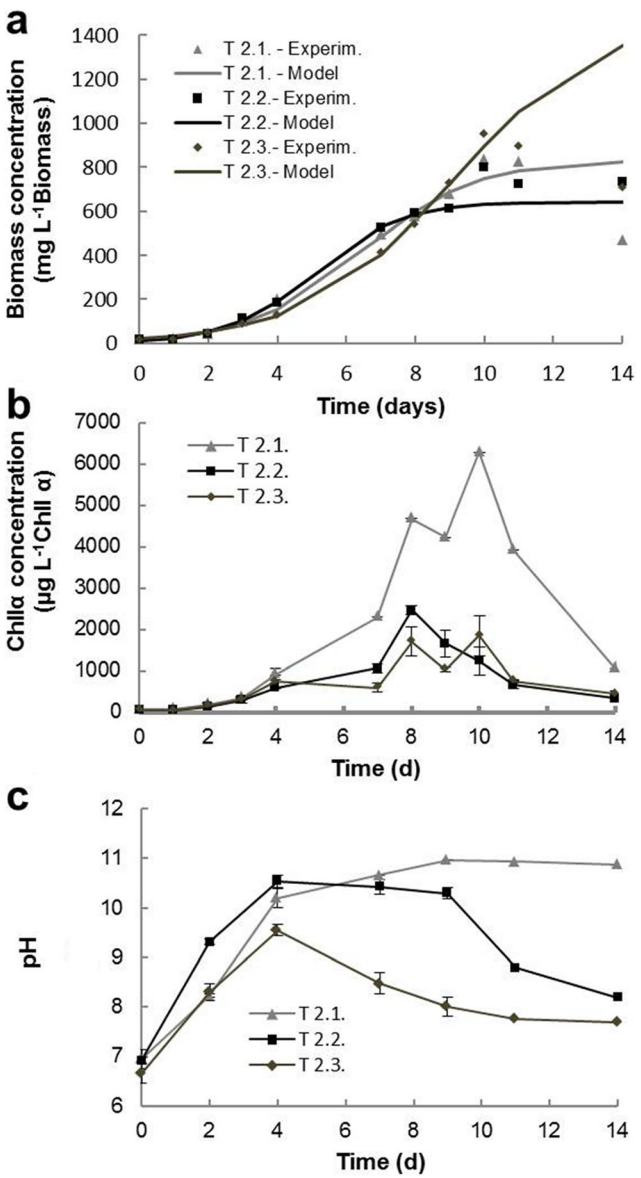

Fig. 6 Variation of biomass growth (**a**), chlorophyll α concentration (**b**), and pH (**c**) of *Chlorella* sp. ACUF_802 growing at different nutrient concentrations at a 5:1 NP ratio. Treatment symbols: T 2.3 (diamonds), T 2.2 (squares), and T 2.1 (triangle). Error bars indicate standard deviation, where $n = 3$

experiment T 2.3 (1488.09 mg L^{-1} and 96.85 mg L^{-1} day^{-1}) than in all the others assays. On the contrary, T 2.2 showed the highest μ_m (0.80 day^{-1}) (Table 3). As expected, due to the different initial nitrogen concentrations, a different incubation time was required to achieve a complete nitrate removal from the media: experiment T 2.1 exhibited a longer nitrate removal time (14 days), whereas a higher nitrogen removal efficiency was achieved in T 2.3 at the 4th day (Fig. 7a). Complete phosphate removal was observed in this assay, but at a different time: experiment T 2.2 showed the highest phosphate removal efficiency in 4 days (Fig. 7b).

Discussion

Species identification

Chlorella (green algae; Chlorophyta) is a cosmopolitan genus living in both aquatic and terrestrial habitats (Masojídek and Torzillo 2008). Furthermore, *Chlorella* and a few other green algae of simple morphology are often summarized as the so-called airborne algae (Sharma and Rai 2010), which are ubiquitous in terrestrial ecosystems (Rindi 2011). In the systematics of coccoid green algae, the identification of the *Chlorella* species is a difficult task (Krienitz et al. 2004). There are a large number of algae from various taxonomic groups showing the same morphological characteristics and the classification of coccoid unicellular algae belonging to *Chlorophyta* requires a molecular approach. However, a recent study carried out on 400 internal transcribed spacer 2 (ITS2) and/or 18S ribosomal RNA sequences of *Chlorella* and related taxa has confirmed that *Chlorellaceae* consist of a *Parachlorella* and a *Chlorella*-clade (Krienitz et al. 2004) and that *Chlorella* is polyphyletic (Heeg and Wolf 2015). For this reason, a clear-cut species designation can be problematic, as in the case of the *Chlorella* strain ACUF_802. The close relation obtained with the ITS phylogenetic tree suggests that our isolated strain can correspond to another *Chlorella* sp. isolated from wastewater (*Chlorella* sp. Iso4) (Electronic supplementary material Fig. S2), which still requires an appropriate taxonomical assignment; therefore, for a cautious designation, our isolate was determined as *Chlorella* sp. ACUF_802.

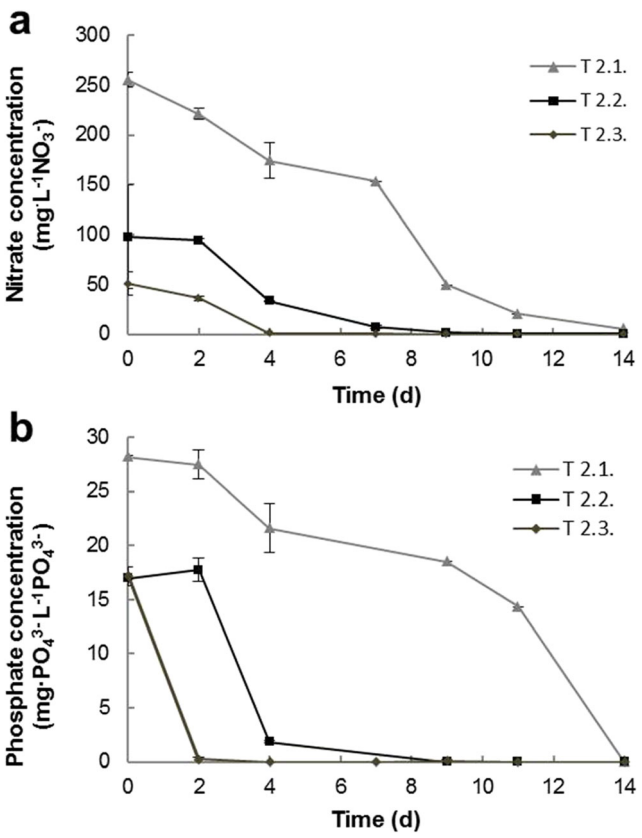

Fig. 7 Nitrate (**a**) and phosphate (**b**) removal of *Chlorella* sp. ACUF_802 growing at different nutrient concentrations at a 5:1 N:P ratio. Treatment symbols: T 2.3 (diamonds), T 2.2 (squares), and T 2.1 (triangle). Error bars indicate standard deviation, where $n = 3$

Growth kinetics and nutrient removal performance on different wastewaters

Chlorella sp. ACUF_802 can change its metabolic pathway according to the supply of organic substrates, suggesting that it can sustain heterotrophic growth in addition to common autotrophic growth (Tan et al. 2014). In our study, the ability of the isolated strain to perform mixotrophic and autotrophic growth was confirmed. This adaptive behavior could be very promising in the field of wastewater treatment as differences in wastewater composition can relatively affect the nutrient removal performed by adaptive microalgae strains. The growth rate in the assay with the SWAA medium was higher than in SWSB. However, SWSB showed a higher μ_m and P_b (Fig. 2; Table 3). In another study, *Chlorella vulgaris* biomass growth was extremely different under diverse carbon supply; in particular, the growth on acetate salts was higher than all other examined carbon sources (Battah et al. 2013). The biomass concentrations of *C. vulgaris* (2.62 g L^{-1}) in mixotrophic growth were 140% higher than autotrophic growth (Mirzaie et al. 2016). Moreover, the total consumption of nitrate from the SWSB medium was obtained at the second day of the assay (Fig. 3), which means that nitrogen depletion was a limiting factor in this experiment.

In this study, the initial pH values were between 6.8 and 8.9 (Fig. 2), which are optimal conditions for the establishment of the cultures (Park et al. 2011). However, the pH values declined at the end of the exponential growth phase in both the SWSB and SWAA experiments, afters day 6 and 8, respectively, due to a decrease in photosynthetic activity (Fig. 2). In addition, HCO_3^- consumption during photosynthesis in autotrophic conditions usually raises the pH value of the growth

medium (Lin et al. 2007; Park et al. 2011). Cell suspensions with high cell densities and without addition of CO_2 gas may attain pH values as high as 11 (Grobbelaar 2007). In this study, pH values as high as 10.5 were observed in SWSB medium without any pH adjustment (Fig. 2). Zhang et al. (2014b) and Su et al. (2012) observed a similar pH trend of algal reactors, including *Phormidium* sp., *Chlamydomonas reinhardtii*, *Chlorella* sp., *C. vulgaris*, and *Scenedesmus rubescens*, which changed from the initial value of 7.0 to around 10.0 after 7 days of cultivation for the five algae species.

The elevated pH can enhance the nitrogen removal from the medium via ammonia volatilization and the phosphorus removal through phosphate precipitation (Craggs et al. 2005). Therefore, the pH can influence both the algal growth and the nitrogen and phosphorus removal efficiency of wastewater treatment systems (Issa et al. 2015). Nevertheless, the highest phosphate removal percentage in SWSB experiments with high P concentration (200 mg L^{-1}) did not exceed 17.8% (Fig. 3). Ammonia volatilization was not observed in the SWAA test due to the lower maximum pH achieved. It is known that the pH rises when sodium or potassium salts of acetate are used as the substrate (Pérez-Garcia et al. 2011). This occurs because the remaining Na^+ or K^+ couple with hydroxyl ions (OH^-) or other anions to form alkali (Pérez-Garcia et al. 2011). This phenomenon was also observed in this study (Fig. 2), although the conditions were not heterotrophic (without dark exposure), but mixotrophic. Nevertheless, the effect on the increase in pH was low compared to the other assay conditions.

Two drawbacks about using bicarbonate as a carbon source have been addressed: the high cost (more than three times that of gaseous CO_2) and the need of specific microalgae species which tolerate high pH and high ionic strength (Markou et al. 2014). However, bicarbonate salts have a higher solubility when compared to CO_2 ($NaHCO_3$ solubility is > 90 g L^{-1} at 25 °C) and it is expected that its application efficiency would be higher than that of CO_2 application. In the study of Chi et al. (2013), the cyanobacterium biomass productivity in a reactor was in the range of 0.11–0.13 g L^{-1} day^{-1}, which is low for lab-scale photobioreactors. In this study, the *Chlorella* sp. strain ACUF_802 gained three times higher (than Chi et al. 2013) biomass concentration using the same carbon source (SWSB) (Table 3), even if the initial carbon source concentration was six times lower (0.024 M) than the lowest concentration (0.16 M) tested by Chi et al. (2013).

A decline of the biomass and chlorophyll α production was observed in the SWSB experiment when the culture reached pH values over 10 (Fig. 2). Simultaneously, a change in the color of the microalgae culture from green to yellow was observed and it became lighter until the end of the assessment (data not shown). The pH affects the growth of the microalgae, and different species and strains have different optimal pH values at which the fastest growth is achieved (Gong et al. 2014). The general optimum pH for most freshwater algal species is between 7 and 9 (Gong et al. 2014). Failure to keep the correct pH can lead to slow growth or the culture to collapse. During the first 3 to 6 days, a fast growth and nutrient consumption in the SWSB experiment was observed that rapidly increased the pH values (Figs. 2 and 5) and caused a reduction of the availability of the carbon source and the precipitation of the phosphate in the medium (Fig. 3).

Wu et al. (2014) reported that *C. vulgaris* is able to grow in nitrogen and phosphorus rich wastewaters. These microalgae can exhibit autotrophic and mixotrophic growth showing differences in biomass production. The authors summarized that for phototrophic growth, the X_m can vary from 700 to 1500 mg L^{-1} and for mixotrophic growth from 400 to 2000 mg L^{-1}. These values were higher compared to the modeled data in this study under SWSB and SWAA conditions, 350.2 mg L^{-1} and 485.21 mg L^{-1}, respectively (Table 3). Additionally, Wu et al. (2014) reported a biomass concentration in the range from 100 to 600 mg L^{-1} for low N and P concentration experiments (12.76 mg L^{-1} NH_4^+·and 2.0 mg L^{-1} PO_4^{3-}·). These values are similar to SWSB in the current study, probably due to the fast assimilation of the nitrogen. The preference for ammonia in the SWAA confirms the observations in other studies: similar results were observed by Liang et al. (2009) in their study on *C. vulgaris* under mixotrophic conditions, which showed improved biomass production with 1 and 2% glycerol, but inhibition with 5 and 10% glucose. Lin et al. (2007) found that an isolated strain of *C. pyrenoidosa* from landfill leachate was well adapted to high ammonium concentrations. Inhibition of biomass growth was observed only in cultures with an ammonia concentration exceeding 135 mg L^{-1} (Lin et al. 2007).

BBM and anaerobic digestion effluent from beef steers cattle waste were tested by Kobayashi et al. (2013) to cultivate three *Chlorella sorokiniana* strains aiming for biomass production (Table 1). The authors found that the biomass productivities in all the strains were 16–21 mg L^{-1} day^{-1}. However, the results indicated that anaerobically digested effluents marginally suppressed the growth of two strains and inhibited the growth of the third one. In this study, the highest maximum biomass productivity and batch productivity were obtained when *Chlorella* sp. ACUF_802 was grown in SWSB (416.51 and 71.43 mg L^{-1} day^{-1}, respectively). On the other hand, for the two *C. sorokiniana* strains studied by Kobayashi et al. (2013), the removal of TP and phosphate was 12% and 24%, respectively, and the nitrogen (TN) removal was 90%. Comparably, Kong et al. (2010) cultivated *Chlamydomonas reinhardtii* during 10 days in a centrated municipal wastewater and found 83% and 15% N and P removal, respectively. Similar nitrate (> 95%) and phosphate (16.2%) removal efficiencies were observed in this study, which suggests a similar tolerance of *Chlorella* sp. ACUF_802 to high nutrient concentrations (Fig. 3). Kobayashi et al. (2013) suggested that the

degree of nutrient removal may be influenced by the effluent type and *Chlorella* strain. Therefore, the low nutrient removal and biomass growth rate of the three *C. sorokiniana* strains tested could be due to the inability of these strains to effectively utilize the available nutrients.

Some strains like *C. pyrenoidosa* generate more ATP from glucose than the autotrophic and mixotrophic cultures with light as energy supply (Yang et al. 2000). In the present study, mixotrophic and autotrophic growths at high nitrate and phosphate concentrations were observed (Table 3). Some microalgae species are not truly mixotrophic but have the ability of switching between phototrophic and heterotrophic metabolism depending on the environmental conditions (Zhang et al. 2014a; Pérez-Garcia and Bashan 2015). This could in part explain the difference in the carbon source preference observed in the SWAA assay.

Low biomass concentration values were measured for *Chlorella* sp. ACUF_802 tested with the different carbon sources (Fig. 2a). On the contrary, high biomass concentration and productivity were observed in axenic cultures of these strains. The authors found for *Scenedesmus obliquus, Chlorella vulgaris*, and *Chlorella kessleri* and a natural bloom a higher biomass concentration in real wastewater effluent than in the synthetic wastewater. After 10 days, *C. vulgaris* showed a maximum biomass concentration of 821 (± 88) mg L^{-1} in synthetic wastewater and 1303 (± 270) mg L^{-1} in real wastewater and 5% CO_2 in air (Arbib et al. 2014). The dissimilarity with the results presented in this study can be related to the low N:P ratios adopted, due to the use of P as a non-limiting nutrient and the pH control applied by Arbib et al. (2014).

The variability in productivity values reported by different authors can be due to the wide multiplicity of the experimental conditions employed: light intensity, light/dark cycles, culture medium, microalgae species, temperature, aeration, source, and proportion of carbon dioxide and photobioreactor type. As mentioned by Arbib et al. (2013), the culture time is different between the studies or the criteria used to stop a test are not indicated, which leads to productivity values depending on the duration of the stationary phase. On the other hand, some authors stop the experiments before the stationary phase; in those cases, the productivities are only partial, and the maximum biomass concentration achieved in the reactors is unknown.

Effect of nitrogen and phosphorus ratios on the growth kinetics and nutrient removal efficiency

It is generally assumed that the standard composition for microalgae is the Redfield ratio $C_{106}H_{181}O_{45}N_{16}P$ (Redfield 1958). This would mean that a culture medium with a N:P ratio (molar) of 16 is required for a balanced growth of microalgae. This formula is usually considered as the baseline of possible nutrient limitation measurements (Arbib et al. 2013). It is noteworthy that many commercial nutrient solutions for growing microalgae are far from this N:P ratio proposed by Redfield. Other researchers report an optimum N:P ratio between 5:1 and 8:1 (molar) for microalgae growth and conclude that the Redfield ratio is not an universal biochemical optimum (Klausmeier et al. 2004; Li et al. 2010). Determination of the proper N:P ratio for a microalgae system for wastewater treatment is a research topic that still requires attention. Table 3 shows the productivity increased four times when adjusting the N:P ratio to 2.79 (T 1.2). This was a higher increase than the one obtained with different concentrations maintaining a N:P ratio range between 5:1 and 8:1, as recommended by the literature (Ammary 2004; Klausmeier et al. 2004).

Chlorella sp. ACUF_802 consumed the nitrogen source fast, resulting in a low biomass productivity (Fig. 4a) and decreasing of photosynthetic activity after day 6 of incubation as observed in the T 1.1 experiment (Fig. 4b). This photosynthetic behavior is supported by the results obtained by Da Silva et al. (2009), where a decrease in the concentration of the nitrogen sources led to a decrease in biomass growth, chlorophyll α concentration and microalgae biomass productivity of a marine microalga (*Rhodomonas* sp.). Nitrogen depletion triggered a rapid decline in nitrogen containing compounds such as photosynthetic pigments (Da Silva et al. 2009), causing strong decline of the photosynthetic efficiency. Wang et al. (2013) reported that among four different initial nitrogen concentrations applied, *Oedogonium* sp. grew most rapidly in the 1/4BG-11 nitrogen (64 mg L^{-1} N) culture medium during the first 3 days. This is in agreement with the trend observed in the T 2.3 predicted growth by the logistic model in the present study (Fig. 4). The logistic curve growth, which represents a sinusoidal curve, fitted the experimental data on the initial day of cultivation until day 11. After that, the predicted model trend showed a higher biomass concentration at day 14. Experimentally, the growth of *Chlorella* sp. ACUF_802 showed a lag phase starting on day zero of cultivation until day 4 and increased linearly until the maximum growth (on day 7of the assay T2.2 and on day 10 in the assays T2.1 and T2.3) was achieved. However, from day 10 onwards, *Chlorella* sp. ACUF_802 growth decreased until day 14. A similar growth trend was reported by Jamaian et al. (2017) and Gani et al. (2017) for other green algae (i.e., *Botryococcus* sp.) utilizing domestic wastewater. The lack of further sampling days during the stationary phase (later than day 10 for the assays T2.1 and T2.3) might be the reason of the higher maximum biomass concentrations predicted at the end of the experiments by the model compared with the experimental data.

The increase of the nitrogen concentration (higher N:P ratios) can enhance the maximum final biomass concentration achieved (Table 3), while the phosphorous increase did not support an enhancement of the final biomass concentration

(Da Silva et al. 2009). This means that not phosphorous, but nitrogen, can be considered as the limiting nutrient in wastewater. The proper N:P ratio for nitrogen removal (usually around N:P = 9) has to ensure simultaneously a high removal efficiency and a short lag phase. Moreover, an important effect of the N:P ratio is the capability of biomass generation. Variations of the N:P ratio of a wastewater greatly affects the nitrogen removal efficiency (Arbib et al. 2013). At N:P ratios between 1 and 13, Arbib et al. (2013) obtained an average nitrogen removal efficiency of 91.6 (± 2.19)%. A similar maximum removal efficiency (95%) was reported for *Scenedesmus dimorphus* in diluted agro-industrial wastewater (N:P = 0.57) by Gonzalez et al. (1997). In this study, it was observed that with a N:P ratio of 5:1, complete removal of nitrogen can be achieved in 14 days using the microalgae strain *Chlorella* sp. ACUF_802 (Fig. 7) when the N and P concentrations were 56 mg N L^{-1} and 16 mg P L^{-1}, respectively (T 2.1).

Conclusions

The strain *Chlorella* sp. ACUF_802, native of a nitrate rich wastewater, is well adapted to high nutrient concentrations, and it can perform nutrient removal from wastewater. Nitrogen in synthetic wastewaters simulating highly concentrated industrial wastewaters was effectively utilized by *Chlorella* sp. ACUF_802 to achieve a batch productivity of 71.43 mg L^{-1} day^{-1}. Bicarbonate-based synthetic wastewater showed a faster nitrate removal, although the maximal P removal efficiency (achieved using the ammonium acetate based synthetic wastewater) was only 16.2%. The productivity of *Chlorella* sp. ACUF_802 could be increased four times by adjusting the N:P ratio to 1.3 (T 1.2). The strain showed a high biomass growth and productivity in the SWAA medium. *Chlorella* sp. ACUF_802 could be used to treat highly concentrated industrial wastewaters, thus combining nutrient removal from wastewater with the reduction of the biomass production cost.

Acknowledgements The authors thank to Niculina Musat and Julian Hofer for their pertinent comments and proof reading of the manuscript.

Funding This study was funded by the European Commission through the Erasmus Mundus Joint Doctorate "Environmental Technologies for Contaminated Solids, Soils, and Sediments, EteCoS3" (FPA 2010/0009).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals (If applicable) N/A. This research did not involve human participants and/or animals.

Informed consent N/A. This research did not involve human participants.

References

Abdel-Raouf N, Al-Homaidan AA, Ibraheem IBM (2012) Microalgae and wastewater treatment. Saudi J Biol Sci 19:257–275

Alachiotis N, Vogiatzi E, Pavlidis P, Stamatakis A (2013) ChromatoGate: a tool for detecting base mis-calls in multiple sequence alignments by semi-automatic chromatogram inspection. Comput Struct Biotechnol J 6(7). https://doi.org/10.5936/csbj.201303001

Ammary B (2004) Nutrients requirements in biological industrial wastewater treatment. Afr J Biotechnol 3(4):236–238

APHA (2005) Standard methods for the examination of water and wastewater, 21st edn. American Public Health Association, Washington, DC 1220 pp

Arbib Z, Ruiz J, Álvarez-Díaz P, Garrido C, Barragan J, Perales JA (2013) Photobiotreatment: influence of nitrogen and phosphorus ratio in wastewater on growth kinetics of *Scenedesmus obliquus*. Int J Phytoremediation 15:774–788

Arbib Z, Ruiz J, Álvarez-Díaz P, Garrido-Pérez C, Perales JA (2014) Capability of different microalgae species for phytoremediation processes: wastewater tertiary treatment, CO_2 bio-fixation and low cost biofuels production. Water Res 49:465–474

Battah MG, El-Sayed AB, El-Sayed EW (2013) Growth of the green alga *Chlorella vulgaris* as affected by different carbon sources. Life Sci J10(1):2075–2081

Boratyn GM, Camacho C, Cooper PS, Coulouris G, Fong A, Ma N, Madden TL, Matten WT, McGinnis SD, Merezhuk Y, Raytselis Y, Sayers EW, Tao T, Ye J, Zaretskaya I (2013) BLAST: a more efficient report with usability improvements. Nucleic Acids Res 41: W29–W33

Bumbak F, Cook S, Zachleder V (2011) Best practices in heterotrophic high-cell-density microalgal processes: achievements, potential and possible limitations. Appl Microbiol Biotechnol 91:31–46

Cai T, Park SY, Li Y (2013) Nutrient recovery from wastewater streams by microalgae: status and prospects. Renew Sust Energ Rev 19:360–369

Cerón García M, García Camacho F, Sánchez Mirón A, Fernández Sevilla JM, Chisti Y, Molina Grima E (2006) Mixotrophic production of marine microalga *Phaeodactylum tricornutum* on various carbon sources. J Microbiol Biotechnol 16(5):689–694

Chi Z, Xie Y, Elloy F, Zheng Y, Hu Y, Chen S (2013) Bicarbonate-based integrated carbon capture and algae production system with alkalihalophilic cyanobacterium. Bioresour Technol 133:513–521

Chinnasamy S, Bhatnagar A, Hunt RW, Das KC (2010) Microalgae cultivation in a wastewater dominated by carpet mill effluents for biofuel applications. Bioresour Technol 101:3097–3105

Craggs R, Sutherland D, Campbell H (2005) Hectare-scale demonstration of high rate algal ponds for enhanced wastewater treatment and biofuel production. J Appl Phycol 24:329–337

Da Silva A, Lourenco SO, Chaloub RM (2009) Effects of nitrogen starvation on the photosynthetic physiology of a tropical marine microalga *Rhodomonas* sp. (Cryptophyceae). Aquat Bot 91:291–297

De la Noüe J, Laliberete G, Proulx D (1992) Algae and wastewater. J Appl Phycol 4:247–254

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Gani P, Mohamed Sunar N, Matias-Peralta H, Abdul Latiff AA, Mohamad Fuzi SF (2017) Growth of microalgae *Botryococcus* sp. in domestic wastewater and application of statistical analysis for the optimization of flocculation using alum and chitosan. Prep Biochem Biotechnol 47(4):333–341

Gargano I, Olivieri G, Andreozzi R, Marotta R, Marzocchella A, Pollio A (2016) Biodiesel production in outdoor cultures of *Scenedesmus vacuolatus*. Chem Eng Trans 49:397–402

Ge S, Champagne P (2016) Nutrient removal, microalgal biomass growth, harvesting and lipid yield in response to centrate wastewater loadings. Water Res 88:604–612

Gong H, Lun S (1996) The kinetics of lysine batch fermentation. Chin J Biotechnol 12:219–225

Gong Q, Feng Y, Knag L, Luo M, Yang J (2014) Effects of light and pH on cell density of *Chlorella vulgaris*. Energy Procedia 61:2012–2015

Gonzalez LE, Canizares RO, Baena S (1997) Efficiency of ammonia and phosphorus removal from a Colombian agroindustrial wastewater by the microalgae *Chlorella vulgaris* and *Scenedesmus dimorphus*. Bioresour Technol 60:259–262

Grobbelaar JU (2007) Photosynthetic characteristics of *Spirulina platensis* grown in commercial-scale open outdoor raceway ponds: what do the organisms tell us? J Appl Phycol 19:591–598

Gupta PL, Choi HJ, Lee SM (2016) Enhanced nutrient removal from municipal wastewater assisted by mixotrophic microalgal cultivation using glycerol. Environ Sci Pollut Res 23:10114–10123

Heeg JS, Wolf M (2015) ITS2 and 18S rDNA sequence-structure phylogeny of *Chlorella* and allies (Chlorophyta, Trebouxiophyceae, Chlorellaceae). Plant Gene 4:20–28

Hongyang S, Yalei Z, Chunmin Z, Xuefei Z, Jinpeng L (2011) Cultivation of *Chlorella pyrenoidosa* in soybean processing wastewater. Bioresour Technol 102:9884–9890

Ibisch R, Austnes K, Borchardt D, Boteler B, Leujak W, Lukat E, Rouillard J, Schmedtje U, Solheim AL, Westphal K (2016) European assessment of eutrophication abatement measures across land-based sources, inland, coastal and marine waters. European Topic Centre on Inland, Coastal and Marine Waters (ETC-iCM), UFZ, Magdeburg

Issa AA, Shaieb FA, Al-Sefat RM (2015) Role of cloacal algae in the treatment of wastewater and their biotechnological applications. J Biosci Bioeng 1(3):48–56

Jamaian SS, Bakeri NM, Sunar NM, Gani P (2017) A Verhulst model for microalgae *Botryococcus* sp. growth and nutrient removal in wastewater. AIP Conf Proc 1870(1). https://doi.org/10.1063/1.4995886

Ji F, Wang YK, Li G, Zhou YG, Dong RJ (2015) Isolation of microalgae with growth restriction and nutrient removal from alkaline wastewater. Int J Agric Biol Eng 8(6):62–68

Klausmeier CA, Litchman E, Daufresne T, Levin SA (2004) Optimal nitrogen-to-phosphorus stoichiometry of phytoplankton. Nature 429:171–174

Kobayashi N, Noel EA, Barnes A, Watson A, Rosenberg JN, Erickson G, Oyler GA (2013) Characterization of three *Chlorella sorokiniana* strains in anaerobic digested effluent from cattle manure. Bioresour Technol 150:377–386

Kong QX, Li L, Martinez B, Chen P, Ruan R (2010) Culture of microalgae *Chlamydomonas reinhardtii* in wastewater for biomass feedstock production. Appl Biochem Biotechnol 160(1):9–18

Krienitz L, Hegewald EH, Hepperle D, Huss VAR, Rohr T, Wolf M (2004) Phylogenetic relationship of *Chlorella* and *Parachlorella* gen. nov. (Chlorophyta, Trebouxiophyceae). Phycologia 43:529–542

Li X, Hu HY, Gan K, Sun YX (2010) Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp. Bioresour Technol 101:5494–5500

Li Y, Chen Y, Chen P, Min M, Zhou W, Martinez B, Zhu J, Ruan R (2011) Characterization of a microalga *Chlorella* sp. well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production. Bioresour Technol 102:5138–5144

Li C, Yang H, Xia X, Li Y, Chen L, Zhang M, Zhang L (2013) High efficient treatment of citric acid effluent by *Chlorella vulgaris* and potential biomass utilization. Bioresour Technol 127:248–255

Liang Y, Sarkany N, Cui Y (2009) Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. Biotechnol Lett 31(7):1043–1049

Lin L, Chan GYS, Jiang BL, Lan CY (2007) Use of ammoniacal nitrogen tolerant microalgae in landfill leachate treatment. Waste Manag 27:1376–1382

Mallick N (2002) Biotechnological potential of immobilized algae for wastewater N, P and metal removal: a review. Biometals 15:377–390

Markou G, Vandamme D, Muylaert K (2014) Microalgal and cyanobacterial cultivation: the supply of nutrients. Water Res 65:186–202

Masojídek J, Torzillo G (2008) Mass cultivation of freshwater microalgae. In: Jørgensen SE and Fath BD (eds) Encyclopedia of ecology. Academic Press, pp 2226–2235

Mata TM, Martins AA, Caetano NS (2010) Microalgae for biodiesel production and other applications: a review. Renew Sust Energ Rev 14:217–232

Mirzaie M, Kalbasi M, Mousavi SM, Ghobadian B (2016) Investigation of mixotrophic, heterotrophic, and autotrophic growth of *Chlorella vulgaris* under agricultural waste medium. Prep Biochem Biotechnol 46(2):150–156

Moheimani NR, Borowitzka MA, Isdepsky A, FonSing S (2013) Standard methods for measuring growth of algae and their composition. In: Borowitzka MA, Moheimani NR (eds) Algae for biofuels and energy. Springer, Dordrecht, pp 265–284

Nichols H, Bold H (1965) Growth media. In: JR S (ed) Hand B. Physiol. Methods. Cambridge University Press, Cambridge, pp 7–24

Nigam S, Rai MP, Sharma R (2011) Effect if nitrogen on growth and lipid content of *Chlorella pyrenoidosa*. Am J Biochem Biotechnol 7(3):126–131

Okonechnikov K, Golosova O, Fursov M (2012) UGENE team Unipro UGENE: a unified bioinformatics toolkit. Bioinformatics 28:1166–1167

Oswald WJ, Gotaas HB, Golueke CG, Kellen WR (1957) Algae in wastewater treatment. Sewage Ind Waste 29:437–455

Park JBK, Craggs RJ, Shilton AN (2011) Wastewater treatment high rate algal ponds for biofuel production. Bioresour Technol 102(1):35–42

Pérez-Garcia O, Bashan Y (2015) Microalgal heterotrophic and mixotrophic culturing for bio-refining: from metabolic routes to techno-economics. Prokop et al. (eds.) Algal biorefineries, Springer. https://doi.org/10.1007/978-3-319-20200-6

Pérez-Garcia O, Escalante FM, de-Bashan LE, Bashan Y (2011) Heterotrophic cultures of microalgae: metabolism and potential products. Water Res 45(1):11–36

Redfield AC (1958) The biological control of chemical factors in the environment. Am Sci 46:205–211

Renuka N, Sood A, Ratha SK, Prasanna R, Ahluwalia AS (2013) Evaluation of microalgal consortia for treatment of primary treated sewage effluent and biomass production. J Appl Phycol 25:1529–1537

Renuka N, Sood A, Prasanna R, Ahluwalia AS (2015) Phycoremediation of wastewaters: a synergistic approach using microalgae for bioremediation and biomass generation. Int J Environ Sci Technol 12:1443–1460

Richmond A (2004) Handbook of microalgal culture: Biotechnol. and Appl. Phycol. Wiley-Blackwell, Oxford

Rindi F (2011) Terrestrial green algae: systematics, biogeography and expected responses to climate change. In: Hodkinson TR, Jones MB, Waldren S, Parnell JAN (eds) Climate change, ecology and systematics. Cambridge University Press, Cambridge, pp 201–227

Ruiz J, Álvarez P, Arbib Z, Garrido C, Barragán J, Perales JA (2011) Effect of nitrogen and phosphorus concentration on their removal

kinetic in treated urban wastewater by *Chlorella vulgaris*. Int J Phytoremediation 13(9):884–896

Sharma NK, Rai AK (2010) Biodiversity and biogeography of microalgae: progress and pitfalls. Environ Rev 19:1–15

Singh M, Reynolds DL, Das KC (2011) Microalgal system for treatment of effluent from poultry litter anaerobic digestion. Bioresour Technol 102:10841–10848

Su Y, Mennerich A, Urban B (2012) Comparison of nutrient removal capacity and biomass settleability of four highpotential microalgal species. Bioresour Technol 124:157–162

Tan X, Chu H, Zhang Y, Yang L, Zhao F, Zhou X (2014) *Chlorella pyrenoidosa* cultivation using anaerobic digested starch processing wastewater in an airlift circulation photobioreactor. Bioresour Technol 170:538–548

Tavaré S (1986) Some probabilistic and statistical problems in the analysis of DNA sequences. In: Miura RM (ed) Lectures math. Life Sci., vol 17. American Mathematical Society, Providence (RI), pp 57–86

Vannecke TP, Bernet N, Winkler MK, Santa-Catalina G, Steyer JP, Volcke EI (2016) Influence of process dynamics on the microbial diversity in a nitrifying biofilm reactor: correlation analysis and simulation study. Biotechnol Bioeng 113:1962–1974

Verhulst PF (1838) Notice sur la loi que la population suit dans son accroissement. Corresp Math Phys Publ Par A Quetel 10:113–121

Wagner EC (1940) Titration of ammonia in the presence of boric acid. Ind Eng Chem Anal Ed 5(6):396–398

Wang H, Xiong H, Hui Z, Zeng X (2012) Mixotrophic cultivation of *Chlorella pyrenoidosa* with diluted primary piggery wastewater to produce lipids. Bioresour Technol 104:215–220

Wang H, Hu Z, Xiao B, Cheng Q, Li F (2013) Ammonium nitrogen removal in batch cultures treating digested piggery wastewater with microalgae *Oedogonium* sp. Water Sci Technol 68:269–275

White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols and applications—a laboratory manual, Publisher. Academic Press, London, pp 315–322

Wu YH, Hu HY, Yu Y, Zhang TY, Zhu SF, Zhuang LL, Zhang X, Lu Y (2014) Microalgal species for sustainable biomass/lipid production using wastewater as resource: a review. Renew Sust Energ Rev 33: 675–688

WWAP (United Nations World Water Assessment Programme) (2015) The United Nations World Water Development Report 2015: water for a sustainable world. UNESCO, Paris

Yang C, Hua Q, Shimizu K (2000) Energetics and carbon metabolism during growth of microalgal cells under photoautotrophic, mixotrophic and cyclic light-autotrophic/dark-heterotrophic conditions. Biochem Eng J 6:87–102

Yang LB, Tan XB, Li D, Chu HQ, Zhou XF, Zhang YL, Yu H (2015) Nutrients removal and lipids production by *Chlorella pyrenoidosa* cultivation using anaerobic digested starch wastewater and alcohol wastewater. Bioresour Technol 181:54–161

Yapijakis C, Wang LK (2006) Treatment of phosphate industry wastes. In: Wang LK, Hung Y, Lo HH, Yapijakis C (eds) Waste treatment in the process industries. Taylor and Francis Group, LLC, USA, pp 399–451

Yeh KL, Chang JS (2012) Effects of cultivation conditions and media composition on cell growth and lipid productivity of indigenous microalga *Chlorella vulgaris* ESP-31. Bioresour Technol 105:120–127

Zhang W, Zhang P, Sun H, Chen M, Lu S, Li P (2014a) Effects of various organic carbon sources on the growth and biochemical composition of *Chlorella pyrenoidosa*. Bioresour Technol 173:52–58

Zhang Q, Wang T, Hong Y (2014b) Investigation of initial pH effects on growth of an oleaginous microalgae *Chlorella* sp. HQ for lipid production and nutrient uptake. Water Sci Technol 70(4):712–719

Zhu LD, Wang Z, Takala J, Hiltunen E, Qin L, Xu Z, Qin X, Yuan Z (2013) Scale-up potential of cultivating *Chlorella zofingiensis* in piggery wastewater for biodiesel production. Bioresour Technol 137:318–325

Zippel B, Rijstenbil J, Neu TR (2007) A flow-lane incubator for studying freshwater and marine phototrophic biofilms. J Microbiol Methods 70:336–345