Growth of three microalgae strains and nutrient removal from an agro-zootechnical digestate

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Marta Franchino[a], Elena Comino[b], Francesca Bona[a], Vincenzo A. Riggio[b]

Abstract

In this paper three microalgae strains (*Neochloris oleoabundans*, *Chlorella vulgaris* and *Scenedesmus obliquus*) were cultivated on an agro-zootechnical digestate in comparable conditions. The material used as growth media was obtained from a pilot plant anaerobic digestor used to digest several mixes of cattle slurry and raw cheese whey. The main aims were to compare the algae growth, their tolerance with respect to the various dilutions of digestate, their nutrient removal efficiency and their role in the transformation of nitrogen compounds. *C. vulgaris* presented the highest elimination capacity of ammonium in 1:10 digestate sample; it was also observed that only 4% of ammonia was removed with stripping, microalgal and bacterial consortium recovered the remaining 96%. The three strains almost completely removed different nitrogen forms and phosphate in 11 d. The results show that microalgal biomass production offers real opportunities for addressing issues such CO_2 sequestration, biofuel production and wastewater treatment.

Keywords Microalgae; Digestate; Nutrients removal; Wastewater recovery

1. Introduction

The continuous increase of oil prices, demand of energy and related environmental issues like global warming have given a strong impetus to the development of renewable alternatives to fossil fuel. Oilseed crops already provide a small fraction of transportation liquid fuels. Recently the potential value of oleaginous microalgae to produce biofuels has also been recognized, as they are more efficient solar energy converters than land plants (Dismukes et al., 2008). Currently, using microalgae to produce biodiesel is too expensive (Norsker et al., 2011). A possible option to reduce the production costs is to use waste CO_2 as source of carbon and wastewater as nutrient supply, see also Pittman et al. (2011) for a review. A wastewater of particular interest is the liquid fraction obtained trough the anaerobic digestion process, which is a primary waste treatment used to reduce organic loading and related noises in agricultural and zootechnical effluents. Long time ago, the use of anaerobic digestion has been spread in Europe, thanks to the support of specific legislative tools aimed at increasing the production of biogas in different economic sectors. One limitation of anaerobic digestion is that it does not significantly reduce the amount of nutrients in the digestate. In fact, it favors more bioavailable nitrogen forms such as ammonium. Several studies have tested algal strains for the treatment of the digestate. The results are still preliminary but promising. Some researchers have studied an association of microalgae and bacteria to treat swine slurry (Molinuevo-Salces et al., 2010 and Gonzàlez-Fernàndez et al., 2011). Wang et al., 2010, Levine et al., 2011 and Yang et al., 2011 have studied the biomass growth and nutrient recovery by the green algae *Neochloris oleoabundans* and *Chlorella* sp. Results showed a high removal efficiency (RE) of main nutrients and they concluded that using microalgae may be an appropriate way of digestate treatment.

The data in the literature concerning the treatment of digestate with microalgae are not comparable, as different microalgae strains, substrate, operational conditions and technologies are used in the different studies. This paper aims to achieve comparable data on growth of different microalgae strains by using the same operational conditions throughout. Three strains were cultured in an incubator with a solution containing digestate coming from a dairy farm, at four different dilutions. This allowed us to compare the data for growth, tolerance to different digestate dilutions and RE of nutrients. Moreover, the contribution of microalgae in this process has been evaluated, since other processes of nitrogen removal can occur, such as ammonia stripping, nitrification and denitrification (Risgaard-Petersen et al., 2004, Gonzàlez et al., 2008, Molinuevo-Salces et al., 2010 and Gonzàlez-Fernàndez et al., 2011). These data are essential for subsequent scale-up treatments of the digestate in the framework of a technological process line aimed at energy production and environmental sustainability.

2. Materials and methods

2.1. Microalgae strains and culture conditions

Three strains of Chlorophyceae known for their productivity and lipid content were selected for this study as possible candidates for sustainable energy production and nutrient removal. The selected strains were *N. oleoabundans* UTEX# 1185 (culture collection of the University of Texas in Austin, USA), *Chlorella vulgaris* CCAP 211/11b (Culture Collection of Algae and Protozoa, Argyll, UK) and *Scenedesmus obliquus* SAG 276-3a (SAG Culture Collection of Algae, University of Göttingen, DE).

They were preserved in BG11 medium (Rippka et al., 1979). Algae were inoculated in 250 mL Erlenmeyer flasks containing 100 mL of liquid medium. The flasks were maintained in an orbital CO_2 incubator (Sanyo CO_2 Incubator Mco-19Aic) flushed with air/CO_2 (97/3, v/v) to support growth and maintain pH within a desired range. In the incubator the temperature was 25 ± 2 °C and the continuous artificial illumination of 200 μmol m^{-2} s^{-1} was provided by daylight Light Emitting Diode. To mix the culture, an orbital shaker with 150 rpm rotation speed was used. Tests were performed in batches, starting from an initial biomass concentration of 0.15 g L^{-1} measured as dry weight in accordance to Chini Zittelli et al. (2000). All the experiments were carried out in duplicate and average values were reported in the results. The cultures were not maintained in a strictly sterile environment, therefore the biomass may have included other microorganisms like bacteria and fungi. Strains were cultivated in batches for 21 d, to allow cultures to reach the stationary phase.

2.2. Growth medium

The material used as growth medium was obtained from the effluent of a pilot anaerobic digestor used to treat several mixes (in volume) of cattle slurry and raw cheese whey. This material was taken from the Fontanacervo farm (Villastellone, Turin – Italy). The growth substrate came from a Digestate Methane Yield (DMY) test performed after a series of experiments that lasted for 229 d described by Comino et al. (2012).

The final stable composition of the mix before the DMY test was 35% cattle slurry and 65% whey. The substrate was stirred every 2 d at 28 rpm for 45 min while biogas analysis was performed. The pH and the temperature inside the reactor, the temperature and the pressure inside the gasometer and the vertical movement of gasometer upper part were constantly monitored, as was the methane concentration inside the biogas. The test system remained sealed during the test duration. After 41 d of Hydraulic Retention Time, the test was terminated and samples were collected for chemical analysis (Table 1).

Table 1. Characterization of wastewaters treated by the anaerobic digester and of its effluent.

Parameter	Cattle slurry	Whey	Digestate DMY test
pH	6.94	4.12	7.49
BOD (mg L^{-1})	39 000	59 000	20 200
COD (mg L^{-1})	120 000	74 400	32 900
Density (g cm^{-3})	0.975	1.012	1.015
105° Residual (%)	11.6	5.08	4.71
550° Residual (%)	2.51	0.559	1.61
Total Volatile Solid (%)	9.1	4.521	3.1
NH_4-N (mg L^{-1})	1.087	57.5	1.634
VFA (mg L^{-1})	<10	<10	<10
Sulfides (H_2S) (mg L^{-1})	0.5	0	0
Alkalinity (meq L^{-1})	140	–	220

A preliminary experiment was performed in order to evaluate algal growth in undiluted or low-diluted digestate. For this experiment *C. vulgaris* was grown in undiluted, 1:2 and 1:5 digestate diluted with tap water, then centrifuged to remove large particles. To exclude a nutrient removal due to centrifugation, ammonium, nitrate and phosphate concentrations were measured before and after this treatment. Data were almost the same and in particular after centrifugation nutrients never decreased (data not shown). Therefore it can be assumed that nutrients were still dissolved in the supernatant and did not precipitate during the centrifugation. The culture medium used for control samples in all the experiments was BG11, as it offers the optimal conditions for the growth of freshwater algae in terms of nutrient concentrations and absence of turbidity, therefore being a good reference to evaluate the algal growth in the digestate.

After this preliminary test, four higher tap water dilutions were selected for the definitive tests with the three strains: 1:10, 1:15, 1:20, 1:25. The feed materials and digestates were stored at 4 °C immediately after sampling and chemical analyses were performed within 48 h by an independent laboratory. BOD was analyzed with the IRSA – CNR n. 5100 A/94 method, COD with the IRSA – CNR n. 5110/94 method, pH with IRSA – CNR Quad 100 met. 2080/94 and directly inside the reactor with the pH probe. Density was calculated with the EMRO/012/1999 method. 105 °C residual and the 550 °C residual as the Total Volatile

Solids were obtained with the IRSA – CNR Quad. 64 n. 2.4.2/84 method. and the volatile fatty acids (C1-C6) were measured with the EMGC 003/1999 method.

2.3. Analytical procedure

Culture growth was estimated experimentally by measuring the optical density (OD) at 750 nm using a spectrophotometer (LKB Biochrom Ultrospec 4050): a linear relationship between OD and dry weight (DW) was determined for each strain. Mean daily biomass productivity was calculated dividing the difference between the dry weights at the end and at the start of the experiment by its duration (d). When a culture entered the stationary phase just before the end of the experiment, its increase in weight was divided by the elapsed time between the start of the experiment and the onset of the stationary phase. Nutrient consumption was evaluated by measuring their variations during the time same interval. For this purpose, culture samples were collected on the first day and the last day of the experiment. Each sample was centrifuged at 3500 rpm for 35 min and supernatant was collected for analysis of ammonium, nitrate and phosphate. Nutrient concentrations were determined by using a spectrophotometer LASA 100-HACH LANGE. The RE of nutrients was calculated by dividing the difference between the initial (C_i) and final (C_f) nutrient concentration by the initial concentration, and then multiplied by 100.

The elimination capacity (EC) was calculated dividing the difference between the nutrient concentration at the start (C_i) and at the end (C_f) of the experiment by its duration (Δt).

Measurements of pH were periodically performed in order to monitor culture conditions (WTW Multi340i pH Electrode SenTix 41).

2.4. Contribute of microalgae to N removal

In order to assess nitrogen transformations during the digestate treatment, we selected one strain for the second experiment. This strain was grown in 250 mL Erlenmeyer flasks containing 100 mL of digestate dairy manure previously diluted 1:10 and centrifugated. Flasks were maintained in an orbital CO_2 incubator under the same conditions of the previous experiment in terms of temperature, pH, irradiance, CO_2 level and rotation speed. Tests were performed in batches for 14 d, starting from an initial algae biomass concentration of 0.1 g L^{-1}. All the tests were carried out in duplicate. We evaluated nitrogen transformations in different biomass treatments: (a) positive controls, in which *C. vulgaris* was inoculated in BG11 culture media; (b) non-sterile samples, in which *C. vulgaris* was added to non-sterile diluted digestate (DD); (c) sterile samples, in which algae were inoculated in diluted digestate previously autoclaved (ADD); (d) negative controls, composed only of diluted digestate, in order to test nitrogen transformations in the absence of algae. OD at 750 nm was used to evaluate culture growth. In all samples bacteria may grow due to non-sterile conditions during the experiment; from the comparison between DD and ADD we aimed at assessing the role of bacteria already present in the digestate (DD sample and negative control) in nitrogen transformation. Chemical parameters measured three times a week were ammonium, nitrate, nitrite, organic

nitrogen, total nitrogen, phosphate and COD. These parameters were valued using the same analytical procedure of the first experiment and previously described. Measurements of pH were daily performed in order to monitor culture conditions.

2.5. Statistical analysis

Generalized Linear Models (GLMs) were used to test the effects of different substrate dilutions at different days on the biomass growth (OD) of three selected strains. GLM was also used to compare the growth of the three strains and to test for differences in productivity of *C. vulgaris* in DD and ADD. GLMs were carried out with R 2.14.0 (R Development Core Team, 2012).

The variables included in our GLMs were: (1) the OD, the response variable; (2) the dilution rate, a categorical predictor variable with five levels (control treatment, 1:10, 1:15 1:20 1:25 dilution ratio), and (3) time, a second predictor variable. Each model thus estimates six parameters: the *Y*-intercept; four regression coefficient for each level of the categorical variable dilution ratio, compared with one level taken as reference level; the regression coefficient for the time predictor variable. In general, the regression coefficients (β) estimated by these models represent the difference in the predicted value of the response variable for each one-unit difference in the predictor variable (e.g. the rate of change of OD with increasing time). For categorical variables such as the dilution ratio, the regression coefficients are the average difference in OD between the reference level (e.g. the control treatment) and each comparison level.

For each β value, the ratio between the estimate and its standard error is used as Wald statistic to finally assess the statistical significance.

GLM was also used to compare the growth of the three strains and to test for differences in productivity of *C. vulgaris* in DD and ADD. GLMs were carried out with R 2.14.0 (R Development Core Team, 2012).

3. Results and discussion

3.1. Comparison among microalgae strains

3.1.1. Algal growth in the digestate

The preliminary test with lower dilutions showed that *C. vulgaris* did not survive at 1:1 and 1:2 dilution ratio, probably due to the high turbidity of the medium. The 1:5 sample survived with an average biomass loss of 87%, in comparison to the control. According to these preliminary results, we chose higher dilution ratio (1:10, 1:15, 1:20 and 1:25). All the three strains survived at all dilutions. Fig. 1 shows growth curves for the three strains.

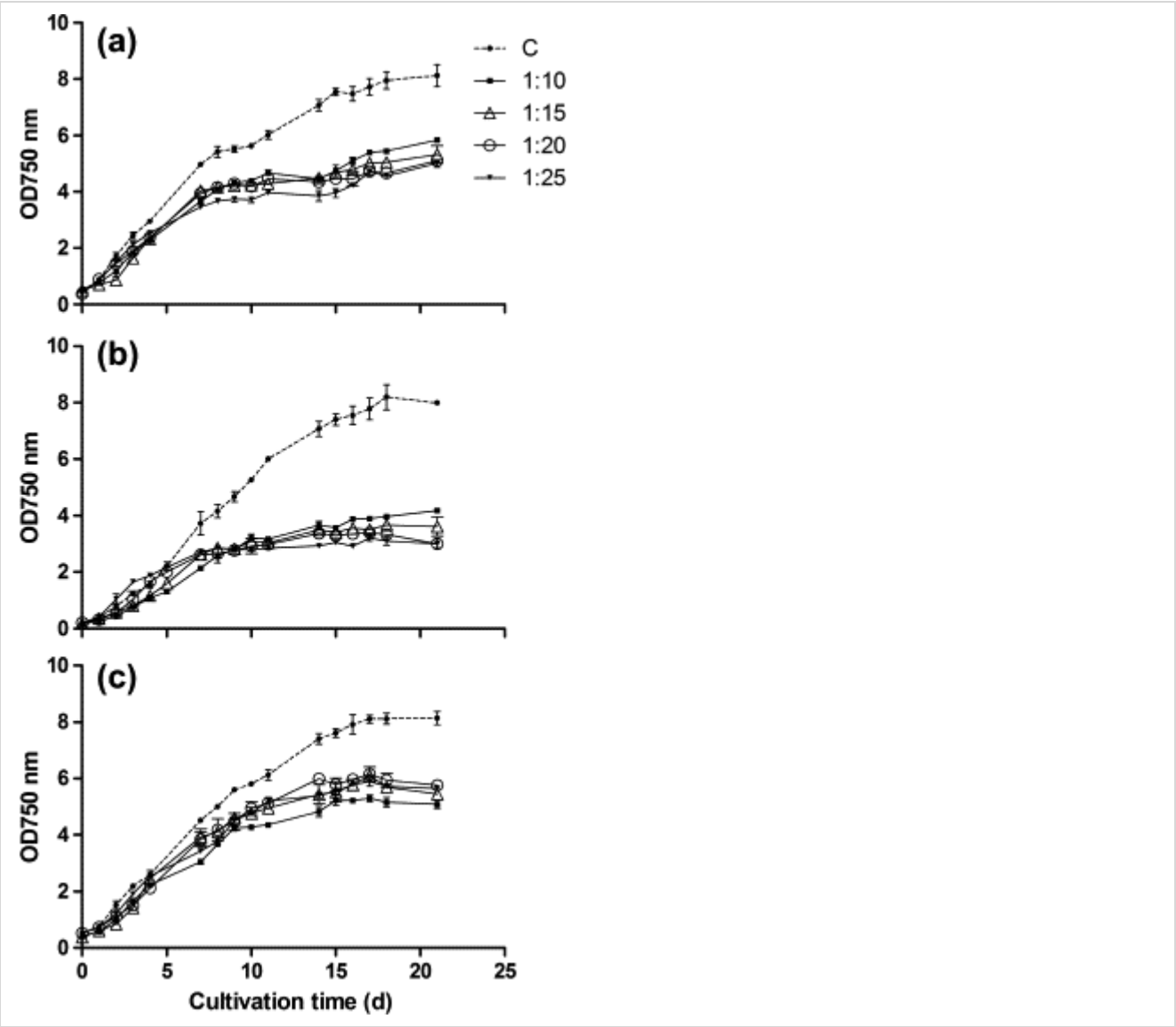

Fig. 1. 750 nm Optical density (OD) variations during time for the three selected strains at different dilution ratio of the digestate. (a) *C. vulgaris*, (b) *S. obliquus*, (c) *N. oleoabundans*. C = control, 1:10 = 1:10 diluted, 1:15 = 1:15 diluted, 1:20 = 1:20 diluted and 1:25 = 1:25 diluted.

In *C. vulgaris* and *S. obliquus* cultures, biomass grew faster in 1:20 and 1:25 diluted samples in the first 7 d. Starting from the 7th day, the growth slowed at all the dilutions, especially in 1:20 and 1:25, possibly due to the lower initial concentrations (Table 1) of digestate and the consequent faster nutrient consumption (Wang et al., 2010) and to the faster increase of the biomass that reduced light penetration. In the final phase of the experiment, samples 1:10 showed the highest biomass. *C. vulgaris* reached the stationary phase at day 7, while *S. obliquus* since day 10 for 1:10 diluted sample and since day 7 for the other dilutions. This trend is confirmed by mean daily productivity data (Table 2). For *N. oleoabundans* cultures we noticed that algae grew faster in 1:20 and 1:25 diluted substrates for the entire duration of the experiment. A slowdown in the growth was noticed in days 7–9, followed by the stationary phase at day 14. For all the three strains mean productivities under the four different dilution ratio showed only slight differences (Table 2), therefore the difference among the initial substrate concentration did not affect algal growth.

Table 2. Growth rates and mean daily productivity of *C. vulgaris*, *S. obliquus* and *N. oleoabundans* on different digestate dilutions.

	C. vulgaris				*S. obliquus*				*N. oleoabundans*			
Parameter	1:10	1:15	1:20	1:25	1:10	1:15	1:20	1:25	1:10	1:15	1:20	1:25
Growth rate (μ) (d^{-1})	0.64	0.52	0.51	0.49	0.49	0.44	0.31	0.23	0.27	0.37	0.30	0.26
Mean daily productivity ($g\ L^{-1}\ d^{-1}$)	0.23 ± 0.04	0.26 ± 0.07	0.25 ± 0.02	0.21 ± 0.04	0.22 ± 0.03	0.25 ± 0.04	0.25 ± 0.06	0.26 ± 0.06	0.20 ± 0.04	0.24 ± 0.07	0.23 ± 0.04	0.21 ± 0.02

Results of GLM applied to algae biomass (Table SM-1 in Supplementary Material (SM)) confirmed that for all the three strains differences in growth were not significant among dilutions ($p < 0.001$). We can therefore assert that 1:10 digestate dilution does not limit microalgae growth. In a full scale plant the 1:10 dilution is likely to support the growth of microalgae, allowing treatment of larger volumes of digestate with an higher initial nutrient concentration. For this reason 1:10 dilution was chosen as the most effective one. Differences are significant, as expected, comparing control (BG11 medium) and digestate and considering time as an explanatory variable. Using linear relationship, the OD of each strain was converted in terms of dry weight in order to compare strains with different morphology. Regarding the comparison among the three strains in 1:10 dilution, GLM did not highlight any significant difference (results not shown). Basing solely on growth data, the three strains are almost equivalent. Moreover, these strains have been proved to be able to accumulate triglycerides in particular culture conditions, like nitrogen starvation (Li et al., 2008, Converti et al., 2009, Gouveia et al., 2009, Gouveia and Oliveira, 2009, Pruvost et al., 2009 and Sialve et al., 2009), therefore it is conceivable to couple wastewater treatment and biofuel production, in order to obtain both economic and environmental benefit.

3.1.2. Nutrient recovery

Most of the nitrogen in the digestate was in the form of ammonium (1634 mg NH_4–N L^{-1}, Table 1). The comparison of nutrient removal in 21 d between different strains is shown in Fig. 2 and Fig. 3.

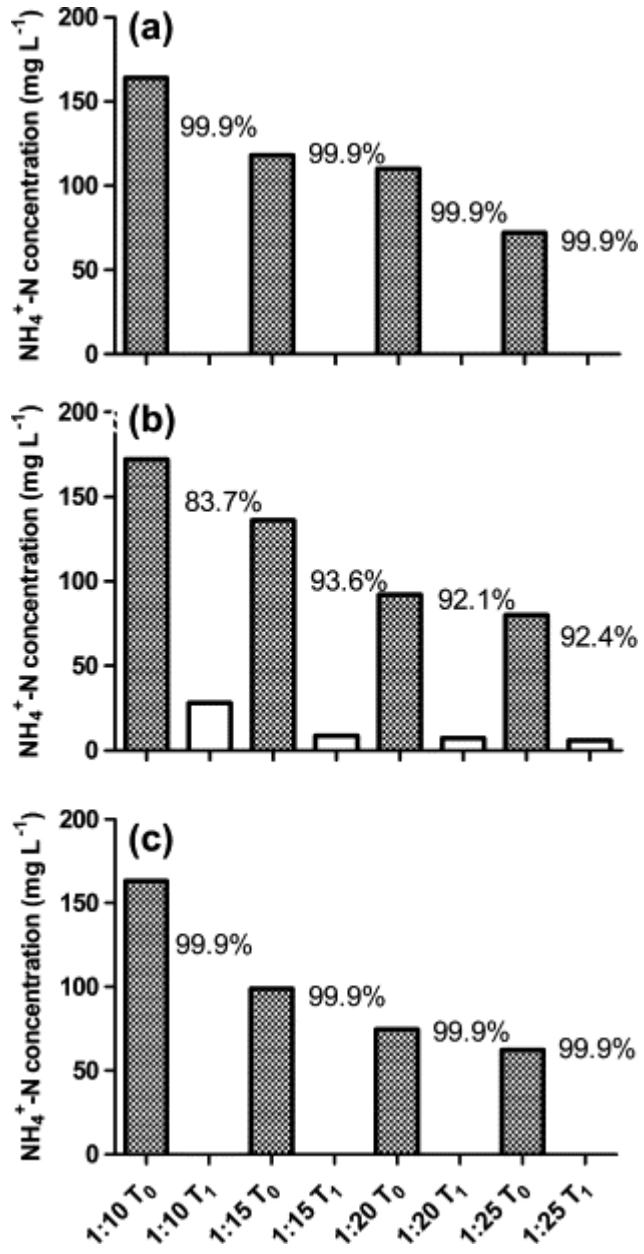

Fig. 2. Initial (T_0) and final (T_1) NH_4^+ -N concentrations (bars) and REs (%) of (a) *C. vulgaris*, (b) *S. obliquus* and (c) *N. oleoabundans* at different dilution ratio of the digestate.

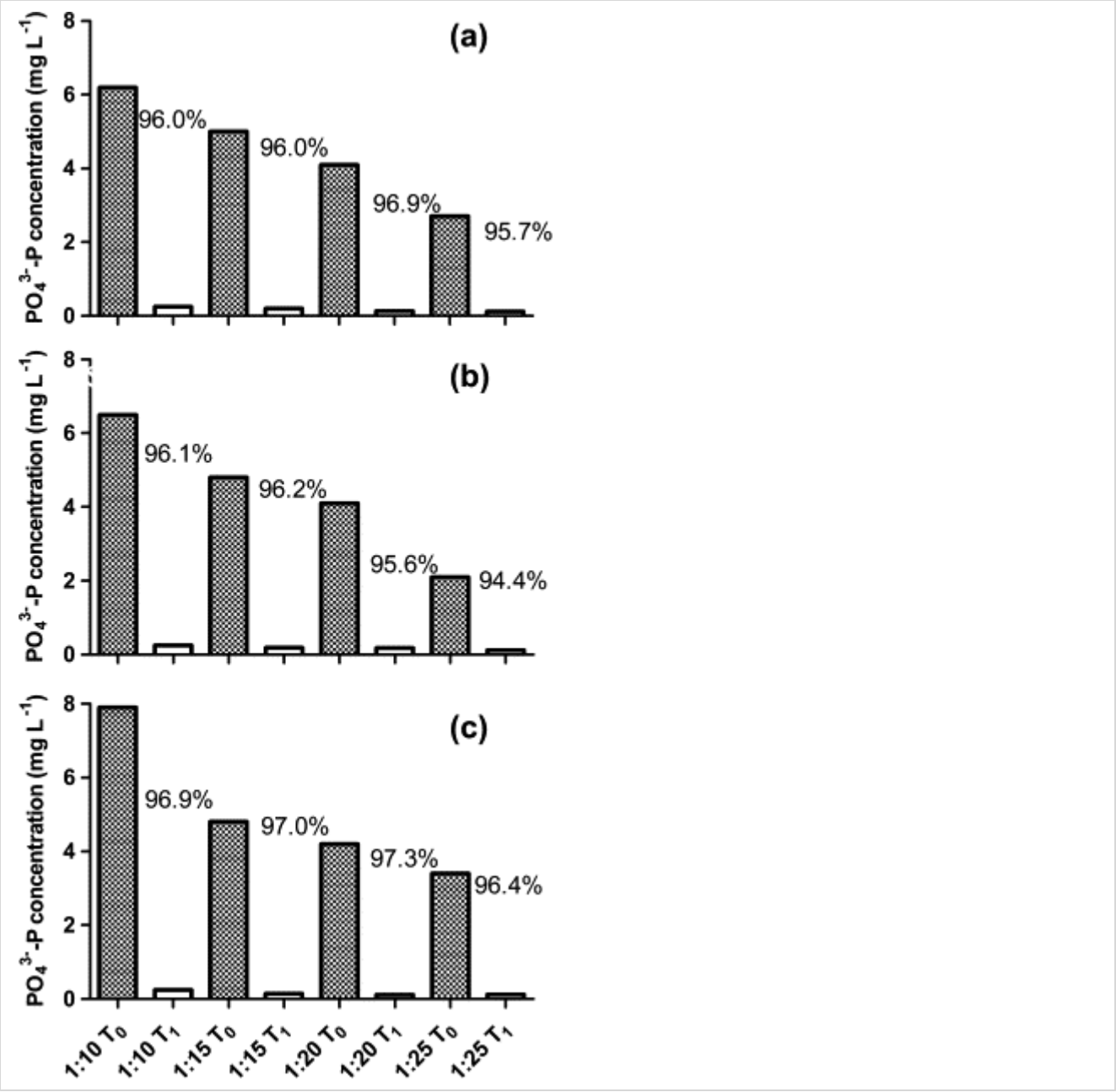

Fig. 3. Initial (T_0) and final (T_1) PO_4^{3-}-P concentrations (bars) and RE (%) of (a) *C. vulgaris*, (b) *S. obliquus* and (c) *N. oleoabundans* at different dilution ratio.

REs of ammonium were more than 83% for *S. obliquus*, with variations according to ammonium initial concentrations, while it was more than 99% for *C. vulgaris* and *N. oleoabundans* regardless how high initial ammonium concentration was (Fig. 2). Nitrogen request of algae generally shows intra- and inter-specific variations. Ammonium is often the preferred N-source for microorganism and the assimilation of either NO_3^- or NH_4^+ by algae is related to the pH of the culture medium (Richmond, 2004). Similar results were observed by Wang et al. (2010) who looked at the growth of *C. vulgaris* in anaerobic digestate dairy manure and found that ammonium was completely removed. Levine et al. (2011) and Yang et al. (2011) showed that *N. oleoabundans* in anaerobic digestate dairy manure can assimilated 90–95% of the initial nitrate and ammonium load. High removal rate of ammonium was observed not only from digestate dairy manure but

also from other substrates: *S. obliquus* was grown in secondary treated wastewater by Ruiz-Marin et al. (2010), *C. vulgaris* was cultivated in primary settled sewage wastewater by Lau et al. (1995) obtaining a removal over 90% of N content and 80% of P content and recently *N. oleoabundans* was tested using agricultural anaerobic and secondary municipal waste effluent (Wang and Lan, 2011 and Yang et al., 2011).

Concerning the final nitrate concentrations (data not shown) slight interspecific variations with a low RE (for 1:10 dilution the highest value was 11% attained by *C. vulgaris*) were detected. In the case of *S. obliquus* final nitrate concentrations were higher than initial ones, with a maximum increased in the 1:20 diluted substrate. For *N. oleoabundans* in 1:10 diluted sample, nitrate concentration increased, while at other digestate dilution it decreased. Thus a clear nitrate removal was not demonstrated, presumably because of the very high ratio $NH4^{+}/NO3^{-}$: in these conditions, microalgae uptake is strongly directed towards ammonium which is their preferred N substrate. However final nitrate concentrations were below the 50 mg L^{-1} limit imposed by European Nitrate Directive.

Also phosphate concentrations decreased significantly, with more than 94% of RE for all the strains (Fig. 3). Indeed phosphorus is essential for algal growth, especially orthophosphate which is the preferred form supplied to algae (Richmond, 2004), as it is involved in many cellular processes although it is less than 1% of the biomass. This phosphate RE was higher than that reported by Wang et al. (2010), who achieved a percentage between 63% and 75%, despite starting at a higher phosphate concentration. Ruiz-Marin et al. (2010) found phosphate REs of 80% for free-cells cultures of *C. vulgaris* and 83% for *S. obliquus* in urban wastewater.

In our experiment a high RE was found in all diluted samples, but EC varied among different dilutions, being related to the initial digestate concentration. EC of ammonium and phosphate were higher in 1:10 diluted samples for all the strains (Table 3). Therefore all the strains were able to remove ammonium and orthophosphate from diluted digestate, but the amount of nutrient removed was greater in samples with higher initial nutrient concentrations.

Table 3. Elimination capacity of $NH4^{+}$-N and $PO4^{3-}$P for the three selected strains, calculated as daily linear difference of nutrient concentration during the cultivation time.

Strains	NH_4^+-N elimination capacity (mg L^{-1} d^{-1})				PO_4^{3-}-P elimination capacity (mg L^{-1} d^{-1})			
	1:10	1:15	1:20	1:25	1:10	1:15	1:20	1:25
C. vulgaris	7.8	5.6	5.2	3.4	0.28	0.23	0.19	0.12
S. obliquus	7.8	4.7	3.6	3.0	0.36	0.22	0.19	0.16
N. oleoabundans	6.9	6.1	4.0	3.5	0.3	0.22	0.19	0.09

3.2. The microalgae contribution to N removal

For the evaluation of nitrogen transformation *C. vulgaris* was selected because it showed the highest productivity and ammonium EC in 1:10 diluted sample. Two treatments were considered: (1) diluted digestate as in the first experiment (DD) and (2) ADD. *C. vulgaris* was also grown in BG11 media, as a positive control. A negative control was composed only of diluted digestate (without microalgae). Initial characterization of DD and ADD is reported in Table 4.

Table 4. Characterization of diluted digestate (DD) and autoclaved diluted digestate (ADD).

Parameter	DD	ADD
NO_3^--N (mg L^{-1})	6.76	8.22
NO_2^--N (mg L^{-1})	0.089	0.05
NH_4^+-N (mg L^{-1})	152	61.2
Organic nitrogen (mg L^{-1})	4.2	3.4
Phosphate PO_4^{3-}-P (mg L^{-1})	7.3	10.4
Total Dissolved Solids TDS (mg L^{-1})	1333	863

After sterilization of the digestate performed in autoclave, ammonium concentration decreased by 60% (from 152 to 61.2 mg L^{-1}) and the pH did not change (7.65 before and after sterilization), presumably due to stripping (Hansen et al., 1998 and Gonzàlez-Fernàndez et al., 2011). The duration of experiment was set at 14 d because, as we ascertained in the previous experiment, 10 d were enough for reaching the stationary phase.

The lag phase lasted the first 2 d (Fig. 4a) and the stationary phase was reached on the 11th day for all treatments. Growth curves were similar between DD and ADD, despite the different initial ammonium concentrations. Results of GLM showed that differences in productivity of *C. vulgaris* grown in ADD and DD were not significant. Therefore we can hypothesized that (a) microorganisms possibly present in the digestate, and (b) physical loss of nitrogen in DD do not affect microalgae growth.

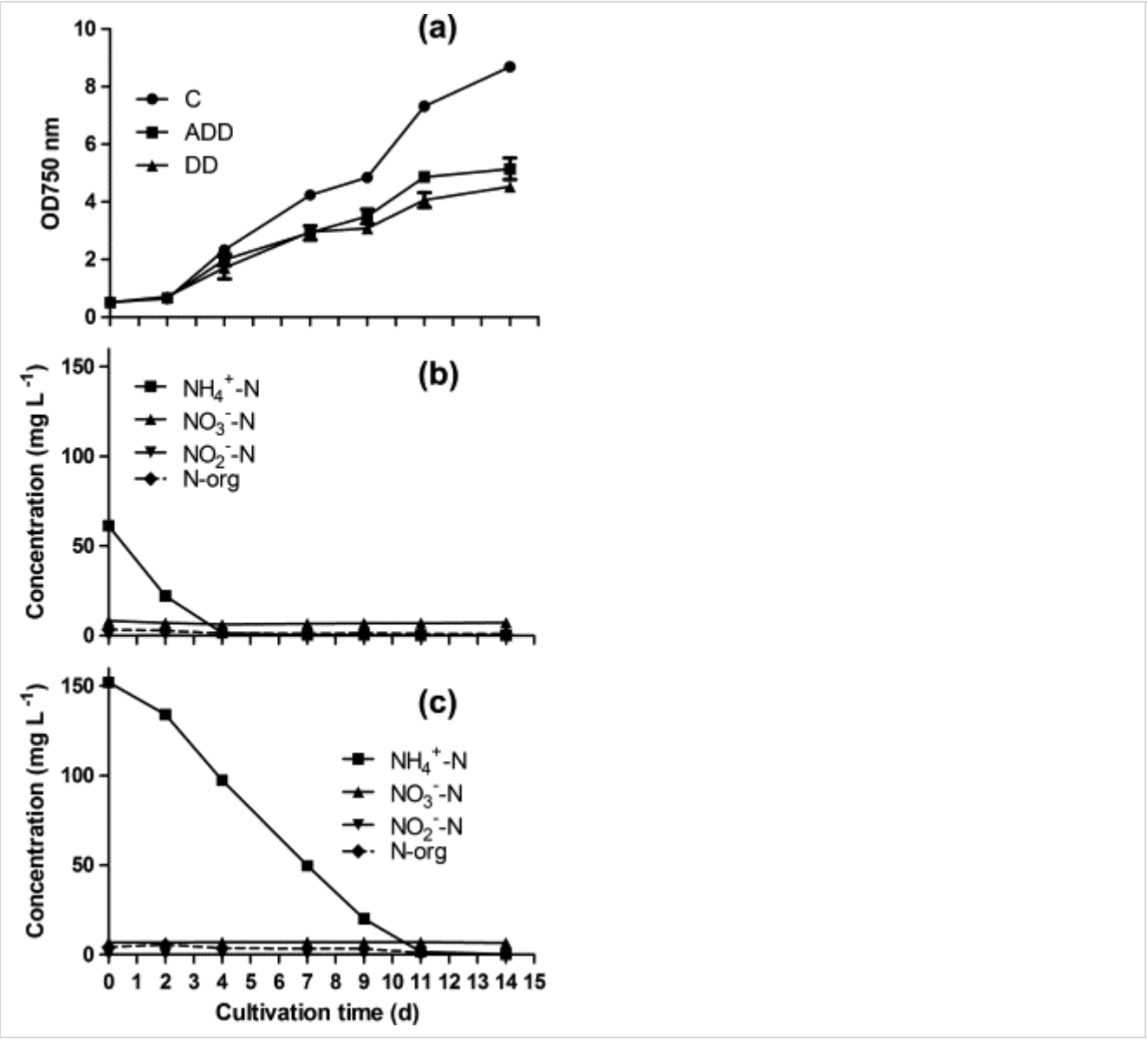

Fig. 4. (a) Growth curves of *C. vulgaris* in control (C), in diluted digestate (DD) and autoclaved diluted digestate (ADD); (b) concentrations of different forms of nitrogen in ADD; (c) concentrations of different forms of nitrogen in DD.

Concentrations of different forms of nitrogen are shown in Fig. 4b and c. Ammonium RE carried out from the ADD only by the algae and bacteria possibly grown in non-sterile conditions (in absence of digestate bacteria), was 97% after 4 d, with a reduction from 61.2 to 1.5 mg L^{-1}, while in DD ammonium RE was 98% after 11 d with a reduction from 152 to 1.6 mg L^{-1}. Total nitrogen followed the trend of ammonium. Nitrite, nitrate and organic nitrogen concentrations remained constant for the duration of the experiment. Trends of different forms of nitrogen were similar in ADD and DD, therefore bacteria possibly still present in digestate did not influence nutrient levels. Despite different initial ammonium concentrations of ADD and DD, growth curves showed similar trends. After the 4th day total nitrogen concentration of ADD was nearly depleted, but OD increased until to 11th day. From days 4 to 11 algae grown in ADD were in nitrogen starvation, therefore the percentage of nitrogen measured at the end of the experiment might be different in biomass grown in ADD in comparison to that grown in DD (Fig. 4b and c).

Phosphate removal efficiency in ADD was 90% after 4 d with a reduction from 10.4 to 0.9 mg L^{-1}, while in DD ammonium RE was 97% after 7 d with a reduction from 7.3 to 0.2 mg L^{-1}. Phosphate starvation began at day 4 in ADD culture (data not shown). COD remained unchanged during the experiment, as expected, since autotrophic metabolism allows mainly nutrient removal. The use of autotrophic strains should be regarded as a secondary wastewater treatment especially when organic loading can be a critical parameter. Concentrations of the most important nutrients were measured also in the digestate without algae. Concentrations of nitrate, ammonium and phosphate were constant during 14 d of experiment, in particular evidencing that ammonia stripping was not significant.

High pH and temperature usually enhance ammonia stripping. This abiotic loss have been described in some other studies concerning microalgae and digestate (Gonzàlez et al., 2008, Molinuevo-Salces et al., 2010, Ruiz-Marin et al., 2010 and Gonzàlez-Fernàndez et al., 2011). In the present study ammonia theoretical abiotic loss was also calculated according to Østergard, 1985. The theoretical ammonia abiotic loss in the control sample was 6.06, 5.77 in ADD and 5.67 mg N L^{-1} in DD, therefore only 4% of total ammonia loss. This is due to the controlled conditions of CO_2 incubator (in particular constant temperature and pH). Biomass growth leads to an increase of temperature and pH, therefore in outdoor conditions is necessary to control these parameters in order to quantify ammonia stripping.

4. Conclusions

The three strains survived at all digestate dilutions and showed high ammonium and phosphate RE. *C. vulgaris* presented the highest EC of ammonium in 1:10 diluted samples, therefore all these strains are promising candidates for digestate treatment. Only 4% of ammonium was removed by stripping, all other nutrient removals were performed by algae and other microorganisms consortium. By up-take, nitrogen can be converted in form of biomass and then used for different purposes, as fertilizer or to produce bioenergy. Microalgal biomass production offers real opportunities for CO_2 sequestration, biofuel production and wastewater treatment.

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Appendix A. Supplementary material

Table SM-1

Estimated coefficient (β), standard error and p-value for the predictor in the model relating the biomass growth (optical density) of the three strains to the different dilution ratio at different days (predictor: time) .For the categorical variable "dilution ratio" we used the control treatment or the 1:10 dilution as reference levels

C.vulgaris					*S.obliquus*				*N.oleoabundans*			
Reference level	Predictors	β	Standard Error	P-value	Predictors	β	Standard Error	P-value	Predictors	β	Standard Error	P-value
control	1:10	-0.3319	0.0644	<0.001	1:10	-0.6173	0.0761	<0.001	1:10	-0.3738	0.0704	<0.001
	1:15	-0.3740	0.0652	<0.001	1:15	-0.6357	0.0766	<0.001	1:15	-0.2785	0.0685	<0.001
	1:20	-0.3833	0.0654	<0.001	1:20	-0.6572	0.0771	<0.001	1:20	-0.2453	0.0679	<0.001
	1:25	-0.4487	0.0667	<0.001	1:25	-0.6545	0.0771	<0.001	1:25	-0.2689	0.0683	<0.001
	time	0.0685	0.0035	<0.001	time	0.0814	0.0042	<0.001	time	0.0771	0.0037	<0.001
1:10 dilution	1:15	-0.0421	0.0677	NS	1:15	-0.0185	0.0794	NS	1:15	-0.0953	0.0742	NS
	1:20	-0.0514	0.0679	NS	1:20	-0.0399	0.0799	NS	1:20	-0.1285	0.0736	NS
	1:25	-0.1168	0.0690	NS	1:25	-0.0373	0.0798	NS	1:25	-0.1049	0.0740	NS
	time	0.0652	0.0040	<0.001	time	0.0721	0.0046	<0.001	time	0.0751	0.0042	<0.001