Outdoor pilot trial integrating a sidestream microalgae process for the treatment of centrate under non optimal climate conditions

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This study was carried out to assess the efficiency of a pilot-scale bubble-column reactor to remove nitrogen in centrate from the biosolid dewatering of a municipal wastewater treatment plant whilst producing biomass for agricultural purposes. The column was inoculated with a mixed community of *Scenedesmus* and *Chlorella* spp. and operated outdoor in batch for 55 days and in continuous for further 130 days. In continuous, the average daily biomass productivity was 40 ± 62 mg TSS $L^{-1} d^{-1}$ and the average NH_4^+-N removal was 20 ± 10 mg $L^{-1} d^{-1}$. Nitrification was fostered by photo-oxygenation leading to the oxidation of 34 ± 27% of the $^-$oxidation of 34 ± 27% of the incoming ammonia nitrogen. Microalgal and bacterial activity inside the column was analyzed by the Generalized Linear Models in order to understand the main factors affecting the process performances. Microalgal growth was affected positively by the NH_4^+-N content in the influent and negatively by the amount of TSS entering the system, probably due to the competition between microalgae and bacteria for phosphorus and other nutrients. The removal rate of NH_4^+-N was positively affected by NH_4^+-N_{in} (influent concentration) and by pH, whose increase fosters stripping, and decreased for increasing NH_3-N concentrations, responsible for inhibiting ni-trifying bacteria. NH_4^+-N oxidation was the result of complex interactions between algae and bacteria and was also affected by flow and solar radiation. No other specific limiting factors have been highlighted. The possibility of improving the process performance by controlling pH, by supplying off-gas as CO_2 additional source, appears as an interesting option. In view of a scale-up, the most relevant expected result would be the energy saving due to the decrease in the oxygen demand for nitrification in the water line. The microalgal biomass grown on centrate was suitable for agricultural use due to its low contamination by heavy metals.

Keywords:
Microalgae
Centrate
Nitrification
Bubble column photobioreactor

1. Introduction

The removal of macropollutants as COD, BOD and nutrients is today effectively achieved in wastewater treatment plants (WWTPs) but resource recovery and energy saving are among the main current challenges in WWTP design and operation to fulfil the circular economy principles.

In conventional WWTPs, the removal of nutrients involves the loss of potentially valuable resources and the production of a mineralized waste sludge having low biomethane potential. Moreover, it is well known that in WWTPs aeration accounts for 45 to 75% of the total operation energy costs [1] and that nitrification contributes sig-nificantly to the aeration demand. Data from Germany, Austria and Italy show that the electric power consumption for wastewater treat-ment accounts for about 1% of the total national consumption, and this figure could probably be extended to the other European countries [2].

In this context, microalgae-based processes seem particularly interesting since they have low energy requirements and offer several possibilities for the recovery of materials/energy from the algal biomass [3]. Several studies reported interesting results on the use of different kinds of wastewater as nutrient source for microalgae culturing. Among them: domestic [4], piggery [5,6], and slaughterhouse [7] wastewaters, as well as municipal centrate [8–12]. Microalgae uptake nutrients from wastewater and produce oxygen by photosynthesis. In microalgae based systems fed on wastewater, algae/bacteria consortia develop, where bacteria take advantage from the oxygen released by microalgae while microalgae use the CO_2 deriving from the bacterial oxidation of the organic matter [13–15]. The most conventional fates of this algal/bacteria biomass are biomethane production in anaerobic digesters [16], and/or agricultural use as fertilizer/biostimulant [17,18].

The main limiting factors for algae-based processes are light and temperature. In Northern Italy, the climate is not so favorable, as during

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winter months solar radiation is indeed faint. According to the data collected from 1991 to 2010 by the Italian Air Force Meteorological Service the average number of hours per day with > 120 MJ m^{-2} ranges between 2 and 4 from November to February. Moreover, in the same time interval, temperature range from 0 to 10 °C (ARPA Lombardia, 2016 [19]).

In this context, microalgae-based treatment cannot be envisaged as a main-stream process but can be integrated into the WWTP scheme as a side-stream process. However, a microalgal process could be applied to the supernatant from sludge dewatering (called centrate if deriving from centrifuging) to reduce the nitrogen load that is brought back to the water line. Since the N-load returned by the centrate may account for 10–20% of the total influent nitrogen load [20], photosynthetic aeration, during the favorable season, would contribute to reduce the energy cost in the overall management of WWTPs.

This research focuses on the integration of a microalgal culturing unit within the conventional scheme of a medium-large wastewater treatment plant. The novelty of the present study consists in the evaluation of the feasibility of applying a microalgal based process within the sludge line to treat directly raw centrate in the non-optimal climate conditions of Northern Italy, while the majority of literature data were obtained on centrate after pre-treatment [21] or enrichment with additional carbon sources [22].

The productivity of microalgae and the removal of nitrogen were monitored for approximately 6 months in an outdoor pilot-scale bubble-column fed on centrate. At present, no definite way of disposal of the algal biomass produced from wastewater (and thus from centrate) is consolidated. The produced algal biomass was analyzed in view of its possible agricultural use, comparing its metal content to the limits set for the agricultural use of sewage sludge at national and European scale. Specific attention was given to nitrite production, for which very scarce data are available in the current literature.

2. Methods

2.1. Wastewater treatment plant

The present work was carried out at a municipal wastewater treatment plant, located in Milano (Bresso, Northern Italy), designed to serve 220,000 inhabitant equivalents (I.E.). The water line consists in: mechanical treatments, primary settling, and secondary treatment by conventional activated sludge (nitrification/denitrification) followed by UV disinfection. The sludge line includes two anaerobic digesters operating under mesophilic conditions (35 °C) followed by one post-digester. On average, the produced biogas is made of 67% CH_4, 31% CO_2, and minor or trace concentrations of other gasses. Biogas is sent to two CHP units producing 220 and 320 kWel, respectively. The digestate is centrifuged and a cationic polyelectrolyte is used to enhance sludge dewatering (EM516GK from SNF Italia S.p.A.).

2.2. Pilot plant and experimental design

A bubble-column (82 L working volume, Ø = 29 cm) was installed outdoor in the area of the municipal wastewater treatment plant of Bresso (Milan, Italy) and run under natural light and temperature conditions. CO_2 and mixing were provided by bubble aeration from the bottom at 20 L min^{-1}.

The reactor was initially filled with 50 L of a mixture of wastewater (25 L of centrate and 25 L of effluent) and 5 L (10% v/v of the total column volume, as reported in [23]) of algal inoculum (1 g TSS L^{-1}) previously cultivated in a laboratory scale reactor (data not shown) fed on real undiluted centrate from Bresso WWTP. Microscopic observation revealed that the inoculum was made of a mixed microalgae/bacteria community including mainly *Scenedesmus* spp. and *Chlorella* spp., in agreement with previous studies [24–27].

The experimentation included a batch mode period (55 days) followed by a continuous trial (130 days). When the algal density was stabilized at 1 g TSS L^{-1}, the column was switched to continuous mode. On the basis of the results from a previous experience with the same substrate and pilot plant [27] the hydraulic retention time (HRT) was maintained around 9 days by using a peristaltic pump (max flow-rate 0.1 L min^{-1}), even if some flow variations occurred.

Microalgal counts, nutrient (PO_4-P, NH_4^+-N, NO_3^--N and NO_2^--N) concentrations, total suspended solids (TSS), optical density (at 680 nm wavelength), conductivity, pH, turbidity and COD were analyzed 1–3 times a week in samples collected from the inlet and outlet flow. Metal concentrations in the centrate and in the algal biomass were measured 4 times during the continuous operation. Elemental analysis (C, N, H and P content) was performed once on the algal biomass sampled at the end of the continuous phase. Daily average values for temperature and irradiance were obtained from the ARPA Lombardia database (data collected from a meteorological station located < 10 km from Bresso WWTP).

2.3. Influent

Centrate and secondary effluent were collected from Bresso WWTP. With respect to centrates from other WWTPs, Bresso centrate has a low concentration of ammonia nitrogen, due to the low percentage of total solids (2.3%) in the waste sludge fed to the anaerobic digestion. The N:P ratio is far from the 16:1 Redfield molar ratio, generally accepted as optimal for microalgae growth [28]. Chemical Oxygen Demand (COD) is low too, due to the high efficiency of anaerobic stabilization, while the high variability of total suspended solids (TSS) and turbidity de-pends on the unstable performance of the solid/liquid separation by centrifuging.

High metal concentrations in the medium might inhibit photosynthesis and cause morphological changes to the microalgal cell [29]. Therefore, the centrate was analyzed for the main heavy metals and the obtained data were compared to the environmental quality standards for surface waters defined as Maximum Acceptable Concentration by EC Directive 2008/105, by the Italian DM 260/2010 and by the US Water Quality Criteria for Aquatic Life (issued by EPA in different years)(Table 1) which were considered as indicators for the potential toxicity of centrate on microalgae.

Table 1
Comparisons between the concentrations of metals in the centrate ($n = 4$) and the environmental quality standards for surface waters defined by EC (Directive 2008/105 EC), by the Italian law (DM 260/2010) and by the US Water Quality Criteria for Aquatic Life (EPA 2004). Concentrations in μg L^{-1}.

Metals	Centrate (mean ± st.dev)	EC EQS (Directive 2008/105)	Italian DM 260/2010	US WQC[b]
Ag	< 1			3.2
Al	380 ± 261			750
As	< 1		10 ([a])	340
Cd	< 1	0.45–1.5[c,b]	0.45–1.5[c,b]	1.8
Cr (III)				570
Cr (VI)			7[a]	16
Cr (total)	< 1			
Cu	6 ± 5			13
Fe	19 ± 15			1000
Hg	< 1	0.07[b]	0.06[b]	1.4
Ni	44 ± 19	20[a]	20[a]	470
Pb	< 1	7.2	7.2[a]	65
Zn	42 ± 60			120

[a] Average annual concentration
[b] Maximum Acceptable Concentration
[c] The value of EQS for Cd varies as a function of the water hardness: for $CaCO_3 < 0.45$ mg L^{-1} EQS ≤ 0.45 μg L^{-1}; for $CaCO_3 = 40 \div < 50$ mg L^{-1} EQS = 0.45 μg L^{-1}; for $CaCO_3 = 50 \div < 100$ mg L^{-1} EQS = 0.6 μg L^{-1}; for $CaCO_3 = 100 \div < 200$ mg L^{-1} EQS = 0.9 μg L^{-1}; for $CaCO_3 \geq 200$ mg L^{-1} EQS = 1.5 μg L^{-1}).

The concentrations of metals in the centrate were all much lower than the standards set by the US. On the contrary, the concentration of Hg and Ni exceeded the European and Italian standards. However, metal toxicity tends to increase with decreasing pH and the significant increase of pH due to photosynthesis makes even more unlikely any toxic effect on microalgae growth.

During the batch period, the column was initially filled with a mixture of secondary effluent and centrate (see Table A1) (50/50% v/v), while, when working in continuous, undiluted centrate was used as feed. The secondary effluent, as it is common, has lower nutrient concentration compared to the centrate (1, 8, 0.5, 30 mg L^{-1} for NH_4^+-N, NO_3^- −N, PO_4-P and COD, respectively).

2.4. Analytical methods

TSS were measured according to Standard Methods [30]. PO_4-P, NH_4^+-N, NO_3^--N and NO_2^--N and soluble COD were analyzed on filtered samples (0.45 μm) using spectrophotometric test kits (Hach-Lange, Germany, LCK 303, LCK 340, LCK 342, LCK 348 and LCK1414, respectively). Conductivity and pH were determined by a portable instrument (XS PC 510 Eutech Instruments, USA). Optical density (OD at 680 nm wavelength in a 1 cm cuvette) and turbidity (at 860 nm in a 5 cm cuvette) were measured by a spectrophotometer (DR 3900, Hach Lange, Germany). Metal analyses were performed by Inductively Coupled Plasma-Mass Spectrometry (ICPMS model 7700×, Agilent Technologies, USA) according to the US-EPA method 200.8 EMMC (version 5.41994). Phosphorus was determined after acid digestion (with H_2NO_3 and H_2O_2) of the dried biomass in a microwave digester (ETHOS 1600, Milestone) according to Green algae procedure (DG-EN-25). Microalgae were counted in 0.1 mL samples of microalgal suspension using a Hemocytometer (Marienfeld, Germany) and an optical microscope 40× (B 350, Optika, Italy). *Scenedesmus* and *Chlorella* algal cells were distinguished according to their morphological characteristics and counted, and the final estimated cell number was obtained from the mean of 6 square (1 mm^2) readings.

Microalgal biomass was analyzed for heavy metals after HNO_3 + H_2O_2 digestion. For the elemental analysis, the samples were dried at 60 °C and analyzed by a Perkin Elmer CHNS/O analyzer 2400 series II.

2.5. Data processing

The specific microalgal growth rate (μmax in d^{-1}) was calculated as the slope of the line fitting the TSS concentration versus time graph.

Mass balances were set to compute the production rates of microalgal biomass (as TSS, r_{TSS}) and of nitrite ($r_{NO2–N}$) and nitrate nitrogen ($r_{NO3–N}$) as well as the removal rates of ammonia (r_{NH4+-N}) and total nitrogen (r_{totN}).

As an example, the TSS production rate r_{TSS} was calculated as:

$$\frac{[TSS]_{t_{i+1}} - [TSS]_{t_i}}{\Delta t} = \frac{[TSS]_{IN} - [TSS]_{t_i}}{HRT} + r_{TSS}$$

where: $[TSS]_{t_i}$ and $[TSS]_{t_{i+1}}$ are the TSS concentrations measured at time t_i and t_{i+1} in the microalgal suspension; $[TSS]_{IN}$ is the TSS concentration in the centrate fed to the microalgal column, and Δt is the sampling interval.

The partitioning of nitrogen species in the effluent was obtained considering residual NH_4^+-N, N in algal biomass, oxidized N (NO_2^--N + NO_3^--N) and stripped N. The percent value of each component was calculated with respect to the concentration of NH_4^+-N in the influent. The amount of nitrogen in the algal biomass was calculated by multiplying the concentration of N measured in the TSS (7.5%, see section 3.4. Characterization of microalgal biomass characteristics) by the TSS concentration in the effluent. Finally, the stripped amount was calculated as difference between NH_4^+-N concentration in the influent and the sum of residual NH_4^+-N, oxidized N in the effluent, and N in algal biomass.

The concentration of free NH_3-N (mg L^{-1}) was computed starting from the total ammonia nitrogen (TAN) [31], as follows:

$$N - NH3 = \frac{1}{1 + 10^{-pH+0.09018+\frac{2729.92}{T(^{\circ}C)+273.15}}} \times TAN$$

Statistical analyses were conducted by the R Project software [32] and the package Hmisc [33].

The r_{TSS}, r_{NO3-N}, r_{NO2-N}, r_{totN}, r_{NH4+-N} obtained during the continuous phase of 2016 experimentation were evaluated by Generalized Linear Models (GLMs) to analyze simultaneously the effects of categorical and continuous variables on response variables that have error distribution models other than a normal distribution. Since the range of values of raw data varied widely and had different units, all response and independent variables were unit-based standardized [(x-min(x))/(max(x)-min(x))].This standardization allows to use the gamma and lognormal link functions (that correspond to a different error adjustment) to the response variables that support only positive number in the GLM function of R. To avoid multicollinearity a stepwise selection of the independent variables using Variance Inflation Factor (VIF) was used [34]. VIF value for each variable was calculated and the variable with the single highest value was removed. This procedure was re-peated until all the VIF values were below 10. The experimental parameters included in the full GLM as independent variables were: cumulative irradiance (CI), cumulative temperature (CT), flow, influent (IN) characteristics (NH_4^+-N, PO_4-P, TSS, turbidity) and algal suspen-sion characteristics (pH and free ammonia). Free ammonia in the sus-pension was considered as “independent” variable, since it could be toxic or inhibiting for microalgal/bacteria community. The full model formula is reported below:

$$X = 1 + \beta_1 * CI + \beta_2 * CT + \beta_3 * Flow + \beta_4 * pH + \beta_5 * NH_4^+ - N_{in} + \beta_6 * PO_4 - P_{in} + \beta_7 * TSS_{in} + \beta_8 * Turbidity + \beta_9 * NH_{3out} + \varepsilon$$

NO_2^--N_{in} and NO_3^--N_{in} were not considered as variables because NO_2^--N_{in} was always below 0.3 mg L^{-1} and NO_3^--N_{in} was slightly higher but still 2 orders of magnitude lower than NH_4^+-N_{in} and its variation range was quite narrow.

The dependent variables represented by X in the model formula were: r_{TSS}, r_{NO3-N}, r_{NO2-N}, r_{totN}, r_{NH4+-N}.

The best link function for each dependent variable was determined comparing AIC_c (Akaike Information Criterion) corrected for small sample size [35] of full models differing only in the link function. Those calculation were made using the AIC_c modavg package [36]. Restricted models were generated by removing variables in a backward-stepwise procedure and compared with the full GLM model by AIC_c tables. This procedure was repeated until the evaluated model had the smallest value of AIC_c. The results of the first 10 days (corresponding to 1 HRT), when the system was not yet stabilized, have not been considered.

The significance of the selected models was evaluated using the table of variance analyses for each model (Table A4).

The 2016 experimental data were then compared to the results of a previous trial (2014), which had been carried out in similar conditions [27], but with the addition of phosphorus to the feed in order to optimize the N:P ratio. As normality and homogeneity of variance could not be achieved for most of the variables, the comparison between the two sets of experimental data (2014 vs 2016) was made by nonparametric procedures. The Kruskal-Wallis test by ranks was used (p value $<$ 0.05). Kruskal-Wallis test by ranks is a non-parametric method for testing whether samples originate from the same distribution, allowing to compare two or more independent samples.

3. Results and discussion

3.1. Microalgal growth and treatment efficiency

3.1.1. Batch phase

Microalgae growth was quantified according to TSS concentration. The maximum growth rate (μ_{max}) achieved during the exponential growth phase was 0.19 d^{-1} and the biomass production was 36 ± 11 mg TSS L^{-1} d^{-1}. At the end of the batch period (55 days), the total biomass was 1 g TSS L^{-1} and the microalgal count was 8.35*10^6 cells mL^{-1}. Table A1 summarizes environmental data and analytical results obtained during the batch phase, while Fig. A1 shows microalgal growth and the nitrogen concentration in the same period. Ammonia, nitrite, and nitrate N were measured during all the batch period. Until day 15, the removal of NH_4-N was mainly due to microalgal assimila-tion, though stripping probably occurred due to the high pH (until day 15 the pH was 8.5 ± 0.5, while it decreased to 7.7 ± 1 afterwards). Later, ammonia oxidation started, with increasing production of NO_2^--N; after day 22 nitrite was partially oxidized, producing NO_3^--N. The maximum production rate of oxidized nitrogen was 3.6 mg NO_x-N L^{-1} d^{-1}. The trends of nitrate and nitrite suggest that ammonium oxidizing bacteria (AOB) developed faster in the bubble-column than nitrite oxidizing bacteria (NOB), which could have been inhibited by the high concentration of free ammonia [37].

3.1.2. Continuous phase

3.1.2.1. Microalgal growth and nitrogen removal. The microalgal community was made of *Chlorella* spp. and *Scenedesmus* spp.(6.4*10^5 ± 1*10^6 cells mL^{-1} and 5.4*10^6 ± 2.0*10^6 cells mL^{-1}, respectively) as shown in Fig. A2 in Appendix. Despite the obvious non-sterile conditions, no other species was found all over the trial. *Scenedesmus* spp. were just slightly predominant in terms of cell counts.

The average biomass production (r_{TSS}) was 40 ± 62 mg TSS L^{-1} d^{-1}, in agreement with our previous findings (50 ± 40 mg TSS L^{-1} d^{-1} [27]) and slightly higher than the one reported by other au-thors (23–40 mg TSS L^{-1} day^{-1} [38]). Moreover, the uncontrolled outdoor conditions did not hamper the resilience of the algal commu-nity. Table 2 summarizes the environmental data and analytical results obtained during the whole continuous phase.

Fig. 1 reports the microalgal counts, the concentrations of NH_4^+-N in the influent and of the different nitrogen species in the effluent. Working outdoor, the environmental conditions were not constant, and temperature and irradiation decreased during the experiment; the same trend was followed also by the microalgal density.

The lowest concentration of NH_4^+-N in the influent was observed in August (from day 58), during summer holidays, when the city population is at the annual minimum. At the same time, microalgal cell density decreases, to increase again at the end of August. The following decrease was related to the seasonal decrease of temperature and irradiance. As in the batch phase, the trend of ammonia, nitrite, and nitrate concentrations in the effluent demonstrate the occurrence of nitrification. As reported in Hernandez et al., [7], NH_4^+-N is assimilated by microalgae or nitrified to NO_3^--N. In the present case, the production of nitrite (concentrations in the effluent ranging between 2 and 111 mg NO_2^--N L^{-1}) was much higher than the production of nitrate (3–37 mg NO_3^--N L^{-1}). Although some NOB activity occurred, as demonstrated by the presence of nitrate in the effluent, the high NH_4^+-N concentration in the feed probably promoted the activity of AOB over NOB.

Other factors could lead to nitrite built up, namely: (i) non negligible free ammonia levels (8 ± 11 mg NH_3-N L^{-1}) which are known to inhibit NOB more than AOB [39], (ii) temperature over 20 °C which correspond to higher specific growth rates for AOB than for NOB [40], and (iii) high irradiance, possibly affecting NOB more that AOB since the former seem to be more photosensitive than the latter [41]. Since NH_4^+-N concentration was just occasionally very low (3 samples out of 35 had concentration < 1 mg N L^{-1}), significant algal uptake of NO_3^--N seems unlikely. Indeed, it is well known that nitrate assimilation occurs when no NH_4^+-N is available [4].

Fig. 2 reports the average apportioning of N in the effluent with respect to NH_4^+-N inlet concentration. The variability of data is indeed high, especially because nitrification has not been active throughout the trial and this has strongly affected the values of the oxidized and residual ammonia fractions. In spite of that, it can be easily seen that the residual fraction of NH_4^+-N is low and that microalgal assimilation and nitrification sum up to 55% on average. However, the contribution of stripping is relevant, and this should be controlled to avoid negative environmental impacts. In fact, NH3 contributes to the formations of aerosol and smog [42], and, after deposition, it may cause acidification in soils, groundwater and surface waters undergoing nitrification [43].

Table 2
Environmental and analytical data recorded during continuous phase. IN = feed (centrate), OUT = effluent (algal suspension).

Parameter (Unit)	Mean ± Standard deviation	
	In	Out
Temperature °C	20 ± 4	
Irradiance (W m^{-2})	214 ± 87	
pH	8.6 ± 0.5	8.7 ± 1.4
Conductivity (mS cm^{-1})	1.4 ± 0.4	1.3 ± 0.3
NH_4^+-N (mg L^{-1})	147 ± 29	14 ± 14
NH_3^-- N (mg L^{-1})	n.d.	7 ± 11
NO_3^--N (mg L^{-1})	1 ± 1	11 ± 7
NO_2^--N (mg L^-)	0.1 ± 0	36 ± 30
COD (mg L^{-1})	115 ± 56	113 ± 53
PO_4-P (mg L^{-1})	3.8 ± 1.2	0.3 ± 0.2
Chlorella spp (cells mL^{-1})	–	6.4* 10^5 ± 1.0* 10^5
Scenedesmus spp (cells mL^{-1})	–	5.4*10^5 ± 2.0* 10^5
TSS (mg L^{-1})	346 ± 410	723 ± 210

3.2. Statistical analyses

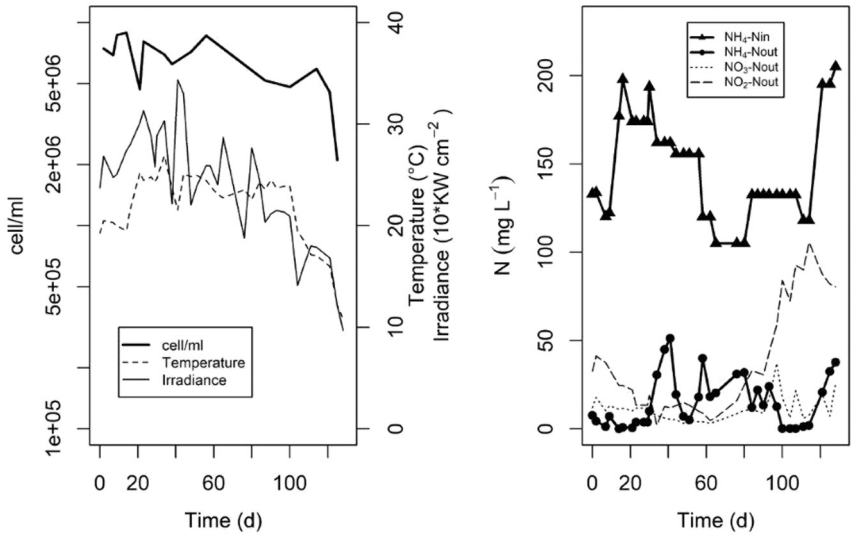
Statistical analyses using GLMs were used to understand the influence of the environmental conditions and feed composition on microalgae growth and nitrogen removal in the experienced variation ranges.

Fig. 3 summarizes the partial effects of the independent variables included in the selected model for each dependent variable. The interpretation of the results must take into account the variety of processes occurring within the column.

The production of algal biomass (r_{TSS}) is negatively related to the amount of TSS entering the system (TSS_{in}), probably due to the competition between microalgae and bacteria for phosphorus and other nutrients (higher TSS_{in} means also higher bacterial count in the influent). On the contrary, a positive relation was observed between r_{TSS} and NH_4^+-N_{in}, easily explained by the role of NH_4^+-N in algal metabolism.

The removal rate of NH_4^+-N (r_{NH4}) was positively affected by its influent concentration and by pH. Actually, the increase of pH depends on photosynthesis and, thus, on algal growth, and, as previously mentioned, algal growth (r_{TSS}) increases with increasing NH_4^+-N_{in}. The positive effect of pH on NH_4^+-N removal rate is also partially due to the increase of stripping. On the other hand, r_{NH4} decreases for increasing NH_3-N concentrations, the latter being responsible for inhibiting nitrifying bacteria.

Nitrate production rate (rNO_3) and nitrite production rate (rNO_2) had opposite relations with the flow (which varied due to operation problems): rNO_2 increased with increasing flow while rNO_3 decreased. That could be explained by a lower growth rate of NOB compared to AOB, resulting in a lower NOB/AOB ratio at higher flow rates (i.e. shorter HRT). As the substrate for nitrification is NH_4^+-N, NH_4^+-N_{in} was positively related to rNO_3.



(a) (b)

Fig. 1. Results of the continuous phase: (a) Microalgal count, temperature and irradiance; (b) NH_4^+-N concentration in the influent and residual NH_4^+-N, NO_2^-N, and NO_3^--N in the effluent.

The negative correlations between TSS_{in} and rNO_2 and between pH and rNO_2 can be explained by the competition between AOB and heterotrophic bacteria for phosphorus and by AOB and microalgae (the latter more active and abundant when pH was higher) for phosphorus and CO_2. The competition between AOB and microalgae could also be one of the reasons for the negative effect of irradiance (IC4 = cumulated irradiance in the 4 days before sampling) which had anyway also an inhibiting effect on nitrifying bacteria. According to Kaplan, 2000 [44] high light intensity could inhibit AOB and NOB ac-tivity.

All such relations are reflected in the positive dependence of the removal rate of total N ($rN_{tot,}$) which was positively related to NH_4^+-N_{in} and to the concentration of phosphorus in the influent (PO_4-P_{in}).

In the end, a positive relation was found between rN_{tot} and temperature (TC4 = cumulated temperature irradiance in the 4 days before sampling) which, of course, enhances all biological activities and also ammonia stripping. The removal of total nitrogen (rN) was the result of the overall effect of algal uptake for growth and ammonia stripping, but not of nitrification.

3.3. Comparison between 2014 and 2016 results

Finally, the results of this experimentation were compared with the results obtained in 2014 [27] in a 160 days continuous trial with the same bubble-column and the same centrate. The main operational difference between the trials of 2014 and 2016 was the addition of P in the centrate. During 2016, no correction was implemented and the N:P in the centrate remained quite high (N/P molar ratio = 85 from data in Table 2) and far from the Redfield value. Nonetheless, comparable algal growth was obtained during 2014 and 2016. This result should be considered in light of the many factors that may affect the actual N:P available for microalgae, which in turn depends on concurrent N and P removal processes taking place in the raceway at the same time as algal growth. Indeed, N is also removed by stripping, while P can precipitate and could be assimilated by other microorganisms, as acknowledged by previous studies [45,46]. Another important aspect is the flexibility of

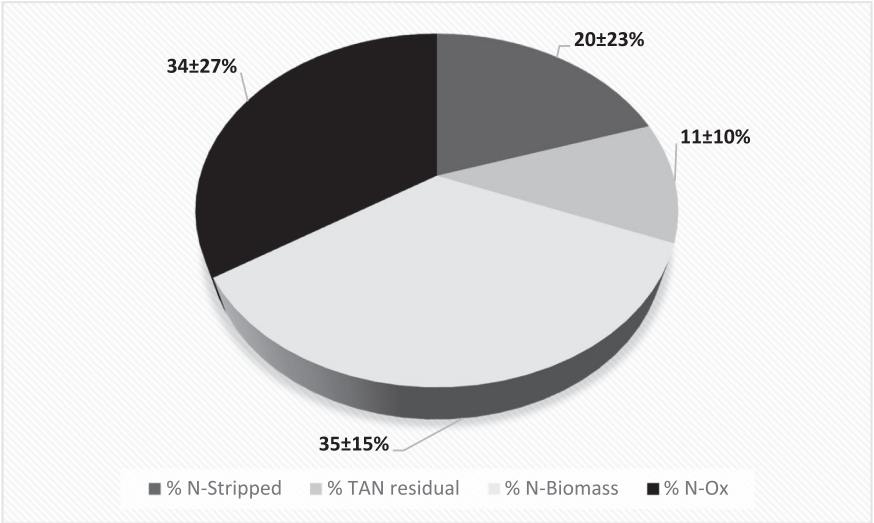


Fig. 2. Nitrogen apportioning in the effluent with respect to the influent.

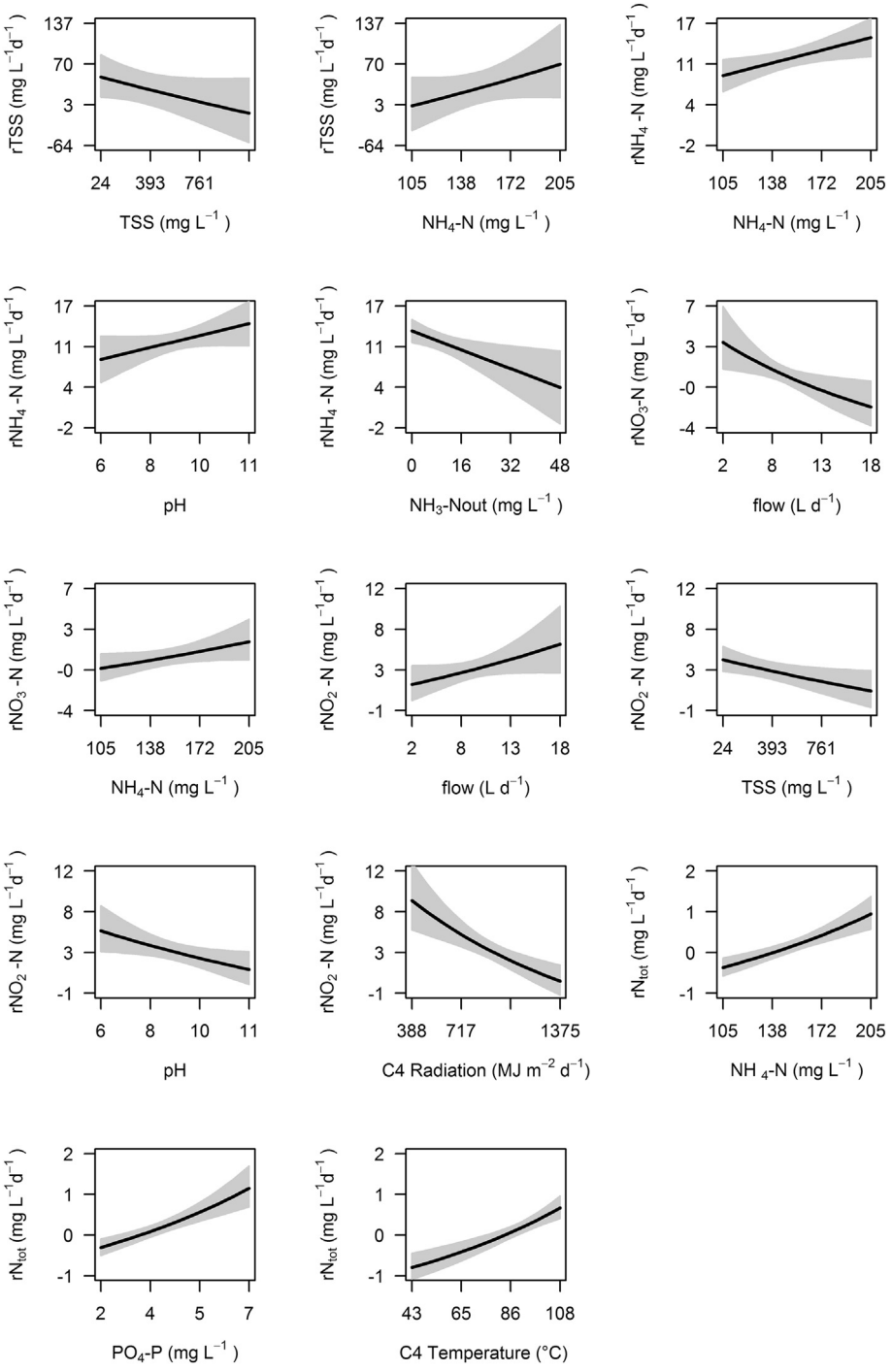


Fig. 3. Partial effects of the independent variables included in the GLMs on the daily production rate of biomass (TSS), NO_2^--N and NO_3^-N and on the daily removal rate of NH_4^+-N and total nitrogen. Lines represent the predicted values, and grey areas represent the 95th-percent confidence interval.

microalgae toward P assimilation, which can exceed their actual need when P is largely available (luxury uptake) and drop when its supply is limited [47] by changing their internal N:P quota [28]. Indeed, the N:P molar ratio of freshwater microalgae has been found to range between 8 and 45 [48]. It is therefore quite complex to identify the optimal N:P in the feed to ensure unlimited algal growth, which must be defined by determining all the relevant biochemical and physical/chemical processes.

Kruskal-Wallis one-way ANOVA for ranks highlighted significant differences (p-value < 0.05) between the two periods for the considered environmental parameters. As shown in Fig. 4 cumulative temperature (°C) and irradiance (W m^{-2}) and concentration of TSS (mg L^{-1}) and pH in the influent were lower in 2014 than in 2016, while the influent concentration of NH_4^+-N (mg L^{-1}) was higher. Fig. 4 shows no significant difference in biomass productivity (as TSS) and concentration of NH_3-N ($p = 0.21$, $p = 0.27$ and $p = 0.26$, respec-tively) between the two experimental periods. The higher NH_4^+-N concentration in 2014 (average = 271 ± 28 mg L^{-1}) was not toxic for microalgae but, on the contrary, allowed an even higher removal rate.

The comparable values of microalgal growth in the two periods show that, in the tested conditions, the variations of cumulative irra-diance and temperature had not significant effects.

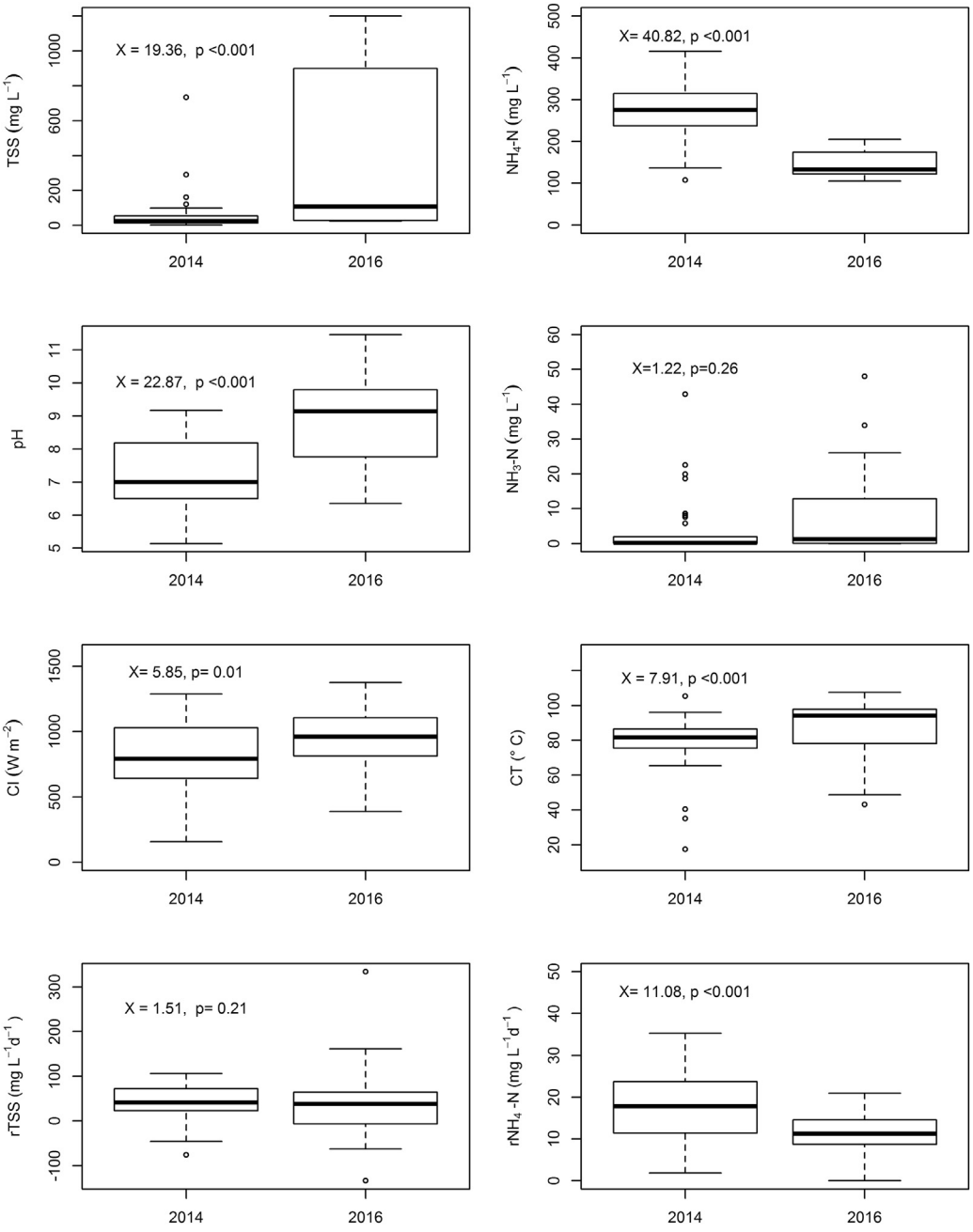


Fig. 4. Boxplot representing the inlet parameters (TSS, NH_4^+-N, pH), the environmental factors (Cumulative Irradiance and Temperature in the 4 days before sampling) and the process performances (biomass productivity, as rTSS, and NH_4^+-N removal rate, as rNH_4-N) in the two experimental periods. The calculated concentrations of NH_3-N in the effluent are also reported. In each boxplot χ^2 and p value are presented. Boxes show the range 25th–75th percentile. The horizontal line indicates the median value, n 2014 = 34, n 2016 = 36.

3.4. Characterization of microalgal biomass

Microalgae are able to accumulate heavy metals and accumulation seems to occur in the short term [49]. This ability can be exploited to remove those contaminants from the liquid phase, but could limit the range of alternatives for the valorization of the microalgal biomass. In view of the possible use of microalgal biomass on soils, e.g. for plant biostimulation, the concentrations of metals in the algal biomass was considered and compared with the limits set by the European Directive of 1986 for the agricultural use of sewage sludge, as no specific reference exists for algal biomass. As shown in Table 3, metal concentrations in the microalgal biomass (mg $kgTS^{-1}$) grown on centrate were always well below the limits (by more than one order of

Table 3
Comparison between metal concentrations in the microalgal biomass grown on centrate and the European limits for agricultural use of sewage sludge (CEC, 1986). $n = 4$.

Metal	Concentration in microalgal biomass (mg kg_{TS}^{-1}) Mean ± st. deviation	Limits (mg kg_{TS}^{-1})
Ni	58 ± 25	300
Cu	16 ± 10	1000
Zn	108 ± 14	2500
Cd	1 ± 0.6	20
Hg	0.7 ± 0.2	10
Pb	5 ± 4	750

magnitude), in agreement with the findings of Solè et al. [50] and, of course, with the low concentrations in the centrate.

In the perspective of agricultural use, the C, N and P content in the microalgal biomass was also determined at the end of the trial, taking into account the minimum thresholds set for C, N and P by the Italian guidelines (D.Lgs.99/1992) (no specific thresholds are reported in the European Directive). The measured values were 45%, 7.5%, and 1.1%on dry matter basis, respectively, in good agreement with literature data [51]. Such values comply with the minimum threshold for agri-cultural use of sewage sludge, where the minimum contents are 20%, 1.5%, and 0.4%, respectively.

3.5. Expected energy savings

In view of a full - scale application, a raceway pond can be assumed as a more cost-effective technical solution compared to the photobioreactor used during the presented experimental study. A calculation of the impact of such a side stream process on the energetic balance of the WWTP can be obtained by assuming to grow microalgae in a raceway pond and by making some further assumptions (see Appendix for details according to Metcalf & Eddy (2014) [52] and Williams & Laurens [53]), the main ones being: (i) to devote 0.5 m^2 $I.E.^{-1}$ (I.E. = inhabitant equivalent) to algae culturing; (ii) a realistic areal productivity of 10 g TSS m^{-2} d^{-1}; (iii) an oxygen production by microalgae of 1.57 g O_2 g TSS^{-1} which supports simultaneous nitrification. Under those assumptions, an energy saving of 11.3 Wh I.E. $^{-1}$ d^{-1} would be obtained due to the reduced oxygen demand for nitrification, since ammonia nitrogen in the centrate is returned to the water line as NOx. This energetic saving corresponds to ca. 11% of the average energy cost for the wastewater treatment at the Bresso WWTP (i.e. 100 Wh I.E. $^{-1}$ d^{-1}). This calculation has to be considered as approximate since it does not compute other operational cost/saving such as the energy request for biomass growth/harvesting, deriving from COD or P removal, the saving in external carbon supply related to the decreased carbon demand for denitrification due to the returning of nitrite instead of nitrate to the denitrification tank. In the conventional treatment scheme denitrification usually starts from NO_3-N, while starting from NO_2-N is not only possible but more convenient, involving a lower carbon request.

4. Conclusions

The applied culturing conditions (centrate as the feed and uncontrolled outdoor conditions) were proven suitable to grow microalgae. Complex interactions of different biological processes involving microalgae and bacteria (especially competition for nutrients and CO_2) and physical and chemical processes were highlighted.

The microalgal productivity in 2016 was comparable to the one reported in a similar study, which had been conducted in 2014, differing mainly by addition of phosphorus, suggesting that such an addition did not result in a relevant effect on microalgal growth. No other specific limiting factors emerged, but the possibility of improving the process performance by controlling pH appears as an interesting option. Keeping pH closer to neutrality would reduce NH_3-N production, thus decreasing stripping, and increase CO_2 availability.

Metal concentrations in the centrate did not limit algal growth and the resulting metal concentrations in algal biomass were acceptable in view of its agricultural use.

In a full scale plant the most relevant expected result would be the energy saving due to the decrease in the oxygen demand for nitrification in the water line, which would be season-dependent. More cost-effective technical solutions could be adopted such as the use of a raceway pond (instead of a bubble-column) where CO_2 supply and mixing would not be based on aeration but on more efficient systems, such as, for instance, off-gas bubbling. In that case, off-gas input could be regulated in order to control pH and would be an additional source of CO_2.

Author agreement

This manuscript describes original work and is not under consideration by any other journal. All authors approved this manuscript and this submission.

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Author contributions

FM collected the data, interpreted the results and drafted the article. MB and SR contributed in the experimental setup, in the implementation of the microalgal cultivation plant, managed and performed the laboratory analyses. RF contributed with data processing and statistics. VM and EF conceived the study and designed of the experimental plan. All authors approved the manuscript revision.

Conflict of interest statement

No potential financial or other interests could be perceived to influence the outcomes of the research.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

Appendix A

Table A1
Environmental and analytical data recorded during batch phase. T0 = Starting day, T55 = Last day of the batch phase.

Parameter	T_0	T_{55}	Average ± Standard deviation
NH_4^+-N (mg L^{-1})	280	34	
NO_3^--N (mg L^{-1})	5	14	
NO_2^--N (mg L^{-1})	0.3	16	
OD	0.063	1.9	
PO_4-P (mg L^{-1})	7	2	
TSS (mg L^{-1})	137	1033	
Temperature °C			15 ± 2
Irradiance (W m^{-2})			226 ± 85
pH			7.9 ± 1.2

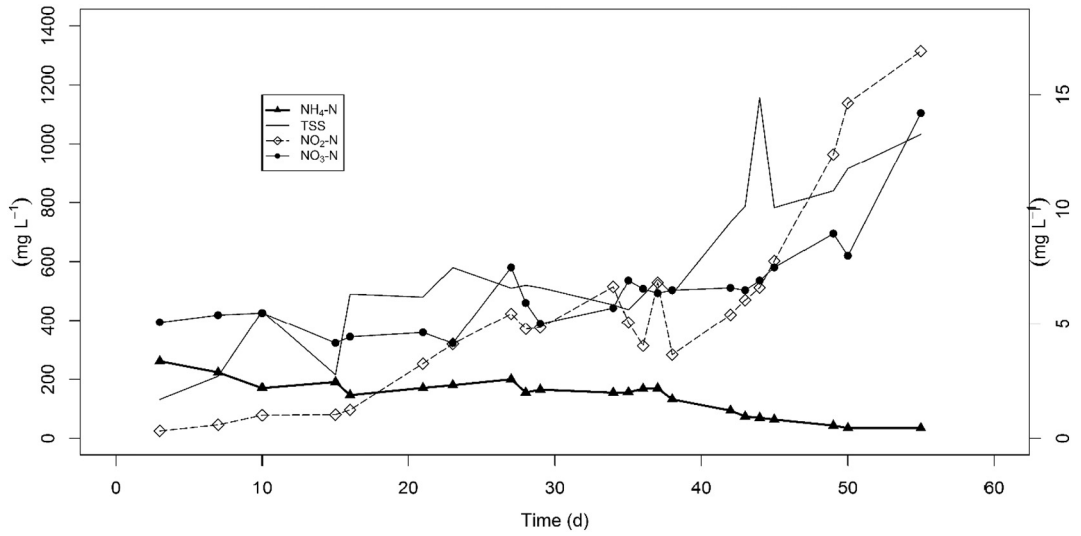

Fig. A1. Microalgal biomass TSS (mg L^{-1}), concentrations of NH_4^+-N, NO_2^--N and NO_3^--N (mg L^{-1}) in the column during the batch phase. TSS and NH_4-N data are in the primary Y axis, NO_2^--N and NO_3^--N in the secondary.

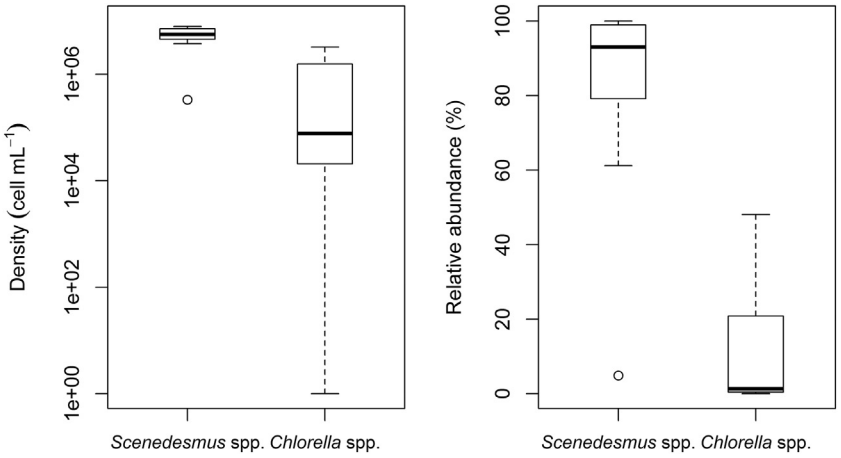

Fig. A2. Microalgal density of *Scenedesmus spp* and *Chlorella spp* (left), and respective relative abundance (right) (cells mL^{-1}), $n = 22$.

Table A2
Assumptions for energy saving calculations.

Parameter	Symbol	Value	UNIT	Reference
Available area for RW	A	0.5	$m^2\ ab^{-1}\ d^{-1}$	Working hypothesis
Areal productivity	Pa	10	$gSSm^{-2}\ d^{-1}$	Williams and Laurens, 2010
N content in algae	N_{SS}	0.1	$gN\ g_{SS}^{-1}$	
Oxygen production from algae	$O_{2\ SS}$	1.57	$gO_2\ g_{TSS}^{-1}$	
O_2 request for nitrification	$O_{2\ NIT}$	3.42	$gO_2\ g_N^{-1}$	Metcalf and Eddy, 2014
Energy request for nitrification	E_{NIT}	4.3	$Wh\ g_N^{-1}$	Metcalf and Eddy, 2014
Energy cost for WW treatment	E_{WWTP}	100	$Wh\ ab^{-1}\ d^{-1}$	Milano-Bresso WWTP data

Table A3
Energy saving calculations.

Parameter	Formula	Value	Unit
Specific algal production (SP)	A*Pa	5	$gTSS\ I.E.^{-1}\ d^{-1}$
N assimilated by algae (Nass)	$SP*N_{SS}$	0.35	$gN\ I.E.^{-1}\ d^{-1}$
O_2 produced by algae	$SP*O_{2\ SS}$	7.85	$gO_2 I.E.^{-1}\ d^{-1}$
N oxidized by photosynthetic aeration (N_{OX})	$SP*O_2\ SS/O_{2\ NIT}$	2.30	$gNox\ I.E.^{-1}\ d^{-1}$
Water line energy saving (E1)	$(N\ ox + N\ ass)*E_{NIT}$	11.3	$Wh\ I.E.^{-1}\ d^{-1}$

Table A4

Analysis of variance of the selected GLMs.

	GLM Family	Estimate	Std. Error	t value	P
ηNH_4^+-N					
Intercept	lognormal	0.158	0.148	1.063	0.297
NH_4-N_{in}		0.181	0.096	1.892	0.069
Residual deviance 1.306 on 27 degrees of freedom					
rNO_3					
Intercept	Gamma	0.391	0.144	2.717	0.011
Flow		0.219	0.104	2.104	0.045
Residual deviance 0.419 on 27 degrees of freedom					
rNO_2					
Intercept	Gamma	−0.066	0.104	−0.636	0.530
pH		0.216	0.052	4.118	< 0.001
IC		0.300	0.054	5.530	< 0.001
Residual deviance 0.312 on 26 degrees of freedom					
rSST					
Intercept	Gamma	0.565	0.080	7.095	< 0.001
ASS_{in}		−0.078	0.042	−1.839	0.077
pH		0.181	0.057	3.185	0.004
Residual deviance 0.288 on 26 degrees of freedom					
ηtotN					
Intercept	Gamma	1.764	0.129	13.712	< 0.001
NH_4-N_{in}		−0.152	0.047	−3.238	0.004
NO_3-N_{in}		−0.306	0.045	−6.729	< 0.001
SST_{in}		−0.168	0.049	−3.400	0.003
ASS_{in}		0.151	0.039	3.387	< 0.001
pH		−0.121	0.059	−2.056	0.052
NH_{3out}		0.210	0.053	3.394	< 0.001
IC		−0.223	0.054	−4.156	< 0.001
Residual deviance 0.089 on 21 degrees of freedom					

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