Research Article

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Disinfection and nutrient removal in laboratory-scale photobioreactors for wastewater tertiary treatment†

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Abstract

BACKGROUND: Secondary effluent wastewaters still contain resources including water and nutrients that must be safely reused and recovered. In this study, the combined role of microalgae as disinfectant and nutrient removal agent was evaluated for the potential application of a microalgae-based process as a cost-effective tertiary treatment. Nutrient removal, biomass productivity and disinfection performances were monitored in laboratory-scale photobioreactors (batch and continuous) fed on a secondary effluent mixed with a 10% on influent collected at a large municipal wastewater treatment plant where tertiary disinfection is performed by UV treatment.

RESULTS: In microalgae-based batch disinfection tests, *Escherichia coli* counts (0.5 ± 0.7 log CFU 100 mL^{-1}) were comparable to those after traditional UV process (0.7 ± 0.84 log CFU 100 mL^{-1}) and lower than in tests where light was applied without microalgae. In the following continuous test, *E. coli* counts were reduced by one order of magnitude and the pathogenic strain of *E. coli* O157:H7/H$^-$, *Salmonella* spp. and indicators such as *Bacteroides* spp. and *Enterococcus* spp. were never detectable in the effluents by molecular tools. Total nitrogen and phosphorus removals reached 93 and 100%, respectively, while the algal biomass productivity of the system averaged 50 ± 30 mg TSS L^{-1} day^{-1}.

CONCLUSIONS: The effluents of the photobioreactors reached quality standards appropriate for water reuse. Moreover, nutrients could be recovered through the generation of algal biomass suitable for further valorization.

Keywords: microalgae-based system; tertiary treatment; nutrient removal; disinfection

INTRODUCTION

Wastewater contains several resources, including water and nutrients, that must be reused and recovered to face the rapidly growing human population, resource depletion and the consequent need for a more sustainable society. Water reuse is a primary objective for arid and developed countries, even when freshwater is not yet considered a limiting resource, i.e. the USA and EU.[1,2] The water industry is therefore facing a drastic shift from systems implemented to remove pollution to those where various value-added products, including water itself, are recovered.[3–5] Nevertheless, the final discharge of wastewater treatment plants (WWTPs), as well as the reused water, must meet stringent water quality standards to preserve the environment and public health.[6] To comply with European legislation (2000/60/EC), especially for the vulnerable and sensitive zones defined in Nitrates Directive 91/676/EEC and Directive 91/271/EEC concerning urban wastewater treatment, additional treatments, also known as tertiary treatments, are often required for advanced nutrient removal/recovery and pathogen elimination.

Conventional tertiary treatments for nitrogen and phosphorus removal involve physicochemical steps such as coagulation, filtration and activated carbon adsorption of organics, while disinfection traditionally is based on chlorine, chlorine dioxide, ozone, peracetic acid, as well as UV radiation.[7] All these processes have the drawbacks of drastically increasing the operational costs of WWTPs and generating residues of chemical disinfectants that are eventually introduced into the environment. Natural and

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biological disinfectant technologies, such as solar radiation and constructed wetlands, are therefore encouraged. Among these, microalgae-based systems offer a valid and cost-effective solution. Microalgae assimilate inorganic nitrogen and phosphorus by growing only with light and CO_2, which can be provided at the WWTP by the off-gas from biogas valorization. Moreover, they produce oxygen and increase pH providing a disinfectant effect. Several goals can therefore be achieved simultaneously and, in addition, the microalgal biomass generated can be used for several purposes, including biofuels, fertilizers and biopolymers.

Energy and nutrient recoveries from different types of secondary treated wastewater using microalgae-based tertiary treatment have been already reported at laboratory and pilot scale. Complete nutrient removal and constant concentration of biomass (1.1 g TSS L^{-1}) could be achieved in a laboratory-scale photobioreactor for the cultivation of a microalgal consortium (mainly *Scenedesmus* spp.) in a mixture of digestate and secondary effluents.[8] Immobilized cells of *Nannochloropsis* sp. removed more than 90% of nitrogen and phosphorus, by producing 0.356 ± 0.097 g L^{-1} lipid, by growing in secondary effluent from palm oil mill in a laboratory-scale fluidized bed photobioreactor.[9] Carbon and nitrate removals, combined with biomass productivity, were observed using a microalgae-based system fed on treated pharmaceutical wastewater.[10]

In contrast, dedicated microalgae-based technologies for disinfection are scarcely tested. In high-rate ponds, Shelef *et al.*[11] have reported a reduction of 99% in total coliform counts, and similar results were also obtained by El Hamouri *et al.*[12] More recently, the disinfection efficiency, based on the counts of *Escherichia coli*, was evaluated in a high-rate algal pond fed on septic tank and facultative pond effluents, demonstrating mean log removal values of 1.75 and 2.75, respectively.[13] Nevertheless, the decay rate of *E. coli* in continuous growth photobioreactors is reported to be strictly related to the dilution rate of the photobioreactor,[14] suggesting that more research on the relationship between operational parameters and disinfection efficiency is also needed.

Most of the disinfectant effect of microalgae-based systems is provided by the tough conditions that the pathogenic bacteria face. Indeed, solar radiation is a potent disinfectant agent, while the photosynthetic activity of microalgae causes an increase in pH, which damages the DNA, and dissolved oxygen concentration, which would increase the effect of photo-oxidation.[15] Also, many algae, like *Chlorella* spp., are known to produce bacteriocins that inhibit bacterial growth.[16]

The scope of the study reported here was to assess the combined role of microalgae as disinfectant agent and in the nutrient removal process. To do this, a preliminary test to evaluate and compare *E. coli* removal efficiency in batch systems, kept under different conditions, was conducted. Then the removal of nitrogen and phosphorus, as well as different types of pathogen indicators, was monitored by standard methods and molecular tools in a laboratory-scale bubble column photobioreactor (PBR) fed continuously on real wastewater.

EXPERIMENTAL

Wastewaters

Samples of raw sewage (S) and secondary treated wastewater prior to the UV disinfection process (pre-UV) were collected from the municipal wastewater treatment plant in Milan-Bresso (Italy) serving 200 000 PE, hereafter named Bresso WWTP. The main physicochemical and microbiological parameters of the two wastewaters are summarized in Table 1.

Origin of inocula

The inocula used in each trial of the batch tests consisted of a microalgal–bacterial suspension developed in an outdoor Plexiglas bubble column (82 L of working volume, 29 cm in diameter) fed on the liquid fraction from digestate dewatering from Bresso WWTP. The microalgal community was composed mainly of *Chlorella* spp., *Scenedesmus* spp. and *Chlamydomonas* spp. The algal suspensions had optical density at 680 nm (OD_{680}) of between 0.6–1.4 and a pH equal to 7.74 ± 0.04.

The inoculum for the continuous test was collected from the above-mentioned column after one month. In this case, the microalgal community was dominated by *Chlorella* spp. The OD_{680} and pH were 0.71 and 7.4, respectively.

Batch tests

Batch tests were performed to assess the role of the microalgae in the removal of *E. coli*. In each trial four different conditions were tested in duplicate (eight in total) and each batch test was run for two days as reported in Table 2. The set-up consisted of 500 mL glass flasks (working volume of 200 mL), which were mechanically stirred and kept at room temperature. Each flask was filled with a fresh mixture of S (10% v/v) and pre-UV (90% v/v) in order to have 3×10^5 *E. coli* CFU 100 mL^{-1}; the main characteristics of the mixture are reported in Table 1. Two flasks (referred to as MA) were inoculated with a blandly centrifuged algal suspension ($OD_{680} = 0.7$) and illuminated artificially (Osram Fluora lamp 6x30W, PAR = 111 µmol m^{-2} s^{-1}) with dark/light 12/12 h cycle; two flasks (referred to as L) were filled with the same mixture of S and pre-UV without microalgae, and kept under the same illumination as MA to evaluate the sole effect of light on *E. coli* removal; two flasks (referred to as D) were prepared as for the L flasks but were kept in the dark, and were used as controls; finally, a further two flasks (referred to as ALK) were prepared and operated as L, but the pH was initially adjusted at 12 ± 0.3 in order to establish the disinfectant effect of the sole high pH. This pH was chosen to simulate the high pH (11.6) achieved in the preliminary batch test inoculated with the microalgae. Three repetitions were performed for D, L and MA, and one for ALK. During the batch test, the number of *E. coli* was monitored in each flask after 24 and 48 h.

Continuous test

Disinfection efficiency and nutrient removal was then evaluated in a continuous test using the influent mixture (IN_{mix} = S (10% v/v) + pre-UV (90% v/v); Table 1) as feed. This test was carried out in a Plexiglas bubble column reactor (height = 100 cm, 14 cm in diameter, working volume of 10 L), which was illuminated with four lamps (PAR *ca* 128 µmol m^{-2} s^{-1}) with a dark/light 12/12 h cycle. The system was constantly mixed by a mechanical stirrer at 200 rpm. A gas mixture, having a composition similar to the off-gas from the cogeneration unit in the WWTP (12% CO_2; 6% O_2; 82% N_2), was sparged from the bottom of the column for 15 min every 4 h to provide CO_2, as well as to control the pH. The column was inoculated with 1 L of algal suspension and 9 L of the feeding wastewater (IN_{mix}). The system was kept in batch for 3 days until the microalgal suspension reached an OD_{680} equal to 0.6. Then, the continuous mode was started by constantly pumping IN_{mix} into the column at fixed flow rate (1.43 L day^{-1}) to maintain a hydraulic retention time of 7 days. The storing tank

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Table 1. Main characteristics of wastewaters (mean ± standard deviation)

Parameter	Raw sewage	Pre-UV	IN_{mix} (batch test)	IN_{mix} (continuous test)
Chemical oxygen demand (mg L^{-1})	111	30.2 ± 13.8	17.7 ± 6	25.5 ± 16.7
pH	7.9 ± 0.9	7.8 ± 0.4	7.18 ± 0.52	7.96 (± 0.30)
NH_4^+-N (mg L^{-1})	22 ± 2.6	0.64 ± 1.42	3.49 ± 0.44	2.94 ± 1.43
NO_3^--N (mg L^{-1})	0.30 ± 0.42	8.1 ± 1.9	5.20 ± 1.63	8.2 ± 0.5
NO_2^--N (mg L^{-1})	<0.03	<0.03	<0.03	<0.03
PO_4^{3-}-P (mg L^{-1})	3.2 ± 1.3	0.57 ± 0.38	0.66 ± 0.21	0.5 ± 0.6
Turbidity (FAU)	184 ± 23	1.1 ± 2.5	2.83 ± 4	3.79 ± 1.61
E. coli (CFU 100 mL^{-1})	$4.7 \times 10^6 \pm 3 \times 10^6$	$1.56 \times 10^4 \pm 2.01 \times 10^4$	$3.12 \times 10^5 \pm 2.30 \times 10^5$	$4.30 \times 10^5 \pm 6.42 \times 10^5$

Table 2. Summary of main conditions and results of each type of batch test (mean ± standard deviation)

	Light (PAR, µmol m^{-2} s^{-1})	Microalgae (OD_{680})	pH 0 h	pH 24 h	pH 48 h	Specific decay rate (day^{-1})
D	–	–	7.6 ± 0	7.4 ± 0.1	7.5 ± 0.1	1.1 ± 0.4
L	111	–	7.7 ± 0.1	7.6 ± 0.2	8.5 ± 0.2	3.5 ± 1.1
MA	111	0.7	7.4 ± 0.33	11.5 ± 0	11.6 ± 0.1	4.7 ± 0.5
ALK	111	–	12 ± 0.3	11.6 ± 0.1	10.4 ± 0.3	5.6 ± 0.9

containing IN_{mix} was kept in the dark at room temperature. The PBR was operated for 23 days, a time corresponding to more than three hydraulic retention times, which is recommended to achieve a steady state in a CSTR. Every three days, a sample of the microalgal suspension and an aliquot of the IN_{mix} (300 mL) were collected to analyse the physicochemical parameters and to perform microbiological analyses by classical and molecular methods.

Analytical methods

The physicochemical parameters were measured in S and pre-UV every time they were collected at Bresso WWTP, and in the microalgal suspensions taken from the column reactor. Concentrations of ammonium (NH_4^+-N), nitrate (NO_3^--N), nitrite (NO_2^--N) and phosphorus (PO_4^{-3}-P) and chemical oxygen demand were determined on filtered samples (0.45 µm) by spectrophotometric test kits (Hach Lange DR6000TM spectrophotometer, Hach Lange LT200 dry thermostat). The total inorganic nitrogen (TN) concentration was calculated as the sum of the concentrations of ammonium nitrogen, nitrate nitrogen and nitrite nitrogen, while PO_4^{-3}-P was considered the sole inorganic P compound present in IN_{mix}. Total and volatile suspended solids (VSS and TSS) were determined in duplicate according to Standard Methods 2540.[17] Conductivity and pH were measured by a portable instrument (XS PC 510, Eutech Instruments, USA), while OD_{680} (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).

Microbiological characterization

Microalgal biomass was evaluated by cell counts and indirect measures (OD_{680} and TSS, VSS). Microalgae were counted using a haemocytometer (Marienfeld, Germany) and an optical microscope 40× (B350 Optika, Italy). *Scenedesmus*, *Chlorella* and *Chlamydomonas* algal cells were distinguished according to their morphological characteristics and counted, and the final estimated cell number was obtained from the mean of six square (1 mm^2) readings.

Optical density was assessed at 680 nm, i.e. around the second chlorophyll absorption peak, which is considered optimal to estimate microalgae levels.[18]

The enumeration of *E. coli* was carried out in duplicate by membrane filtration according to the Italian APAT-IRSA standard methods[19] that derive from the APHA methods.[17] Serial dilutions of each sample were made and filtered through nitrocellulose membranes of 0.45 µm pore size (47 mm in diameter; Whatman, Maidstone, UK). Membranes were then placed on chromogenic *E. coli* agar (EC X-GLUC AGAR, Biolife, London, UK) and incubated at 44 ± 1 °C. After 18–24 h the *E. coli* colonies were counted. The detection limit of *E. coli* counting by this method is one *E. coli* per sample volume or dilution tested (100 mL).

DNA was extracted from microalgal suspension collected from the continuous flow column and from the water sample of the IN_{mix} according to the method reported by Ahmed *et al.*[20] with minor modifications. A known volume of sample (100 mL) was filtered through Nuclepore polycarbonate membranes of 0.45 µm pore size (47 mm in diameter; Whatman, UK). Filters were then aseptically transferred in 2 mL sterile tubes and stored at −20 °C. Half filter of each sample was then placed in the beads tube provided by the Powersoil DNA isolation kit (Qiagen, Italy) and vigorously shaken for 10 min in a mini bead beater (Biospec Bartlesville, OK, USA). The resulting slurry was then centrifuged at 13 000×*g* for 3 min and the DNA was extracted from the supernatant according to the manufacturer's manual.

Qualitative end-point PCR was used to detect *Salmonella* spp. and *E. coli* O157:H7/H^-. For *Salmonella* spp., PCR was conducted with OriC designed primer for *Salmonella* spp.[21] The pathogenic strain of *E. coli* was detected by PCR with primers designed by Jinneman *et al.*[22] In both cases DreamTaq DNA polymerase (Thermo Fisher Scientific, USA) was used as follows: 25 µL final volume, 1× DreamTaq buffer, 0.3 µmol L^{-1} of each primer, 200 µmol L^{-1} of each dNTP, 1.2 U of Taq polymerase, 3.0 µL of template DNA. PCR products were checked by electrophoresis on 1.5% agarose gel stained with 0.5 µg mL^{-1} ethidium bromide. Both methods used had a limit of detection of 2 log cell eq. 100 mL^{-1}, as stated in laboratory trials conducted in previous works with artificially inoculated water

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samples with target pathogens *Salmonella enterica* CECT 409 and *E. coli* O157:H7 CECT 4267.[23,24]

Quantitative real-time PCR (qPCR) was conducted on total bacteria, *Enterobacter* spp. and *Bacteroides* spp. Total bacterial 16S rDNA gene were determined by qPCR.[25] Bacterial synthetic 16S rRNA derived from *Pseudomonas putida* ATCC 12633 Genebank sequence (450 bp) was supplied by Geneart service (Thermo Fisher, Italy) embedded in a construct vector. The constructs were linearized and standard DNA stock solutions of 10^9 copies of plasmid per microlitre were prepared. The qPCR reaction was conducted with 2.0 µL of template DNA using an ABI 7300 real-time PCR system. The standard curve was generated by tenfold dilution of standard plasmid up to 10^2 copies per microlitre. The final amplification efficiency was 95% and R^2 was 0.998. Powerup SYBR Green master mix (Applied Biosystems, Italy) was used in 25 µL final volume reactions, 3 µL of template DNA, 0.3 µmol L^{-1} of each primer, and run on an ABI 7300 real-time PCR system, as above. Melting curve analysis was performed at the end of PCR cycles. *Bacteroides* spp. and *Enterococcus* spp. were assessed by qPCR by the methods previously described.[26,27] In both cases, the protocol was the same as for universal bacterial method, with different thermal programme (annealing temperatures of 60 and 57 °C for *Bacteroides* and *Enterococcus*, respectively) and different efficiency and R^2 (105% and 0.999 for *Bacteroides* and 109% and 0.995 for *Enterococcus*, respectively) were obtained. Quantitative data were converted to cell equivalent mL^{-1} water on the basis of the number of operons of 16S gene of bacterial domain, *Bacteroides* spp. and *Enterococcus* spp. (4.3, 5.5 and 4.0, respectively) as reported on the *rrn* database.[28]

Data processing

During the batch test the net specific growth rate (μ, in day^{-1}) of the microalgae in MA flasks was determined as the slope of the line fitting OD_{680} data in a semi-log plan:

$$x = t, \quad y = \ln\left(\frac{OD_{680,t}}{OD_{680,t0}}\right)$$

where $OD_{680,t}$ and $OD_{680,t0}$ are the OD_{680} values measured at time t and at the beginning of the test, respectively.

The decay rate of *E. coli* (K_d) was calculated as the slope of the line fitting cell counts in a semi-log plan:

$$x = t, \quad y = \ln\left(\frac{N_{t0}}{N_t}\right)$$

where N_t and N_{t0} are the *E. coli* counts per 100 mL measured at time t and at the beginning of the test, respectively.[29]

The removal efficiencies were expressed in terms of log units removed (LUR) according to the following equation:

$$\mathrm{LUR} = \log\left(\frac{c_f}{c_i}\right)$$

where c_f and c_i are the concentrations of the considered microbial indicator after and before the disinfection treatment.[30]

During the continuous test, mass balances were set by assuming the column as a CSTR in order to compute the removal rate of the total nitrogen (rTN) as a sum of ammonium (rNH_4^+-N), nitrite (rNO_2^--N) and nitrate (rNO_3^--N), as well as the production rate of microalgal biomass (as TSS, rTSS). As an example, the TSS production rate (rTSS) was calculated using the following equation:

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

where $[TSS]_{t_i}$ and $[TSS]_{t_{i+1}}$ are the TSS concentrations measured at time t_i and t_{i+1} in the column; $[TSS]_{IN}$ is the TSS concentration in the IN_{mix}; Δt is the sampling interval; and HRT is the hydraulic retention time.

Statistical analyses

Analysis of variance (ANOVA) was performed to determine whether the differences in the disinfection observed in the batch tests were significant. Simple linear regressions were computed to understand the correlation between the number of algal cells and the optical density at 680 nm, as well as the relationship between the microalgal density and the LUR. All statistical analyses were performed with the function Data Analysis Tools in Excel 2016.

RESULTS

Batch tests

The results of the batch tests are reported in Table 2 and shown in Figs 1 and 2. These data suggest that the removal of *E. coli* over time was highly dependent on the operational conditions. In MA, the presence of active microalgae ($\mu_{max} = 0.2 \pm 0.1$ day^{-1}) did increase the decay rate of *E. coli*, which was four times higher than in the control ($P < 0.001$, ANOVA) (Table 2). In ALK test, the maximum removal was observed after 24 h (1.18 ± 0.00 *E. coli* log CFU 100 mL^{-1}) and remained stable until the end of the test ($P = 0.19$, ANOVA). After 24 h, more than 3.2 ± 0.6 and 4.1 ± 0.2 LUR could be removed in MA and ALK, respectively, and the counts of *E. coli* were similar to the ones of the final effluent of the WWTP (after the UV disinfection process) (0.7 ± 0.84 log CFU 100 mL^{-1}).

The pH of D reactors did not change over time, while higher values could be observed in L after 48 h. The phototrophic growth of the microalgae increased the pH to 11.6, which was similar to the pH of the tests after 24 h ($P > 0.40$, ANOVA). The highest decay of *E. coli* occurred at very alkaline pH (ALK). Such condition is not easily achieved in tertiary treatment unless chemicals are added. However, photosynthetic activity (MA) increased pH not far from that measured in ALK, which is likely to play an important role in the observed disinfection, masking the potential contribution of bactericide algal metabolites. On the other hand, light alone (L) also increased LUR with respect to the dark condition (D).

Continuous test

Nutrient removal and microalgal productivity

In spite of the normal variations, occurring particularly in the first days of the test, as shown in Fig. 3, the TN removal reached 93%. As for TP, the concentration in the IN_{mix} was below 1 mg L^{-1} for most of the experimentation and the averaged concentration in the effluent was 0.08 ± 0.17 mg L^{-1}, corresponding to an average removal efficiency of 99 ± 1%.

The biomass concentration in the column and the productivity of the system averaged 451 ± 57 mg TSS L^{-1} and 50 ± 30 mg TSS L^{-1} day^{-1} (VSS/TSS = 77 ± 13%), respectively. The algal density was not constant over time as shown in Fig. 4. The total algal number ranged between 5.4×10^6 and 2×10^7; *Chlorella*

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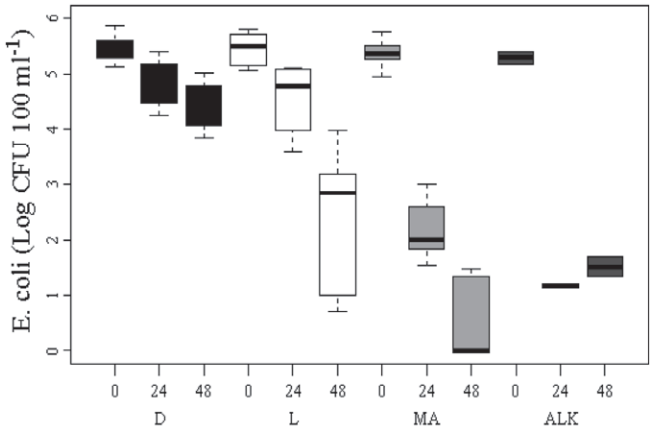

Figure 1. Counts of *E. coli* detected in the different batch tests at time 0 and after 24 and 48 h. D (black blocked boxes) are the tests kept in the dark without the microalgae, L (white blocked boxes) are the tests kept illuminated without the microalgae, MA (light grey blocked boxes) are the tests which were inoculated with microalgae and kept under light, ALK (dark grey blocked boxes) are the tests in which the pH was adjusted to 12 and kept under light.

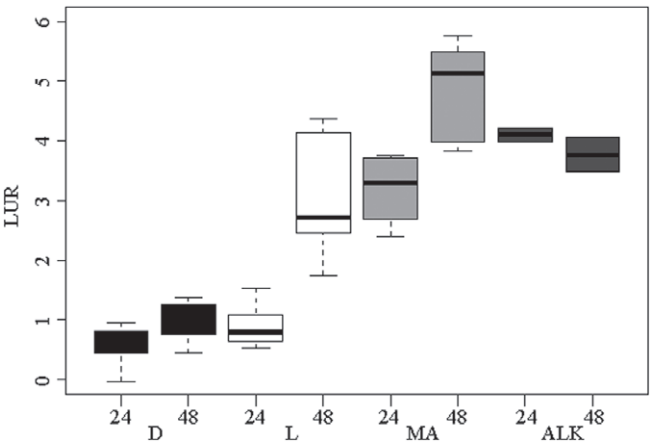

Figure 2. Logarithmic unit removal (LUR) of *E. coli* in the batch tests after 24 and 48 h. D (black blocked boxes) are the tests kept in the dark without the microalgae, L (white blocked boxes) are the tests kept illuminated without the microalgae, MA (light grey blocked boxes) are the tests which were inoculated with microalgae and kept under light, ALK (dark grey blocked boxes) are the tests in which the pH was adjusted to 12 and kept under light.

spp. remained the dominant species in the system, but an increase of *Scenedesmus* spp. count could be observed at the end of the test (up to 30% of the total community). *Chlamydomonas* spp. could be observed sporadically in the system, accounting for less than 0.4% of the total community. The trend of the microalgal counts agreed with the observed OD values ($OD_{680} = 4 \times 10^{-8}$ algal cell $mL^{-1} + 0.31$, $R^2 = 0.7996$, $P = 0.04$).

The decay of microalgal biomass after 7 days was probably due to nutrient shortage, especially of phosphorus, the concentration of which in the influent was barely detectable. As regards N, the microalgae, which were inoculated in the PBR, were previously grown on digestate, where the main N form was the ammoniacal nitrogen, while in the IN_{mix} the majority of N was in the form of nitrate. It is plausible that during the first 7 days, the microalgae were not so efficient in assimilating nitrate (nitrate removal rate of 0.7 mg L^{-1} day^{-1}), so they used for survival and growth their

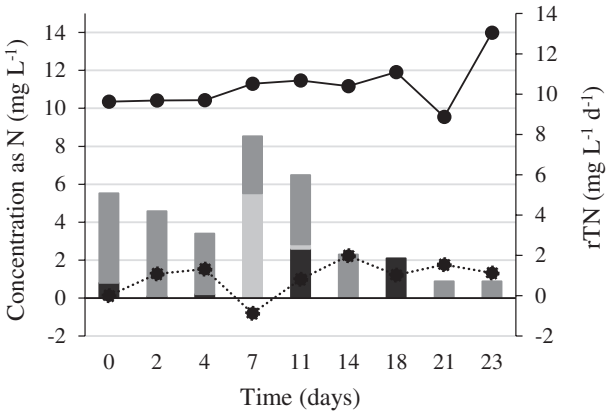

Figure 3. Total inorganic nitrogen (TN) concentration in the influent (solid line), N compounds in the effluent (bars, NH_4^+-N in black, NO_2^--N in light grey and NO_3^--N in dark grey) and nitrogen removal rate (rTN; dashed line) detected in the PBR over time.

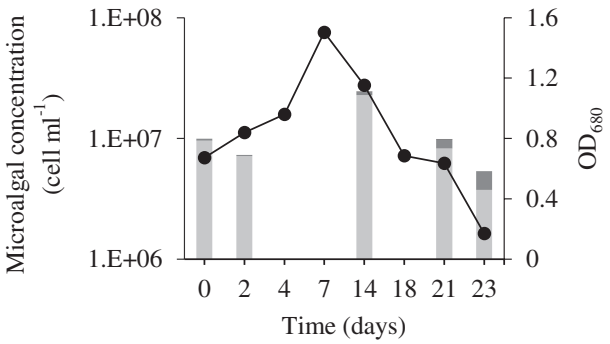

Figure 4. Distribution of the main algal species (*Chlorella* spp. in light grey, *Scenedesmus* spp. in dark grey and *Chlamydomonas* spp. in black) and algal density expressed at OD_{680} (solid line) detected over time in the PBR. Data of microalgal number at days 4, 7 and 18 are not available.

internal source of N and the low content of NH_4^+-N available (ammonia was not detected in the effluent until day 11), reaching a biomass productivity of about 50 mg TSS L^{-1} day^{-1}. Given that, it might be possible that, after day 11, the algal biomass adapted to assimilate more nitrate (nitrate removal rate *ca* 0.9 mg L^{-1} day^{-1}), as well as NH_4^+-N, with the result of higher N removal. However, the nitrogen loading rate of the PBR (1.4 mg L^{-1} day^{-1}) was not high enough to sustain a stable algal growth of such density, which would have required *ca* 3.5 mg N L^{-1} day^{-1} (if considering the cell being composed of 7% of N), so it is also likely that when the internal N of microalgae was depleted the algal biomass decayed. In addition, the dead microalgal cells release organic matter, N and P. As no oxygen limitation occurred, due to photosynthetic activity, the oxidation of the ammonium to nitrate by nitrifying bacteria was probably favoured, as well as the growth of heterotrophic bacteria, which could contribute to nutrient removal by uptake.

Disinfection

A reduction of more than one order of magnitude of *E.coli* count could be observed until day 14, though the number of faecal indicators was below the levels recommended by the World Health Organization[6] only during the first 10 days of the trial (Fig. 5). The removal efficiency was correlated to the optical density of the microalgae in the PBR ($y = 0.32x + 0.46$, $R^2 = 0.54$, $P = 0.038$), suggesting an active role of the microalage in the inactivation

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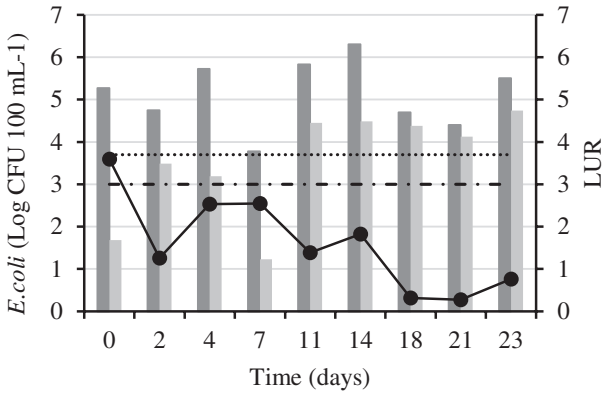

Figure 5. Number of *E. coli* in the influent (bars, dark grey) and in the effluent of the PBR (bars, light grey) counted over time and the corresponding logarithmic unit removal (LUR, black marked solid line). The two lines represent the set limits for the discharge in water bodies located in sensitive areas (dotted line) and for agriculture reuse (dashed line).

of faecal indicators, probably due to the concurrent effect of microalgae and light. In the continuous test, the CO_2 supply was sufficient to keep the pH below 8 most of the time (7.9 ± 1.8), thus reducing the disinfecting effect observed in the batch tests as a consequence of the strongly alkaline pH. Better disinfection performances could have been likely achieved by letting the pH rise to higher values as during the above-described batch tests and as reported in a similar study, which was conducted in a continuous flow photobioreactor where the pH was not controlled (*E. coli* LUR of 2–4).

Concerning pathogenic bacteria, the PCR analyses revealed that in the IN_{mix} *E. coli* O157:H7/H$^-$ was sporadically present (five samples resulting positive out of seven), while it was never detected in the column samples. *Salmonella* spp. were never detected by the molecular method applied in any analysed sample. Quantitative PCR results showed that the removal efficiency of the faecal indicators of *Bacteroides* spp. and *Enterococcus* spp. was high throughout the experiment (Fig. 6). Variable level of both indicators could be detected only in the IN_{mix} samples, while all effluent samples had a non-detectable level of contamination. The number of total bacteria in the IN_{mix} was quite high (7.1 ± 0.33 log cell mL^{-1}), and it was found to be reduced by about 1 log in the PBR effluent (0.93 ± 0.34) (Fig. 7).

DISCUSSION

In this study, the potential use of microalgae-based systems as tertiary treatment for the concurrent removal of nutrients and pathogens was evaluated. In the presence of active microalgae the effluents of both batch and continuous growth tests were characterized by values of chemical and microbiological parameters suitable for discharge in sensitive areas, and, in some cases, also for reuse in agriculture (European Directive 91/271/CE and 2000/60/CE).[6] The implementation of a microalgal process in existing WWTP seems therefore to be a promising alternative to traditional physicochemical treatment, with the great advantage of combining disinfection and nutrient removal. The obtained results must be evaluated even more positively considering that the experimental tests were carried out on a mixture of influent and effluent, having thus higher bacterial counts (of about an order of magnitude) and nutrient concentrations with respect to usual secondary effluents. However, it must be acknowledged that the microalgae require temperate climate, and algal ponds have a large footprint. Moreover, the *E. coli* decay rate is much lower than the UV disinfection, leading to much longer contact times.

The microalgae activity seemed to play a key role in the disinfection process. Usually the disinfectant effect is reported to be due to the harsh environment created by the photosynthetic organisms for the survival of pathogens and to photo-oxidation.[15] Indeed, the results of the batch tests demonstrated that the strongly alkaline pH values and light exposure favoured the removal of *E. coli* as shown by the higher decay rates. This is not surprising as the survival of *E. coli* at pH higher than 8.5 is strongly inhibited.[31,32] However, only in flasks where the microalgae were actively growing were the counts of *E. coli* comparable to the one in the final effluent of the WWTP (after UV disinfection), suggesting that the presence of the microalgae enhanced the disinfection efficiency. In these tests, the synergetic effect of high pH and dissolved oxygen concentration probably enhanced the photo-oxidation process as previously suggested.[15] In the continuous growth test a positive correlation was observed between the *E. coli* LUR and algal density similarly to other studies conducted in eutrophic lake and in a continuous flow PBR fed on effluent from an up-flow anaerobic sludge blanket reactor and operated with different dilution rates.[14,29] As the pH of the PBR was controlled by CO_2 sparging, it could not contribute to the bacterial abatement, while the increase of dissolved oxygen concentration (up to 20 mg L^{-1}) and the probable release of secondary metabolites such as such as fatty acids terpenoids, carbohydrates, peptides, polysaccharides and alkaloids by the microalgae might have an effect on the inactivation of *E. coli*.[16,33,34] Even though these antimicrobial compounds were not analysed in the study, *Chlorella* sp. and *Scenedesmus* sp., the dominant species detected in the PBR, are known to have potent antibacterial activity against both Gram-positive and Gram-negative bacteria.[33,35]

The disinfection efficiency of the microalgae-based process was also confirmed by the PCR-based analyses, which demonstrated that the numbers of all tested pathogenic and faecal indicator strains were below the detection limit (2 log cell eq. 100 mL^{-1}) in the samples collected from the microalgal suspensions. However, it should be noted that the presence of the pathogens in the influent was rare too. Molecular techniques are quick and more sensitive but not recognized as standard methods for testing disinfection efficacy, since they cannot distinguish between live and dead cells, so the positive results must be taken with caution. On the other hand, PCR-based methods offer a fast and sensitive tool to assess bacterial pathogens whose isolation and cultivation are time-consuming, and also can consider sub-lethally injured target cells so as viable but not culturable (VBNC) in which many pathogenic bacteria can enter by counteracting harsh environment derived by environmental factors, including water treatments.[36] The sporadic presence of pathogen-positive samples from PCR analyses in the influent did not allow an adequate evaluation of the pathogen removal potential, though the absence of positive signals in the effluents is encouraging.

On the other hand, the quantification of the faecal indicators by qPCR confirmed the disinfection efficiency of the photobioreactor observed by traditional plate. Indeed, the variable quantity of both *Bacteroides* spp. and *Enterobacter* spp. in the influent (ranging from 2 to 4 og cell eq mL^{-1} and from 1.4 to 2 log cell eq mL^{-1}, respectively) was reduced to below the level of detection of the method (1 log cell eq mL^{-1}) in all effluent samples. The total number of bacteria in the influent was quite constant and only 1 log reduction could be observed in the system. This is not surprising as the quantitative methods applied can detect also photosynthetic bacteria, like cyanobacteria and *Chloroflexi*, commonly coexisting

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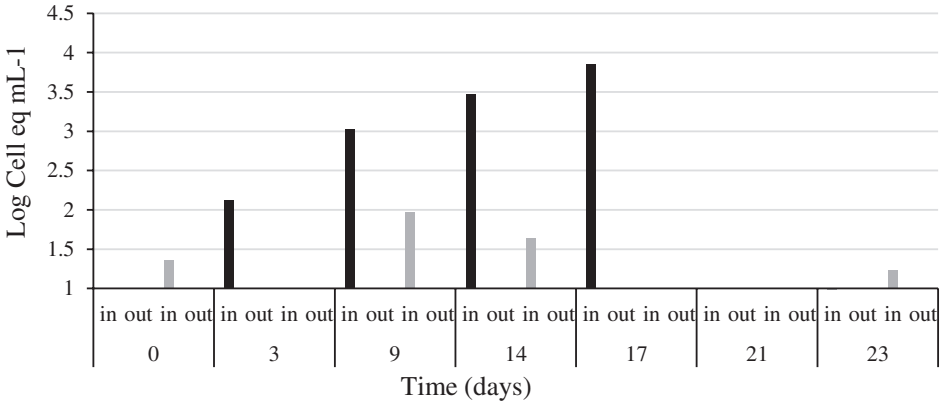

Figure 6. Concentration of *Bacteroides* spp. (black bars) and *Enterococcus* spp. (grey bars) detected in the influent and in the effluent of the PBR over time.

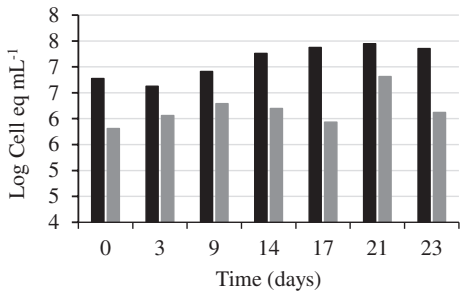

Figure 7. Concentration of total bacteria in the influent (black bars) and in the effluent (grey bars) of the PBR detected over time.

with the microalgae in these types of system.[37] However, more analyses addressing the bacterial composition, and its dynamics, should be performed to confirm this hypothesis.

The effluent of the PBR was safe to be discharged and/or reused, but also residual nutrients could be recovered from the generated biomass. Although the microalgal growth seemed to be inhibited or limited in the batch tests as demonstrated by the low specific growth rate observed, the biomass productivity (about 50 mg TSS L^{-1} day^{-1}) in the PBR was in line with data reported in similar studies.[8,13,38] However, the microalgal N content (dry weight basis) inferred by the biomass nitrogen assimilation rate was lower (1.5%) than that commonly reported (3–12%).[39] This might be due to the low nutrient load to the PBR as demonstrated by a similar study assessing the correlation between the N load and N biomass content of a microalgal biofilm fed on a synthetic municipal wastewater effluent containing 10 mg L^{-1} NO_3-N and 1.1 mg L^{-1} PO_4-P.[40] Most likely the low concentration of nutrients in the IN_{mix}, i.e. total inorganic P in the influent, was below the detection limit for several days, and the unbalanced supply of N and P did affect the microalgal metabolisms. Indeed, nutrient starvation conditions reduce the cell growth and redirect the carbon fixed through photosynthesis to the synthesis of carbohydrates or lipids. Although the concentration of lipid in the microalgae was not measured, it might be possible that the biomass produced in this study could be suitable for the generation of bioenergy (i.e. biodiesel and biogas). However, PBR configurations featuring a solid retention system and allowing operation at solid retention time higher than the hydraulic retention time, such as sequencing batch reactors, membrane reactors or biofilm systems, are recommended to enhance the volumetric nutrient removal efficiency and the specific biomass productivity.

CONCLUSIONS

The potential use of microalgae-based processes as a cheap tertiary treatment for simultaneous pathogen removal and nutrient recovery was successfully demonstrated. The disinfection efficiency of the systems, which was demonstrated by both classical and molecular methods, was strictly related to the photosynthetic activity of the microalgae. Nevertheless, the low and unbalanced nutrient content of the secondary effluent used in the study was not always enough to sustain an intense microalgal growth, though it might be possible that nitrogen starvation induced the accumulation of lipids in the algal biomass. Therefore, more studies are needed to understand which are the operational parameters and reactor configuration that could improve the reliability of this promising technology.

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