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Removal of pharmaceutical compounds from the liquid phase of anaerobic sludge in a pilot-scale high-rate algae-bacteria pond

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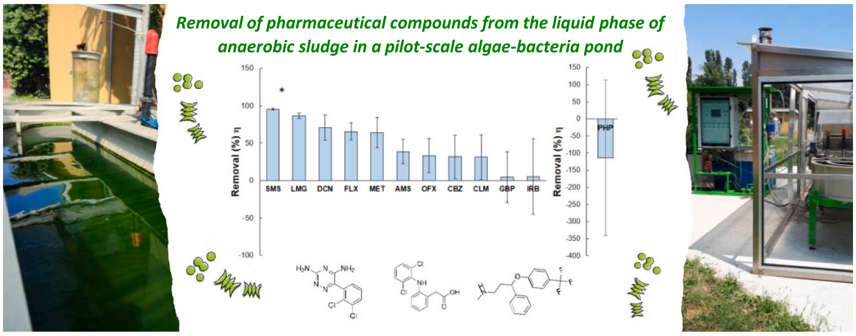
HIGHLIGHTS

- Microalgae-bacteria removed some PhACs from the liquid phase of anaerobic sludge.
- Lamotrigine and Diclofenac removal efficiencies were over 70 %.
- Fluoxetine and Metoprolol removal efficiencies were over 60 %.
- Propyphenazone and Irbesartan were not removed.
- Microalgae-bacteria synergy and global radiation contributed to the overall removal.

GRAPHICAL ABSTRACT


Removal of pharmaceutical compounds from the liquid phase of anaerobic sludge in a pilot-scale algae-bacteria pond

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ABSTRACT

This study evaluates the effectiveness of a pilot-scale high-rate algae-bacteria pond (HRAP) to remove pharmaceutical compounds (PhACs) from municipal centrate. The studied PhACs belonged to different classes of synthetic active compounds: antihypertensives, antiepileptics, antidepressants, neuroprotectors, and anti-inflammatory drugs. The HRAP, growing a mixed microalgal consortium made of *Chlorella* spp. and *Scenedesmus* spp., was operated in continuous mode (6 days hydraulic retention time) from May to November 2021. Removal efficiencies were high (>85 %) for Sulfamethoxazole and Lamotrigine, promising (65–70 %) for Metoprolol, Fluoxetine, and Diclofenac but low (30–40 %) for Amisulpride, Ofloxacin, Carbamazepine, and Clarithromycin. Propyphenazone and Irbesartan were not removed, and their concentrations increased after the treatment. The combination of abiotic and biotic drivers (mostly global radiation and the synergy between microalgae and bacteria metabolisms) fostered photo and biodegradation processes. Overall, results suggest that microalgae-based systems can be a valuable solution to remove PhACs from wastewater.

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1. Introduction

Pharmaceutical compounds (PhACs) are a heterogeneous group of substances whose production and continuous use by humankind has caused their spread and presence in all the environmental compartments. Since they are synthesized to ensure effectiveness and resistance before being metabolized in the organism, they are characterized by a recalcitrant chemical structure. Nowadays, with increasing dependence on PhACs used to cure or alleviate several types of diseases, their resistant nature is a crucial problem as they have been detected in trace concentration (from ng·L^{-1} to μg·L^{-1}) even in remote areas of the world (Couto et al., 2019).

The effectiveness of conventional wastewater treatments in removing these substances is known to be limited, variable, and often unreliable since wastewater treatment plants (WWTPs) were not originally designed for this goal (Gusmaroli et al., 2020). Advanced chemical oxidation processes and adsorption on activated carbon are considered as the best available technologies for the removal of pharmaceuticals from wastewater on the basis of both pilot and full scale studies (Eniola et al., 2022). However, operational costs still remain high in comparison to removal efficiencies, which strongly depend on the type of PhACs (Eniola et al., 2022). That is why the selection and optimization of alternative treatments based on biological processes to remove pharmaceutical compounds is still a field of great significance in the scientific community (Rizzo et al., 2019).

The interest in microalgae for the remediation of PhACs from wastewater has grown in the last two decades and it still represents a trend in water research (Sutherland and Ralph, 2019). Microalgae-based processes are efficient in nutrient removal by assimilation; moreover, the microalgal biomass can accumulate many organic and inorganic pollutants, thus removing them from the liquid phase (Xiong et al., 2018). In many cases, the degradation of organic molecules also occurs, due to the synergy of different factors in algal cultivation systems. Some evidence already exists confirming the potential of microalgae-based systems to remove PhACs, but they mostly derive from lab-scale experiments (Sutherland and Ralph, 2019) and, due to the complexity of the subject, more data are needed to assess the full-scale feasibility of the process. These cost-effective systems are designed to maximize light exposure. Usually, they are built outdoors to exploit sunlight, promoting algal biomass growth and facilitating the photodegradation of PhACs (Matamoros et al., 2015). Different organic compounds, such as PhACs, can undergo sorption mechanisms as the cell wall components of microalgae can bind them. Further, the oxygen released by photosynthesis increases the redox potential, promoting oxidation, and is available for aerobic bacteria to perform degradation processes. Precisely, the combination of different biotic and abiotic drivers coexisting in HRAPs makes this treatment interesting and potentially effective (Jiménez-Bambague et al., 2020).

The present paper aims to evaluate the effectiveness of a pilot-scale HRAP operated in continuous mode to remove different PhACs from municipal centrate. In the climatic conditions of Northern Italy, where the research has been carried out, microalgae-based systems cannot be adopted as main biological treatments for wastewater, due to light and temperature constraints. So, the process has been adopted as a side-stream process to provide both an option to decrease the environmental footprint of the WWTP during nitrogen remediation and a new technology for removing PhACs. The obtained results have been contextualized and compared with data from similar applications, while the effect of both abiotic and biotic factors has been statistically analyzed to understand the possible mechanisms leading to the removal of the targeted compounds.

2. Material and methods

2.1. Wastewater treatment plant and wastewater characteristics

2.1.1. Wastewater treatment plant

The Bresso-Niguarda WWTP, located in the suburbs of Milan (Italy) hosted this experiment. As previously described in Mantovani et al. (2020) and Rossi et al. (2023) the plant, serves 220,000 population equivalents (P.E.), and is organized in conventional water and sludge lines. The influent wastewater is subjected to mechanical treatments, primary settling, and secondary treatment by activated sludge. Filtration serves as a tertiary treatment and is followed by UV disinfection. Anaerobic digesters (operating under mesophilic conditions at 35 °C with 25 days HRT) are fed on the sludge from the primary and secondary settling to produce biogas. The resulting digestate is processed by post-thickening and solid/liquid separation by centrifugation. The centrifugation performance is fostered by the addition of cationic polyelectrolytes, allowing for a better separation of the solid fraction from the supernatant (referred to as centrate). The produced biogas from anaerobic digestion is upgraded to biomethane by a hollow-fiber membrane filtration.

2.1.2. Characteristics of the liquid phase of anaerobic sludge

As described elsewhere (Mantovani et al., 2020; Rossi et al., 2023) the centrate is used to feed a HRAP to test and validate the efficiency of a side-stream algae/bacteria treatment for nutrient removal. The relatively low Total Ammoniacal Nitrogen (TAN) concentrations allow microalgae to grow without inhibition even if the N/P value does not respect the Redfield molar ratio. Moreover, the low turbidity and total solid concentration favor light penetration making the centrate suitable for microalgal-based applications (more detail in Table 1S in the Supplementary material). As indicated by Rossi et al. (2023), the centrate is supplemented with sodium bicarbonate to counterbalance the low total alkalinity and inorganic carbon content, thus decreasing the competition between microalgae and nitrifying bacteria for inorganic carbon. The presence of pharmaceuticals in the centrate is influenced by the efficiency of the upstream treatment units in the WWTP. The primary and secondary sludge, generated during wastewater purification, as well as the excess sludge from the activated sludge treatment, contribute to the digester's feed. Consequently, the presence of pharmaceuticals in the digestate depends on the effectiveness of sorption onto sludge flocs and biodegradation. Furthermore, some PhACs can undergo biotransformation or partial degradation during anaerobic digestion (Azizan et al., 2021). Those processes are usually linked to co-metabolism with other substrates (present in higher concentrations) that can sustain the growth of bacteria. Finally, the characteristics of the residual individual contaminants, including polarity and hydrophobicity, influence their distribution between the solid phase of the sludge itself and the centrate (Genz and Reemtsma, 2022).

2.2. Microalgae cultivation

Outdoor pilot-scale microalgal cultivation was carried out in an 870 L HRAP for eight months (from May to November 2021) as further

Table 1
List of the independent variables included in the Full GLMs and their variability during the sampling time window.

Independent Variables	Unit	Variability range
Average Global Radiation (R_{MED})	W·m^{-2}	12–337.6
Minimum daily temperature (T_{MIN})	°C	12–25
Total Ammoniacal nitrogen (TAN)	mg N·L^{-1}	144–245
Chemical oxygen demand (COD)	mg O_2·L^{-1}	76.5–340
Turbidity	FAU	63.5–580
Counts	M Cells·mL^{-1}	0.5–4.2
Dissolved Oxygen (DO)	mg DO· L^{-1}	2.2–11.8

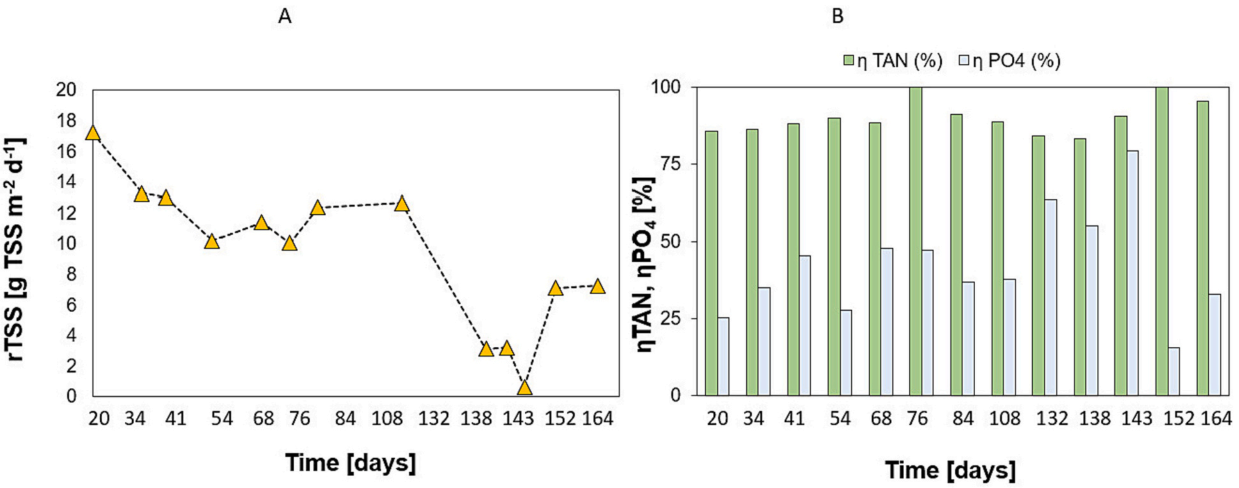

Fig. 1. A) Biomass areal productivity (g TSS·m^{-2}d^{-1}) and B) nutrient removal efficiency (η TAN and ηPO$_4$, %).

detailed in Rossi et al. (2023). The pilot plant (Fig. 1S of Supplementary material) had a total surface of 5.8 m^2 and a gravity overflow of 0.15 m. A paddlewheel, operating at 4 RPM, was used to continuously mix the algal suspension. The HRAP is covered by a greenhouse, protecting the culture from heavy rain events. However, during spring, summer and autumn the lateral wall panels are removed to favor sunlight exposure. A mixed microalgae-bacteria consortium, containing mainly *Chlorella* spp. and *Scenedesmus* spp. was provided by Istituto Spallanzani (Rivolta d'Adda, CR, Italy). At first, it was grown in the laboratory on Bresso centrate and then it was inoculated in the HRAP in tap water: centrate (1:2) mix and maintained in batch mode for two weeks. Then the system was switched to continuous mode and fed on undiluted centrate with a hydraulic retention time (HRT) of 6 days. The centrate was stored in 1m^3 containers and was fed by a peristaltic pump. The rejected CO_2 stream from the biogas upgrading line of Bresso WWTP was used as an additional carbon source to sustain the microalgal photosynthesis as well as for pH control. The gas was sparged from the bottom of the HRAP through porous stones. The HRAP was connected to a Programmable Logic Controller (PLC), allowing the control of the CO_2 input by a compressor as a function of the pH in the algal suspension. The pH set point was 7.5. The PLC was also connected to online probes for measuring temperature, pH, turbidity, Dissolved Oxygen (DO), Total Ammoniacal Nitrogen (TAN) and NO_X concentrations, and solar radiation.

2.3. *Sampling and routine analyses*

The HRAP performance was monitored routinely (once per week) as described in Rossi et al. (2023) by evaluating microalgal growth and nutrient consumption. Conventional wastewater quality parameters, including Total Ammoniacal Nitrogen (TAN), oxidized nitrogen forms (N-NO_2^- and N-NO_3^-), orthophosphate (P-PO_4^{3-}), soluble chemical oxygen demand (COD) were analyzed on filtered samples (cutoff 0.45 μm), using Hach-Lange kits for spectrophotometric quantitative measurements (test codes: LCK303, LCK342, LCK339, LCK348, and LCK314, respectively). Gravimetric analyses were carried out according to Standard Methods (APHA, AWWA, WEF, 2017) to determine total suspended solids (TSS) and volatile suspended solids (VSS). Optical density (OD) was measured at 680 nm by a spectrophotometer (DR 3900, Hach Lange, Germany). Microalgae cell counts were also performed by an optical microscope 40× (B 350, Optika, Italy) and a hemocytometer (Marienfeld, Germany). The main microalgae species have been distinguished based on their morphology and size. Further details on the routine analyses of the HRAP are available in Rossi et al. (2023). In addition, the ability of the microalgae-bacteria consortia to remove pharmaceutical compounds from the centrate was studied. To this aim, influent and effluent grab samples were collected every two-three week from the influent storage tank and the HRAP, respectively, stored in 1000 mL amber glass bottles, and transported to the laboratory. There, the samples were filtered at 0.45 μm to separate the biomass and solids from the supernatants and stored at −20 °C until further analysis.

2.4. *PhACs and analytical procedure*

The target molecules are shown in Table 1S (Supplementary materials), which also provides information on some chemical-physical properties that can influence their mobility in the water compartment. Amisulpride (AMS), Ofloxacin (OFX), Metoprolol (MTP), Sulfamethoxazole (SFX), Clarithromycin (CLM), Gabapentin-Lactam (GBP), Carbamazepine (CBZ), Irbesartan (IRB), Diclofenac (DCN), Lamotrigine (LMG), Fluoxetine (FLX) and Propyphenazone (PHP) were chosen as they are common pharmaceuticals that can be detected in municipal wastewaters, and they belong to different classes of active compounds. All of them have similar molar masses except for CLM, which is a larger molecule. CBZ, IRB, DCN, LMG, FLX, OFX, and CLM are hydrophobic molecules while GBP, SFX, MTP, PHP, and AMS have a higher affinity for the water phase. Native and isotopically labeled standards (IS) were purchased from Sigma-Aldrich (St. Louis, MO, US) in their solid form. Stock solutions were prepared for every PhAC by dissolving about 1 mg of each standard in about 1 mL of methanol straight in autosampler vials. The preparation of the standards was carried out using a 5-decimal place analytical balance and PIPETMAN® air-displacement pipettes (having different capacities from 0.2 μL to 10 mL). The proper volume of methanol was added in the vial with the pipettes, according to the precise weight of the standard, to reach the desired concentration of 1 mg mL^{-1}. The vials were then put in an ultrasound bath for 20 min to allow the dissolution of the standards. 10 μL of each stock solution was added in a single vial with 880 μL of methanol to prepare an intermediate solution containing all the analytes at 10000 μg·L^{-1} (solution A). After the ultrasound bath, 100 μL of solution A were diluted with ultrapure water (UW) to obtain a 1000 μg·L^{-1} stock solution (solution B). That was used to prepare the calibration curves for each compound, having seven calibration points between 0.5 and 50 ng·mL^{-1}. Limits of detection (LODs) and quantification (LOQs) were estimated as threefold and tenfold the standard deviation of the lowest standard, as indicated in the ISO 6107:2021 Standard (ISO 2021) (More information can be found in Supplementary material, Table 3S–5S). A IS working solution of 1000 μg·L^{-1} of fluoxetine-d6 was prepared in the same way. The inlet and outlet water samples from the HRAP were first thawed and filtered at 0.2 μm. Later, 120 mL were collected for each of them and spiked with

2.5 μL of the IS solution. The same process was carried out for procedural blanks using UW. Water samples were finally solid-phase extracted on Oasis® HLB cartridges (200 mg/6 mL, Waters, Milford, MA). The pre-concentration steps included: cartridges conditioning (using 6 mL of methanol and 6 mL of UW); sample extraction under vacuum filtration at 5–10 mL·min^{-1}; cartridges washing with 6 mL of UW; air-drying for 15 min; samples elution using 6 mL of methanol. Finally, the extracted samples were concentrated under an N_2 stream to remove the solvent, resuspended in 1 mL of UW, and recovered in autosampler vials. Separation was carried out by an XSelect CSH C18 XP chromatographic column, 130 Å, 2.5 μm, 3 mm × 100 mm, with a mobile phase made of solvent A (UW with ammonium acetate 1 mM and 0.10 % formic acid) and solvent B (methanol with ammonium acetate 1 mM and 0.10 % formic acid). Extracts were analyzed through ultra-performance liquid chromatography (Acquity UPLC H-class, Waters, USA) coupled with a QDa detector (Waters, USA). The elution rate was set according to the compounds to be analyzed. The flow rate was 0.5 mL·min^{-1}.

2.5. Data processing

The data obtained during the monitoring of the HRAP were used to set mass balances for each parameter of importance. Complete stirring was assumed in the pilot reactor due to the paddlewheel and a hydraulic balance was set as follows:

$$Q_{OUT}(ti) = Q_{IN}(ti) - Q_{EV}(ti) \tag{1}$$

Q_{OUT}(ti) is the effluent flow rate at the time step ti, Q_{IN}(ti) is the influent flow rate ensured by the feeding pump, and Q_{EV}(ti) is the evaporation rate estimated by the Buckingham model, as detailed in Pizzera et al. (2019). Since the HRAP was always covered by the rooftop of the greenhouse, rainfall was neglected in the balance. Q_{OUT}(ti) and Q_{IN}(ti) were used to calculate the production rate and/or the consumption rate or relevant processes as follows:

$$R_X(t_i) = \frac{\Delta X}{\Delta T} - \frac{Q_{AVG,IN}(t_i).X_{AVG,IN}(t_i)}{V} + \frac{Q_{AVG,OUT}(t_i).X_{AVG,OUT}(t_i)}{V} \tag{2}$$

Where: $R_X(t_i)$ [mg X·L^{-1}·d^{-1}] is the removal or production rate for the general element X at the time t_i, $\Delta X = (X_i - X_{i-1})$ [mg X·L^{-1}] is the variation in the concentration of the element X in the time interval $\Delta t = (t_i - t_{i-1})$, $Q_{AVG,IN}(t_i)$ and $Q_{AVG,OUT}(t_i)$ [L·d^{-1}] are the average influent and effluent flowrates along the time interval Δt, respectively, $X_{AVG,IN}(t_i)$ and $X_{AVG,OUT}(t_i)$ [mg X·L^{-1}] are the average concentrations of X in the influent and effluent in the time interval, respectively, and V is the volume of the microalgal suspension inside the HRAP.

The removal efficiencies of nutrients and pharmaceuticals (η_X) were computed on a mass flow basis, according to the following formula:

$$\eta_X = \frac{(Q_{IN} \cdot X_{IN}) - (Q_{OUT} \cdot X_{OUT})}{(Q_{IN} \cdot X_{IN})} \tag{3}$$

Where: Q_{IN} and Q_{OUT} [L·d^{-1}] are the actual influent and effluent flowrates, X_{IN} and X_{OUT} [mg X·L^{-1}] are the concentrations of X in the influent and effluent, respectively.

2.6. Statistical analysis

Statistical analyses were conducted using the R project software (R Core Team, 2021). Pearson's correlation coefficients were used to explore significant linear relationships between environmental and operational parameters and HRAP efficiencies in the removal of nutrients and PhACs. Global radiation, Air Temperature, Algal Temperature, COD, TAN, P-PO_4^{3-} concentrations in the centrate, Algal counts, TSS concentration, Turbidity, and Dissolved Oxygen concentration in the microalgal suspension were the independent variables. When no linear correlations were detected, Spearman's rank correlation coefficient analysis was performed on the same variables based on their ranks. Relationships having *p* values <0.05 were considered statistically significant. Generalized linear models (GLMs) were used to study the simultaneous effect of continuous and categorical variables on the removal of pharmaceutical compounds. Variance Inflation Factor (VIF) was used to choose the independent variables to be added to the GLM to avoid or minimize multicollinearity. At first, nutrient concentrations (TAN, N-NO_2^-, N-NO_3^-, P-PO_4^{3-}, COD) both for centrate and microalgal suspension; microalgal productivity (rTSS) and concentration (Algal counts, Optical Density, Turbidity, and Total Suspended Solid), environmental conditions (Average, Minimum and Maximum daily Temperature and Global radiation) and Dissolved Oxygen concentration were considered as potential variables to build the GLM. The pH of the microalgal suspension was not included since it was controlled during the campaign through CO_2 bubbling and its variability was negligible. VIF factor was calculated for each variable and the one having the single highest value was rejected. The procedure was repeated until all the remained variables had VIF below 10 (Kutner et al., 2004). The GLM was finally built with the following experimental parameters (also listed in Table 1) as independent variables: average global radiation (R_{MED}), minimum daily temperature of the microalgal suspension (T_{MIN}), Total Ammoniacal Nitrogen concentration (TAN), soluble Chemical Oxygen Demand (COD) in the centrate, microalgal and solid concentration (Counts and Turbidity) and Dissolved Oxygen (DO) concentration in the microalgal suspension. The full GLM model is here reported:

$$X = 1 + \beta_1 \cdot R_{MED} + \beta_2 \cdot T_{MIN} + \beta_3 \cdot TAN + \beta_4 \cdot COD + \beta_5 \cdot Turbidity + \beta_6 \cdot Counts + \beta_7 \cdot DO + \varepsilon \tag{4}$$

Starting from the full model, a backward-stepwise procedure was used to remove the non-significant variables, generating restricted models that were compared with the full GLM model by ANOVA. Computed models were used to predict the effect of the independent variables on the removal efficiency of each pharmaceutical compound.

3. Results and discussion

3.1. Microalgal growth and nutrient removal

The whole monitoring campaign of the HRAP reporting the microalgal productivity, the evolution of the microalgal consortium composition, and its effectiveness in nutrient removal are described in detail in Rossi et al. (2023) who compared the two experimental campaigns of 2020 and 2021. Here, a summary is reported, focusing on the data collected during the 2021 trial, concurrently with the sampling campaign performed for monitoring the pharmaceutical compounds removal. The *Chlorella* spp. and *Scenedesmus* spp. consortium grew well on the centrate with an average productivity of 8.4 ± 5.7 g TSS·m^{-2}·d^{-1} from May to November. Environmental conditions affected the microalgal growth as the productivity was stable during the first 120 days till September (12.1 ± 4.2 g TSS·m^{-2}·d^{-1}), then it started to drop in autumn concurrently with the decrease of temperature and solar radiation. During the final period, the average productivity dropped to 5.2 ± 4.8 g TSS·m^{-2}·d^{-1}.

The microalgal community was always dominated by *Chlorella* spp. while *Scenedesmus* spp. was present in lower concentrations. On average the microalgae counts ranged between 1.7 and 4.2 Mcells·mL^{-1}. The proportion of the two strains was maintained until September when algae flocculation occurred in response to the appearance of microalgae predators (protozoa). Except for the protozoa contamination, the presence of other microalgae species was negligible.

Fig. 1 shows the trend of microalgal productivity (rTSS), highlighting in yellow the data corresponding to the sampling days for the study of pharmaceutical compounds. As already observed in previously mentioned studies (Mantovani et al., 2020; Rossi et al., 2023), the HRAP was effective in removing TAN, with an overall efficiency of 85.4 ± 8.1 %, due to the combination of microalgal uptake and nitrification. The addition of sodium bicarbonate in the feed seemed important in reducing the competition between microalgae and nitrifiers, also allowing to balance the acidification of the suspension due to the nitrification process.

Nitrite and nitrate were produced in the HRAP, by the activities of Ammonia Oxidizing Bacteria (AOB) and Nitrite Oxidizing Bacteria (NOB) that could exploit the oxygen produced by the microalgae during the photosynthesis. AOB were always more active than NOB and caused nitrite accumulation in the effluent of the HRAP while nitrate concentration was always below 60 mg N L^{-1}. Only at the end of the campaign, the nitrification was almost complete. The observed trend was already described in microalgae-based systems: nitrite accumulation also happened during the experimental campaign of 2017 when the HRAP was fed with an HRT of 10 days (Mantovani et al., 2020). Bani et al. (2020) observed a similar scenario in two outdoor cultivation systems (PBR and HRAP) treating digestate. AOB and NOB can be affected differently by environmental and operative conditions, however explanation of the AOB/NOB dynamic is still lacking. Regarding phosphorus, the removal of orthophosphate was less effective (35.8 ± 18.7 % on average) as often reported in similar case studies (Mantovani et al., 2020; Sutherland et al., 2020).

3.2. PhACs removal

Table 2 shows the average concentrations of PhACs in the collected samples and the flow rates regulating the operation of the HRAP, while Fig. 2 represents PhACs overall removal efficiencies, calculated on a mass balance basis using the eq. (3). A high variability can be noted in both the inlet (centrate) and outlet (microalgal suspension) concentrations, probably related to differences in the loads of these compounds entering in the WWTP during the year. All the targeted compounds were always found in the centrate except for Sulfamethoxazole, which was detected only in three samples but never in the microalgal suspension. In those cases, the percentage removal efficiency (η (%)) was high (95 ± 2). Interestingly, the absence of Sulfamethoxazole in both the inlet and outlet of the HRAP aligns with previous findings by Mezzanotte et al. (2022), who performed a preliminary study on the same HRAP in October 2020. It could be possible that in Bresso WWTP this antibiotic is biodegraded by activated sludges where bacteria can use it as a carbon source for their growth (Müller et al., 2013; Yan et al., 2022), or during the anaerobic digestion, even if with some variability so that a residual concentration can sometimes be detected in the centrate. The potential of microalgae-bacteria to remove Sulfamethoxazole was already highlighted at the laboratory and pilot scale, with efficiencies ranging between 54 and 85 % (da Silva Rodrigues et al., 2020; Chu et al., 2022; García-Galán et al., 2020).

The highest removal efficiencies were observed in the Bresso HRAP for Lamotrigine and Diclofenac, achieving 87 ± 3 % and 71 ± 17 %, respectively. These results, while noteworthy, were slightly lower than those reported by Mezzanotte et al. (2022), who found removal efficiencies of 94 % and 98 %, respectively. In both cases, the removal is higher than reported in the literature. The result is particularly remarkable considering Lamotrigine, as activated sludge processes seem not equally effective (Krzeminski et al., 2019). This compound is quite persistent and not particularly degraded by photolysis (Keen et al., 2014). Literature data concerning microalgal-based applications for Lamotrigine removal are scarce. However, a 47 % removal for Lamotrigine was obtained in Colombia, during a pilot scale study with an HRAP fed on domestic wastewater and with 3 days HRT (Jiménez-Bambague et al., 2020). However, the experimental conditions differed from those in this study (the HRT is half the value used in Bresso) and the Tropics are much more suitable for microalgal cultivation than Northern Italy, preventing direct comparisons. As for Diclofenac efficiencies between 40 and 60 % were obtained with a similar microalgal strain (*Chlorella sorokiniana)* on both batch lab cultivation and continuous HRAP operation (García-Galán et al., 2020; de Wilt et al., 2016). Photodegradation is a well-known phenomenon affecting Diclofenac, which was observed also in other HRAP applications (Matamoros et al., 2015) thus suggesting that it could have an important role in the Bresso pilot HRAP.

Efficiencies over 60 % were found for Fluoxetine and Metoprolol (66 ± 11 %, and 64 ± 20 %, respectively). Fluoxetine is quite recalcitrant and conventional WWTPs seem not able to eliminate it (Metcalfe et al., 2010). Evidence on the potential of microalgae on Fluoxetine removal from wastewater does exist, but mainly on lab batch systems working with high inlet concentrations (10–200 μg·L^{-1}). Under those circumstances, removal efficiencies were high (77–100 %) and biodegradation was indicated as the most important removal pathway (Silva et al., 2022; Xie et al., 2022). Metoprolol is not completely removed by WWTPs, as the efficiencies of both activated sludge and even advanced systems like membrane bioreactors (MBR) are not over 54 % (Lacey et al., 2012; Rubirola et al., 2014). High variability is reported also for microalgal-based systems (40–100 %) treating centrate (Mezzanotte et al., 2022) and urine (de Wilt et al., 2016).

Amisulpride, Ofloxacin, Carbamazepine, and Clarithromycin were removed by Bresso HRAP with efficiencies over 30 % (39 ± 16 %, 33 ± 23 %, 32 ± 29 %, and 31 ± 30 %, respectively). The removal of Amisulpride was slightly higher than reported in Mezzanotte et al. (2022) and had a lower variability. To the best of our knowledge, studies focusing on Amisulpride removal by microalgae are scarce. Bollmann et al. (2016) found that Amisulpride was particularly recalcitrant to treatment with activated sludge performed at the lab scale for 14 days.

Ofloxacin is removed by conventional WWTP up to 60 % (Li and Zhang, 2010; Verlicchi et al., 2012), which is clearly better than the result obtained in this paper. Variability in microalgal-based studies can be noted: Ofloxacin was not removed in Mezzanotte et al. (2022), while Hom-Diaz et al. (2017) observed 67 % removal from toilet wastewater growing microalgae in a multitubular photobioreactor (PBR). There are conflicting data regarding the removal of Carbamazepine using microalgae. In a 5-day batch test, the co-cultivation of microalgae and bacteria within a biofilm showed overall removal rates ranging from 18 % to 51

Table 2

Average inlet (centrate), outlet (microalgal suspension) concentrations, and flow rates. Data are shown as average ± standard deviation ($n = 13$ for all the compounds except for SMS, for which $n = 3$).

Chemical	Inlet (ng·L^{-1})	Outlet (ng·L^{-1})	Q_{IN} (m^3·d^{-1})	Q_{EV} (m^3·d^{-1})	Q_{OUT} (m^3·d^{-1})
Amisulpride (AMS)	38 ± 14	25 ± 8	0.144	0.019	0.019
Ofloxacin (OFX)	156 ± 67	109 ± 39	0.144	0.015	0.015
Metoprolol (MTP)	38 ± 19	16 ± 9	0.144	0.007	0.007
Sulfamethoxazole (SFX)	2.6 ± 4.0	0.5 ± 0.5	0.144	0.020	0.020
Clarithromycin (CLM)	13 ± 3	9 ± 2	0.144	0.030	0.030
Gabapentin-Lactam (GBP)	77 ± 16	78 ± 26	0.144	0.018	0.018
Carbamazepine (CBZ)	9 ± 6	5 ± 4	0.144	0.008	0.008
Irbesartan (IRB)	2337 ± 1359	2176 ± 1038	0.144	0.022	0.022
Diclofenac (DCN)	300 ± 125	71 ± 32	0.144	0.015	0.015
Lamotrigine (LMG)	3145 ± 33	19 ± 6	0.144	0.015	0.015
Fluoxetine (FLX)	52 ± 8	20 ± 7	0.144	0.020	0.020
Propyphenazone (PHP)	5 ± 3	7 ± 4	0.144	0.006	0.006

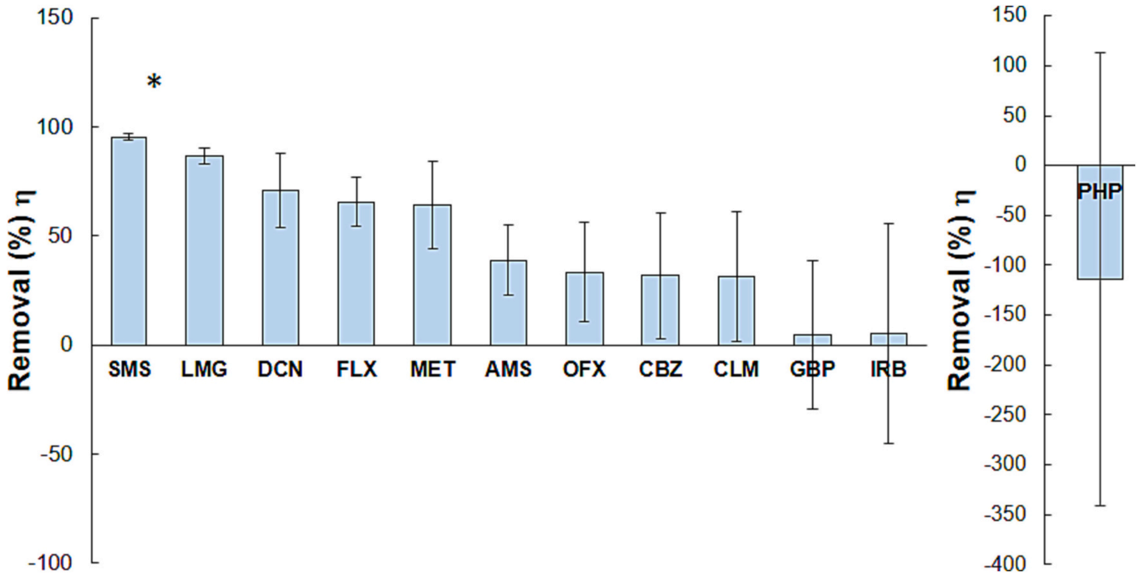

Fig. 2. Percent removal of Sulfamethoxazole (SMS), Lamotrigine (LMG), Diclofenac (DCF), Fluoxetine (FLX), Metoprolol (MET), Amisulpride (AMS), Ofloxacin (OFX), Carbamazepine (CBZ), Clarithromycin (CLM), Gabapentin-Lactam (GBP), Irbesartan (IRB) and Propyphenazone (PHP). Data are calculated on a mass balance basis and are presented as average and standard deviations, $n = 13$, except for Sulfamethoxazole (as indicated by the * $n = 3$). Negative values correspond to higher concentrations in the effluent than in the influent.

%. Light conditions, biomass concentration, and the composition of the synthetic wastewater affected those results (Akao et al., 2022). Meanwhile, Bai and Acharya (2017) achieved only a 13 % removal efficiency in a batch test where *Nannochloris* sp. was cultured in lake water spiked with 10 μg·L^{-1} of Carbamazepine. In contrast, García-Galán et al. (2020) reported no removal in their pilot-scale study, while Matamoros et al. (2015) noted seasonal variations in the efficiency of their High-Rate Algal Ponds (HRAP), which were further influenced by HRT. Concerning Clarithromycin, Gentili and Fick (2017) obtained a 90 % removal by growing a mixed microalgae community (in which *Dictyosphaerium* sp. was the prevailing taxa) in an open pond fed with municipal wastewater. The test, however, consisted of a 7-day batch, and data on continuous cultivation systems are currently not available in the literature. Clarithromycin removal didn't occur in Bresso HRAP in the 2020 campaign (Mezzanotte et al., 2022) as the concentrations in the effluents were even higher than the ones in the influent.

In the present research, the removal of Gabapentin-Lactam was <10 % (5 ± 34 %) and in some cases, the concentrations increased after treatment. The explanation for such an increase, also observed for some other compounds (Propyphenazone and Irbesartan), could be linked to the mechanism known as product-to-parent transformation, where metabolites present in the wastewater are transformed into the parental compound (Verlicchi et al., 2012). However, the variability in both inlet and outlet concentrations for these compounds is especially high making it difficult to deduce a reliable explanation. *t*-tests for paired data showed no significant differences between the average inlet and outlet loads both for Irbesartan and Propyphenazone in the HRAP. The microalgal treatment had variable effectiveness, resulting in quite high standard deviations except for Sulfamethoxazole, Lamotrigine, and Fluoxetine. This is often a problem when dealing with the remediation of pharmaceutical compounds (Couto et al., 2019). Overall, differences were noted with respect to data presented in Mezzanotte et al. (2022), even if it is not easy to explain them. One substantial factor is that in 2020 the sampling campaign had been carried out in October when the microalgae density started to drop due to the worsening of the environmental conditions. However, in 2021 samples were collected in a wider time window (from June to October 2021) and a more active and denser microalgal community was assessed. This could have explained the better result in the 2021 trial for Amisulpride, Ofloxacin, and Carbamazepine but not the big difference for Clarithromycin. However, in 2021 thirteen inlet and outlet samples were analyzed, while in the preliminary study of 2020, the number of samples was much lower.

3.3. Statistical analysis

Spearman's rank correlation coefficient was computed to assess the presence of a monotonic correlation between nutrient removal efficiencies (η TAN and η P-PO_4) and key factors potentially affecting the HRAP. As displayed in Fig. 3, both η TAN and η P-PO_4 exhibited a positive and statistically significant correlation with the daily average temperature in the pond (Temperature of the microalgal suspension), the TAN concentration in the centrate, and the Dissolved Oxygen concentration in the pond. Warmer temperatures were associated with higher microalgal growth rates (Pearson's correlation indicated a linear positive correlation, *p*-value <0.001), resulting in increased oxygen production. Under these conditions, the activities of Ammonium-Oxidizing Bacteria (AOB) and Nitrite-Oxidizing Bacteria (NOB) were promoted. They consumed the oxygen produced by microalgal photosynthesis to oxidize the available TAN into N-NO_2 and N-NO_3, respectively. While the relationship between η TAN and TAN concentration in the centrate is straightforward, the correlation between η P-PO_4 and TAN concentration in the centrate might be less intuitive. However, a higher nitrogen availability could reduce competition between microalgae and bacteria, resulting in an improved HRAP treatment efficiency.

Pearson correlations were also analyzed to understand if and how the main chemical-physical properties of PhACs could have influenced the HRAP treatment. As indicated in Table 1S (Supplementary materials), molecular weight (MW), octanol-water partition coefficient (LogK_{OW}), and acid dissociation constant (pKa) were considered. The analysis of data has been carried out considering all PhACs and only the subgroup positively removed by the HRAP treatment. Sulfamethoxazole was not included in the analysis as it was detected only in three samples. No correlations were found considering MW and pKa as independent variables (*p*-value was 0.7 and 0.95). However, a significant correlation was found between η (%) and Log K_{OW}, on the subgroup of PhACs positively removed by the HRAP treatment. As shown in Fig. 4, the higher the LogK_{OW} (and the hydrophobicity of the PhACs), the higher the removal efficiency (p-value = 0.048).

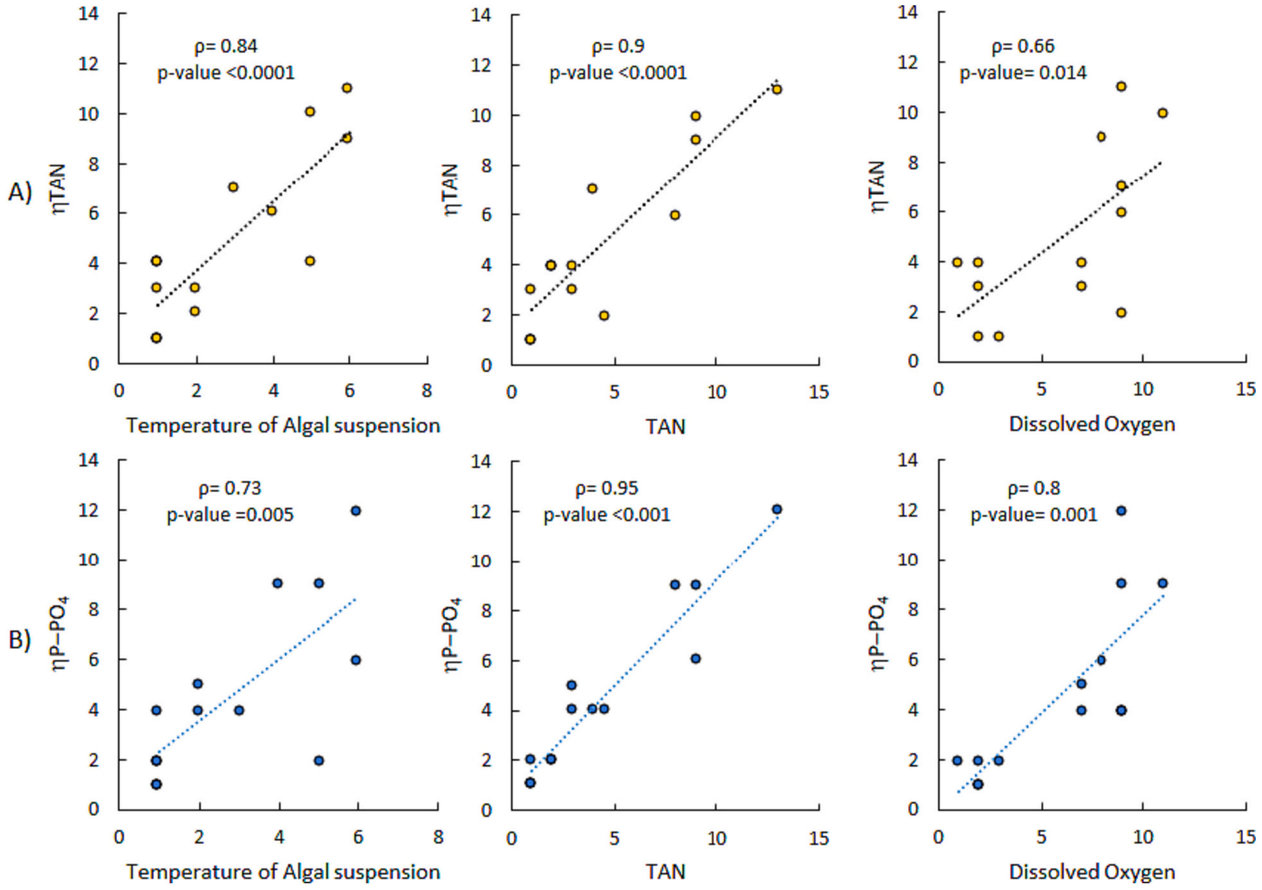

Fig. 3. Spearman's rank correlation coefficient (ρ) analysis. Nutrient removal efficiencies (η TAN (A) and η P-PO$_4$ (B)) were the dependent variables while Temperature of algal suspension, TAN concentration and Dissolved Oxygen in the HRAP were the independent variables.

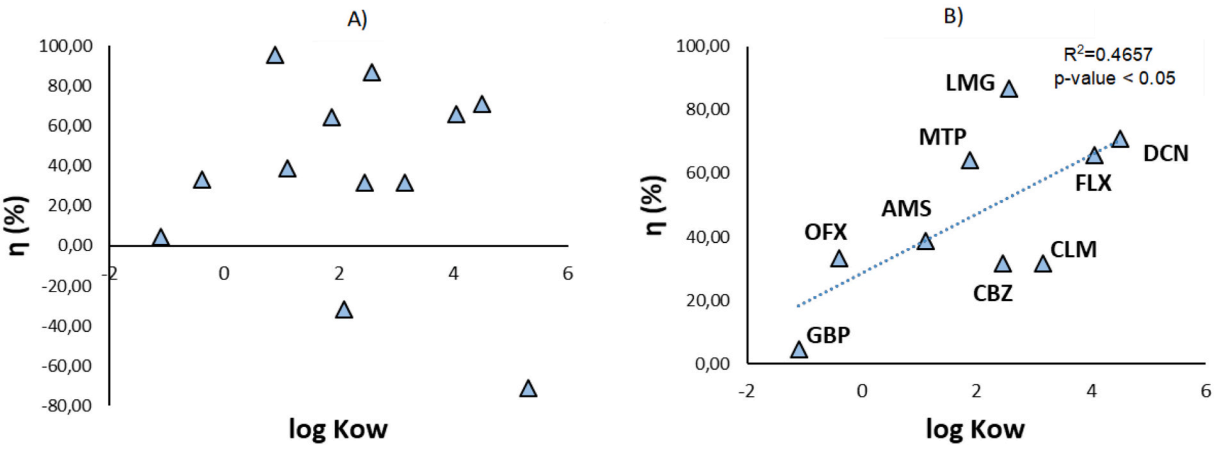

Fig. 4. Linear regression between average removal efficiency (η (%)) and Log K$_{OW}$ considering all the compounds (A) and the ones that were positively removed (B).

The importance of hydrophobicity for the removal of pharmaceutical compounds by a microalgae-bacteria-based system was to be expected. The chemical composition of microalgae cell walls can be quite variable from one species to another. However, it is known that functional groups contained in polysaccharides and proteins forming the cell walls can bind different molecules (in this case PhACs) thus removing them from the water compartment. The most hydrophobic PhACs have probably undergone sorption processes, leading to their accumulation in the biomass. Considering the trend of biomass productivity (rTSS) shown in Fig. 1, this removal pathway could have been relevant throughout the experiment. On the other hand, sorption processes are not the only possible explanation for the fate of PhACs in the HRAP. Exploratory analyses, through Spearman correlations, were performed to get preliminary information on the influence of environmental conditions, feed characteristics, and microalgae-related factors on the removal efficiencies of the studied compounds. The significant results can be found in Table 3, showing Spearman coefficients (ρ) and their significance (*p*-value).

OD680 had a negative effect on Diclofenac removal (*p*-value <0.05) suggesting that higher efficiencies were achieved in HRAP when the algal suspension was less dense, probably favoring photo-degradation. Turbidity of the algal suspension and Oxygen concentration had a

Table 3
Results (coefficients and level of significance) of Spearman's rank correlation coefficient (ρ) analysis on PhACs removal.

Independent variables	Dependent variables								
	ηAMS	ηOFX	ηMET	ηCLM	ηGBP	ηCBZ	ηDCN	ηLMG	ηFLX
OD680							−0.65 *		
Turbidity									0.70**
Oxygen			0.63*						0.67*
Algal Temperature			0.69**			0.69**		0.69**	
Air Temperature	0.58*		0.74**					0.72**	
TAN centrate	0.64*		0.88***	0.60*				0.59*	0.74**
$P\text{-}PO_4$ centrate				0.67*					
COD centrate							0.82***		
rNOB		−0.69**			−0.56*				

Significance levels (code) referred to Spearman test: $p < 0.001$ (***), $p < 0.01$ (**), $p < 0.05$ (*), $p < 0.1$ (').

positive correlation (p-value <0.01 and < 0.05, respectively) with Fluoxetine removal, suggesting an active role for both microalgae and bacteria.

The temperature in the HRAP (Algal Temperature) had a positive effect on Metoprolol, Carbamazepine and Lamotrigine removals, showing p-values <0.01. Similarly, Air Temperature had a positive effect on Metoprolol and Lamotrigine removals (*p*-values <0.01) and on Amisulpride removal (p-value <0.05). Warmer days could have fostered the activity of microalgae and bacteria, leading to higher effectiveness in the treatment of centrate. Nutrient availability had a positive effect on many PhACs. TAN concentration in the centrate was positively correlated to the removal of Metoprolol (p-value <0.001), Fluoxetine removal (p-value <0.01), Amisulpride, Clarithromycin and Lamotrigine (p-values <0.05). $P\text{-}PO_4$ concentration in the centrate had a positive effect on Clarithromycin removal (p-value <0.05). In the HRAP microalgae and bacteria do compete for nitrogen and phosphorous, so it could be reasonable to say that their growth and their ability to remove PhACs is higher when primary nutrients are not limiting. COD in the centrate showed a positive correlation with Diclofenac removal (p-value<0.001). Nitrite oxidizing bacteria growth rates (rNOB) showed a negative correlation with Ofloxacin and Gabapentin-Lactam removal (p-values <0.01 and < 0.05, respectively) but a reasonable explanation is still lacking.

At least one significant correlation was found for each pharmaceutical removal and the considered independent variables. However, it must be noted that Spearman's rank correlation uses only one independent variable at a time meaning that it can't predict how the system is affected by their interaction. Therefore, GLMs were used to understand the simultaneous influence of environmental conditions, feed characteristics, and microalgae-related factors on the removal

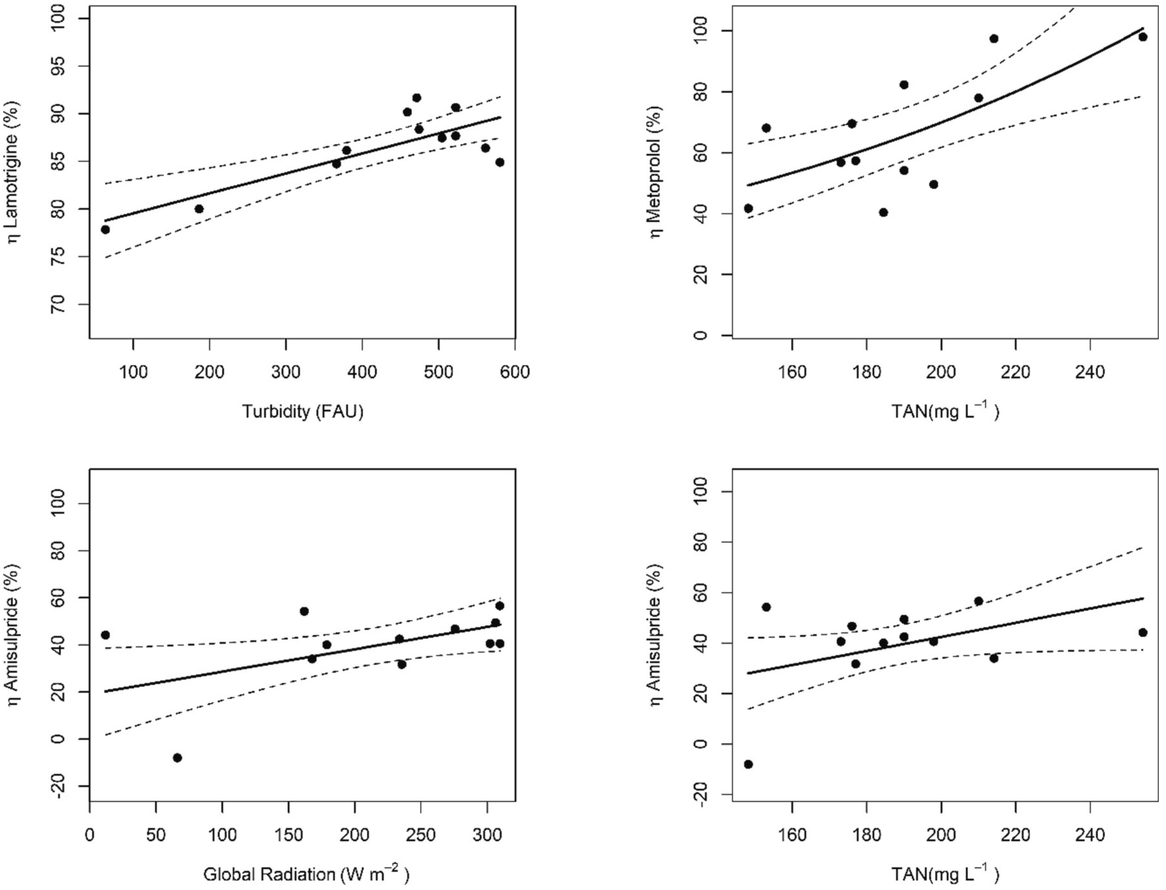

Fig. 5. Partial effects of the independent variables included in the GLMs on the removal efficiencies of Lamotrigine, Metoprolol, and Amisulpride. Dots represent experimental data, solid lines represent the predicted values, and dashed lines represent the 95th-percent confidence interval.

efficiencies of the studied compounds. The interpretation of the output is not always straightforward; the complexity of the system and the variety of processes and phenomena occurring in the HRAP must be considered. Indeed, in many cases, the high variability of the removals makes it difficult to perceive clear trends, also by considering the limited variation of the environmental and operational parameters during the monitoring period. Fig. 5 summarizes the partial effects of the independent variables included in the restricted model on specific pharmaceuticals, while Table 6S (Supplementary materials) summarizes the results, providing coefficients and level of significance. Lamotrigine removal (ηLMG) showed significant and positive correlations with the Turbidity of the microalgal suspension. This is an interesting finding and suggests an active role of microalgae (and bacteria) in the elimination of this specific compound. While sorption mechanisms on the microalgae-bacteria biomass could be justified by Lamotrigine's Log K_{OW}, a high Turbidity could have also other roles. Besides producing oxygen that is used by aerobic bacteria during pollutant degradation, microalgae can generate other strong oxidants like hydroxyl radicals that can chemically degrade different PhACs (Fatta-Kassinos et al., 2011). Nonetheless, no significant effect of the DO level in the system on the PhACs removal was observed. The Amisulpride removal (ηAMS) was positively correlated to Global Radiation (R_{MED}) and the concentration of Total Ammoniacal Nitrogen (TAN) in the centrate. Those trends could have multiple explanations: higher sun exposure, typical of summertime, leads to higher microalgal growth but can also foster photo-degradation. Wastewaters do contain dissolved organic matter and inorganic ions such as NH_4^+ and NO_3^- that, in sunlit water environments can generate •OH radicals, possibly enhancing the photodegradation of many pharmaceutical compounds (Fatta-Kassinos et al., 2011). The correlation between ηAMS and TAN concentrations is also interesting as some microorganisms can degrade non-growth-related organic compounds when primary substrates are available (Fernandez-Fontaina et al., 2014). The same consideration can be made for Metoprolol, whose removal showed a positive correlation with TAN concentration. As already observed by Wang et al. (2022), high ammonia loading rates foster nitrification-based cometabolism, thus enhancing benzotriazole removal. The ability of ammonia-oxidizing bacteria (AOB) to partially biodegrade Metoprolol (10 $\mu g \cdot L^{-1}$ in synthetic wastewater) was observed by Velázquez and Nacheva (2017) in batch experiments, who measured 64 % removal after 24 days. The trends highlighted by the GLMs for Amisulpride and Metoprolol removal could suggest an active role of nitrifying bacteria also in the Bresso HRAP, where they compete with microalgae for TAN availability. The synergy between microalgae and bacteria seems quite interesting, suggesting that microalgae-bacteria systems for the removal of pharmaceutical compounds from municipal wastewater should be at least deepened with further research. Indeed, additional studies are needed to provide more insights in the removal mechanisms. The chemical extraction from the algal and bacterial biomass and the subsequent analysis of the extracts could help to single out the role of adsorption and/or absorption on the biomass. Moreover, the role of photo-oxidation compared to biodegradation cannot be distinguished from available results, so parallel lab-scale tests are needed to understand the role of each potentially active degradation route.

3.4. *Biomass valorization*

The fate of the microalgae grown on wastewaters is a key point to make microalgae-bacteria-based systems feasible. Finding the best valorization strategy for the microalgae is challenging as the accumulation of PhACs and other pollutants in the biomass can pose some limitations. Anaerobic digestion (co-digestion with municipal sludges) could increase the biogas production of the WWTP. The development of a cost-effective pretreatment of the biomass to weaken the cell walls of microalgae would be important to increase their degradability and biomethane potential. Hydrothermal carbonization is an interesting conversion process for microalgal biomass grown on wastewater such as municipal centrate. Microalgae are rich in carbon, nitrogen and phosphorus (assimilated from the centrate) and can be used to produce hydrochar, or novel modified adsorbents for the removal of emerging compounds and other pollutants from wastewater (Mantovani et al., 2022). HTC itself was recently proposed to degrade some PhACs from municipal sludges, as its temperature and pressure conditions (up to 225 °C and 30 bar) can degrade many organic compounds (Miserli et al., 2022). The same fate should occur to many PhACs or other pollutants accumulated on the Bresso microalgal biomass when treated through HTC. Studies on the produced hydrochar are thus needed to confirm this hypothesis.

4. Conclusion

The outdoor HRAP confirmed its efficacy as an alternative biological side-stream treatment of the centrate, by fostering the nitrification of TAN by bacteria, which benefit from the microalgal photo-oxygenation. Furthermore, the microalgal-based system offers an interesting potential in the treatment of emerging compounds such as pharmaceutical compounds, that are in general poorly and variably removed by conventional biological processes. The best results were achieved in the elimination of Lamotrigine, Diclofenac, Fluoxetine, and Metoprolol from the centrate (with removal efficiencies of 87 ± 3 %, 71 ± 17 %, 66 ± 11 %, and 65 ± 20 %, respectively). Even if Sulfamethoxazole removal was almost complete (95 ± 2 %), this compound was only detected in three samples of centrate during the whole study. Amisulpride, Ofloxacin, Carbamazepine, and Clarithromycin were not particularly affected by the microalgal treatment, still being removed with efficiencies over 30 % (39 ± 16 %, 33 ± 23 %, 32 ± 29 %, and 31 ± 30 %, respectively). Despite the observed performance was not sufficient for these targeted pollutants, overall, the data seem consistent with literature studies concerning microalgae, while the conventional activated sludge process does not offer more reliable results. Different mechanisms regulating the removal of pharmaceutical compounds by the HRAP could be hypothesized. Photodegradation, fostered by sunlight exposure, direct adsorption and/or absorption by the microalgal cells, chemical degradation, and biodegradation (from microalgae, heterotrophic bacteria, and nitrifying bacteria) can occur at the same time and have a combined effect. Some of these effects could be partially explained through the application of a generalized linear model. The obtained results are certainly promising and could pave the way for further studies to be carried out at the pilot scale. The obtained results are indeed promising and could pave the way for further studies to be carried out at the pilot scale. The physico-chemical characteristics of the compounds are strong drivers that regulate the effectiveness of the HRAP, however environmental conditions (global radiation and temperature) and operation parameters still have a crucial role. A strong positive correlation was also found between the removal of PhACs and their hydrophobicity, suggesting the risk of accumulating these compounds in the microalgal biomass. This aspect can pose challenges in finding the best valorization option for microalgae grown on wastewater. Anaerobic digestion, including co-digestion with the WWTP sludges, could boost biogas production even if cost-effective pretreatments of the biomass should be identified to enhance microalgae degradability. Another possibility is biomass conversion through hydrothermal carbonization, producing algal-based hydrochar or modified adsorbent for the removal of a wide range of contaminants from wastewater. These strategies not only address PhACs accumulation but also enhance microalgae's resourcefulness in wastewater treatment, envisioning a key role for microalgae in both wastewater treatment and resource recovery.

CRediT authorship contribution statement

Marco Mantovani: Investigation, Visualization, Writing – original draft. **Simone Rossi:** Writing – review & editing. **Elena Ficara:** Conceptualization, Writing – review & editing. **Elena Collina:**

Supervision, Conceptualization, Writing – review & editing. **Francesca Marazzi:** Writing – review & editing. **Marina Lasagni:** Supervision, Conceptualization, Writing – review & editing. **Valeria Mezzanotte:** Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.167881.

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