Microalgae wastewater treatment: Pharmaceutical removal and biomass valorization.

Abstract

In comparison to activated sludge biological treatments, microalgae-based photobioreactors entail a more environmental sustainable approach to sewage treatment, while performing similarly in terms of contamination removal, including pharmaceutical residues. Thus, the present study aims to investigate (i) the removal of pharmaceuticals in microalgae-related wastewater treatment systems, (ii) the variation of biomass productivity and nutrient recovery as a function of the presence of these compounds, and (iii) the feasibility of using the produced biomass in agriculture as a biostimulant. Experiments were performed in pilot-scale thin-layer reactors fed in continuous mode by spiking the inlet wastewater with up to six pharmaceuticals (sulfamethoxazole, trimethoprim, metronidazole, ofloxacin, ciprofloxacin, and diclofenac), selected by its relevance. Results demonstrated that the presence of these compounds does not influence biomass productivity or the fluorescence of chlorophylls as an indicator of the status of the cells. Either, the presence of pharmaceuticals does not reduce the recovery of nutrients, both biomass productivity and nutrient recovery being linearly related. On average, the removal of CECs was higher in summer (90%) than in winter (74%), with variations depending on the pharmaceutical tested. Sulfamethoxazole was the most effectively removed (>91%), while trimethoprim had the lowest removal rate (>44%). Most importantly, on average less than 3% of the pharmaceuticals remains in the biomass, trimethoprim being the contaminant most retained in the biomass (up to 6%), thus confirming that they are mainly degraded into the mixed liquor. Bioassays performed to evaluate the biostimulant capacity of produced biomass confirm that no negative effects were observed, moreover acting as plantpromoting agents. These results confirm the capacity of microalgae-related wastewater treatment processes to remove pharmaceuticals and to produce safe water and biomass for its final use in agriculture.

Keywords: Microalgae, wastewater, pharmaceuticals, biomass productivity, nutrients recovery, biostimulants

1. Introduction

Mankind is facing significant challenges related to (i) the exploration of innovative production methods based on renewable energies, (ii) the mitigation of greenhouse gas emissions, and (iii) the assurance of accessible clean water for all. All these elements are directly related to global pollution. Wastewater discharge is among the leading contributors to water pollution; thus there is an urgent need for efficient, sustainable and economically feasible wastewater treatment processes (Wang et al. 2024).

Sewage undergoes purification processes in wastewater treatment plants (WWTPs). Activated sludge systems are the most studied and extensively implemented secondary treatment for urban wastewater (Bui et al. 2022). They are dominated by bacteria, although eukaryotes (protozoa and fungi), archaea and viruses are also present in this type of process. This process requires mechanical aeration for adequate oxygenation of the water using blowers or turbines, which involves high operating costs due to the associated high energy requirements. In conventional wastewater treatment processes, the removal of organic matter has been well-optimised. However, carbon and nitrogen removal are carried out via volatilisation into the atmosphere whereas phosphorous is lost with the solids produced in the process. Indeed, the organic matter present in wastewater is converted to CO_2, which is a greenhouse gas, whereas nitrogen is either converted into N_2 through nitrification and denitrification processes or volatilized as NH_3 while phosphorous precipitates as phosphates and is removed with solids (Morillas-

España et al., 2020). Today, the removal of nitrogen and phosphorus demands increased energy expenditure and the installation of supplementary equipment and procedures. These three compounds are the main nutrients for some microorganisms including microalgae.

As an alternative, the use of microalgae for wastewater treatment has been proposed, thus industrial facilities already in operation demonstrate the feasibility of this technology. Among other advantages, such as lower investment and operation cost, the processes based on microalgae allow for the recovery of the nutrients contained in the wastewater (C, N, P) as microalgae biomass that can be used to generate several products, making the process economically viable (Siddiki et al., 2022). When produced using wastewater, the most studied application of the microalgae biomass is the production of agricultural products, mainly plant biostimulants (Morillas-España et al., 2022a). Thus, microalgae-related wastewater treatment offers the opportunity to transform wastewater into reusable water and biomass for agricultural uses, thus helping to increase the sustainability of food production systems.

However, in addition to regular contaminants such as organic matter, urban wastewater also contains relevant concentrations of compounds of emerging concern, among them pharmaceuticals (Maryjoseph and Ketheesan, 2020). Urban wastewater treatment plants receive pharmaceutical residues either through human excretions (in their original form or as metabolites) or through direct disposal of unused pharmaceuticals into drains, besides the discharges from hospitals and drug manufacturing industries. These compounds have become a family of ubiquitous pollutants that are routinely detected not only in the influents entering these treatment plants, but also in the treated effluents, which are discharged into surface waters (rivers, seas) and reach groundwater, not only through infiltration processes, but also through the application of sludge from treatment plants as fertilizer on agricultural lands. The surface and groundwaters, also containing residues from livestock farms, are transformed into drinking water for human consumption making possible its excretion again, and reentering in a cycle (Fatta et al., 2007; Paiga et al., 2016). These compounds play a relevant role in aquatic ecosystems and negatively affect biodiversity, therefore their removal is needed (van Dijk et al., 2023). Conventional treatment processes partially mitigate the occurrence of these compounds. The removal rates depends on many factors such as geographic location, weather, population density, water supply, physico-chemical properties of the pharmaceutical and treatment system parameters. In very general terms, a 50% global removal rate could be considered but the removals can vary notably in the same facility over the time. Even, it has been pointed out that the concentration in the effluent can be higher than that in the influent for certain compounds particularly resistant. For instance, the following removal rates have been reported after conventional treatments for some pharmaceuticals: from -36% (higher concentration in the outlet) to 100% for sulfamethoxazole, from -150% to 45% for ofloxacin, -89% for ciprofloxacin, from -89% to 98 for trimethoprim, from -110% to 70% for metronidazole, and 10-70% for diclofenac (di Marcantonio et al., 2020; Lindberg, R.H., Matesun et al., 2024; Molnarova et al., 2024; Neczaj, 2020; Wang et al., 2016; Yang et al., 2017; Zhang et al., 2023). As an alternative, the use of advanced oxidation processes is suitable for removing compounds of emerging concern, but their implementation and operation are expensive (Barbosa et al., 2016; Yang et al., 2017; Wang et al., 2020). The occurrence of pharmaceuticals in the environment, and particularly in surface waters, entails a list of adverse effects such as ecological damage, reproduction disruption, endocrine disruption, metabolic disruption, genotoxic effects, neurotoxicity, physiological effects, impact on aquatic flora and fauna, and altered soil microbial communities (Matesun et al., 2024). Thus, at relatively high concentrations in surface waters, stress and toxicity effects caused by pharmaceuticals such as sulfamethoxazole (Texeira and Granek, 2017), diclofenac (Zhang et al., 2019) and metronidazole (Hena et al, 2020) have been described for microorganisms, including microalgae. However, at the usual concentrations in surface waters the phenomena of acute toxicity are rather limited, being more worrying the possible chronic and synergistic effects, which are not well defined nowadays (Bolong et al., 2009; Xin et al., 2021).

Microalgae have been reported to be also feasible for the removal of these compounds from wastewater by a combination of mechanisms such as adsorption, direct or indirect photodegradation, bioaccumulation, biodegradation, volatilisation and hydrolysis (Wang et al., 2017; Leng et al., 2020; Hena et al., 2021). Some mechanisms lead to the degradation and complete removal of the compounds from the medium, while others result in their adsorption into the biomass, potentially compromising its final use. However, very scarce works have been performed in this respect, especially in real operation conditions. In this context, the goal of this study is to determine the feasibility of microalgae-related processes to remove pharmaceuticals from wastewater and the influence of the presence of these compounds both (i) on the production of biomass/nutrients recovery, and (ii) on further uses of treated water and biomass. For that, experiments were performed outdoors in pilot-scale thin-layer cascade photobioreactors fed in continuous mode in winter and summer. The removal and the influence of up to six pharmaceuticals was evaluated, they were selected because of their widespread occurrence in European wastewater treatment plants (Rodriguez-Mozaz et al., 2020). Results from this study are highly relevant for advancing industrial microalgae-based wastewater treatment processes, addressing significant gaps noted in current literature (Li et al., 2022). These findings also support regulatory changes for the use of products, such as water and biomass, from wastewater treatment systems in agriculture.

2. Materials and methods

2.1. Strain

The microalga used in this study was *Scenedesmus almeriensis* (CCAP 276/24) available at the University of Almeria, Spain. This strain is resilient to the environmental conditions of the region and exhibits a high growth rate throughout the year. It can tolerate temperatures up to 45 °C and pH ranges from 7 to 10, although its optimal conditions are around 30 °C and pH 8.0 (Villaró et al., 2022). In this study, the pH was controlled at 8 by on-demand injection of carbon dioxide.

2.2. Photobioreactor and operation mode

In this research, two thin-layer photobioreactors were utilized, located at the SABANA demonstration plant (www2.ual.es/sabana/) at the IFAPA Research Centre, next to the University of Almería (Almería, Spain). The reactors consisted of two fibreglass thin-layer reactors with identical characteristics, formed by a shallow channel together with a 0.5 m^3 polyethylene tank. The reactors had a total area of 10 m^2 and were operated at a 0.02 m constant depth, which means that the total volume of the culture was 0.45 m^3. At the bottom of the tank, a plate membrane diffuser injected CO_2 on demand with a flow rate of 2.2 L min^{-1} (Rodríguez-Torres et al., 2021). The culture medium used was wastewater obtained after a secondary treatment at the wastewater treatment plant "El Bobar" located in Almería, Spain. The biomass was produced in semi-continuous mode at a dilution rate of 0.3 d^{-1} from Monday to Friday. This means that every day (from Monday to Friday) 30% of the total volume of the culture was removed and replaced with secondary wastewater. The biomass was harvested by centrifugation, frozen and stored at -20 ºC until further analysis (usually within 3 weeks).

One of the reactors was used as control; hence, it was operated using unspiked wastewater. The water entering the other reactor was spiked with known concentrations of selected pharmaceuticals. Six pharmaceuticals were selected among 17 antibiotics detected at least once in the final effluents of 4 wastewater treatment plants located in 7 European countries (Rodriguez-Mozaz et al., 2020). The pharmaceuticals selected were sulfamethoxazole, trimethoprim, metronidazole, ofloxacin, ciprofloxacin, and diclofenac. These compounds were added daily to the inlet effluent at a concentration of 2.9-5.0 $ng \cdot L^{-1}$ to mimic real concentrations reported in the bibliography for these compounds.

The experiments were carried out in two different seasons, winter and summer. Six aliquots of effluent, influent, and microalgal biomass were daily taken from the spiked and non-spiked (control) reactors to monitor the occurrence of the target compounds.

2.3. Analytical determinations

2.3.1. Biomass concentration

The biomass concentration was assessed by dry weight after drying the biomass in an oven at 80 °C for 24 h. Biomass productivity was calculated by multiplying the biomass concentration by the dilution rate (0.3 day^{-1}). The Fv/Fm ratio, indicative of the maximum quantum yield of PSII chemistry, was measured daily using an AquaPen AP 100 fluorimeter (Photon System Instruments, Drásov, The Czech Republic). Before measurements, microalgal cells were dark-adapted for 15 minutes.

2.3.2. Nutrients

The officially approved standard procedures of the Spanish Ministry of Agriculture were used to assess the nitrogen and phosphorus composition of the culture medium and the supernatant once the biomass was harvested. Briefly, N-NO_3^- was quantified using a Genesys 10S spectrophotometer (Thermo Fisher Scientific, Barcelona, Spain) by measuring absorbance at 220 and 275 nm. In addition, N-NH_4^+ was determined using the Nessler reactive method and P-PO_4^{3-} was evaluated using visible spectrophotometry via the phospho-vanado-molybdate complex. The nutrient content of the medium (after removing the biomass) was analysed at the beginning (day 0), before inoculation, at the end of the batch phase, and after completion of the semi-continuous production (Acién Fernández et al., 2018). Three determinations were made for each sample.

2.3.3. Pharmaceuticals

Wastewater samples were analysed by a fast direct-injection LC-MS/MS analytical procedure already described by de la Serna Calleja et al., (2023). Briefly, 100 ml samples were filtered through 0.45 µm nylon membranes and spiked with internal standards at

1,000 ng L^{-1}. Then, 2 mL of the resulting solutions were filtered by 0.22 µm PTFE syringe filters just before instrumental analysis. Filtrates were collected in 1.5 mL amber vials. The content of pharmaceuticals in algal biomass was determined using an analytical method previously developed for veterinary drugs (López-Serna et al., 2022). Briefly, lyophilized microalgal samples (0.3 g) were extracted twice, with 15 and 10 mL, respectively, of a 90:10 (v/v) water/methanol mixture, assisted by ultrasonic shaking in the presence of primary and secondary amine (PSA) (0.3 g). The supernatants separated by centrifugation were combined. Then, ethylenediaminetetraacetic acid disodium salt (EDTA) was added to achieve a concentration of 0.1% (w/v), and the resulting solution was diluted to 100 mL with ultrapure water before instrumental analysis.

An Exion LC AD liquid chromatograph coupled to a triple quadrupole 6500+ mass spectrometer, both from AB Sciex (Framingham, MA, USA), was used. The chromatograph was fitted with an EVO C18 column (50 × 2.1 mm, 1.7 µm) from Phenomenex (Torrance, CA, USA) at 40 °C. The mobile phase flow rate was 0.5 mL·min^{-1} and it consisted of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in methanol (B). The analyses were done in a gradient mode: from 5% B (kept for 1 min) to 95% B in 2 min, kept for 3 min. The re-equilibration time was 4 min. The injection volume was 400, 200 and 10 µL for the effluent, influent, and biomass samples, respectively.

MS/MS data were acquired by an Analyst (AB Sciex) software with an electrospray ionisation interface in the positive mode, working in selected reaction monitoring (SRM) mode. Two SRM transitions were monitored for each compound, between the precursor ion and the two most abundant fragment ions. A custom-made standard addition calibration method was developed for each type of matrix. Aliquots of each sample type were spiked with increasing pharmaceutical concentrations. The lyophilised microalgae samples were allowed to stand at room temperature and in darkness for 12 hours to evaporate the organic solvent and embed the analytes in the sample matrix. Peak areas of the most intense SRM transitions, which were measured in the chromatograms by using the OS software (AB Sciex), were used for quantification. The detection limits of the analytical methodology for aqueous samples were close to 5 ng·L^{-1} for sulfamethoxazole, 6 ng·L^{-1} for trimethoprim, 10 ng·L-1 for metronidazole and ciprofloxacin, 5 ng·L^{-1} for ofloxacin, and 50 ng·L-1 for diclofenac. The detection limits in biomass samples were about 1 ng·g^{-1}

2.4. Biostimulant capacity

The evaluation of the biostimulant capacity of the collected microalgal biomass was conducted after disrupting the cell wall using an UP 400 S ultrasonic processor (Hielscher Ultrasonics, Germany) as described elsewhere (Morillas-España et al., 2022b). *In vitro* bioassays were performed to measure the germination index of watercress seeds (*Lepidium sativum L*.), induction of adventitious soybean roots (*Glycine max L*.), and the expansion of excised cucumber (*Cucumis sativus L*.) as described previously (Navarro-López et al., 2020). Distilled water was used as a negative control, and the hormones 6-benzylaminopurine (BAP) and indol-3-butyric acid (IBA) were used as positive controls. Extracts were evaluated in triplicate at biomass concentrations of 0.5 and 2.0 g·L^{-1} and commercial hormones were evaluated at a concentration of 0.3, 1.0 and 5.0 mg·L^{-1}.

2.5. Statistical analysis

Variations between seasons and culture conditions were assessed using analysis of variance using Centurion XVI statistical software. The criterion for the least significant difference (LSD) was established at $p<0.05$ in all cases. The relationships between different variables were examined using bivariate Pearson correlation ($p<0.05$).

3. Results and discussion

3.1. Production of biomass

Experiments were performed in Almería (South of Spain), where the environmental conditions are favourable for microalgae production all through the year. However, environmental conditions significantly impact the performance of microalgae (Morillas-España et al., 2020). Therefore, trials were conducted during both summer and winter seasons (Figure 1). The mean daily solar global radiation during the experiments was 280 and 440 $W{\cdot}m^{-2}$ in winter and summer, respectively, whereas the average temperatures ranged from 15 to 25 °C. Regarding solar radiation, the maximum levels reach up to 700 and 870 $W{\cdot}m^{-2}$ in winter and summer, respectively, whereas maximum and minimum temperature values were 10-19 ºC in winter and 21-29 ºC in summer.

For all the season the photobioreactors were operated at a constant dilution rate of 0.3 day^{-1}, which was found to be the optimal rate for this strain and location irrespective of the season (Morillas-España et al., 2020). Despite the constant dilution rate, the variation in environmental conditions also provokes changes in the culture conditions such as pH, dissolved oxygen and temperature, which finally influence the performance of the culture such as biomass productivity and the status of the biological system such as fluorescence of chlorophylls (Figure 2). Results show that biomass productivity increases from winter to summer but no differences were observed when adding pharmaceuticals. On average the biomass productivity in winter was 8.2 $g{\cdot}m^{-2}{\cdot}day^{-1}$ whereas it increases up to 10.4 $g{\cdot}m^{-2}{\cdot}day^{-1}$ in summer. This increase was much lower than previously reported due to the limitation of nutrients that take place in these experiments because of the use of wastewater after secondary treatment. Similar results were obtained in previous studies when working with secondary wastewater and thin-layer cascade reactors (Villaró et al., 2022). Productivities of 30 $g{\cdot}m^{-2}{\cdot}day^{-1}$ have been reported in previous work using thin-layer reactors and commercial fertilizers as the nutrient source (Zurano et al., 2020). The lack of nutrients has also a negative impact on the status of the biological system thus the fluorescence of chlorophylls reduces from winter to summer time, with mean values decreasing from 0.57 to 0.32. The fluorescence of chlorophylls for *S.*

almeriensis cultures at optimal conditions is 0.7, with values below 0.5 indicating adverse culture conditions.

Regarding culture conditions, the pH was maintained at 8.0 through on-demand CO_2 injection, ensuring both optimal pH and carbon availability. However, maximum dissolved oxygen and temperature varied significantly between seasons, though these changes were not influenced by the presence of pharmaceuticals. Dissolved oxygen concentration in winter achieves values up to 265 %Sat, whereas in summer the maximum value was 300 %Sat., indicating the existence of photooxidation conditions in larger extension in the summer time. The maximum water temperature was also higher in the summertime, up to 32 ºC versus 24 ºC achieved in the wintertime, also indicating larger damage by the excess temperature in this period. Thin-layer reactors showed inhibitory problems caused by the accumulation of dissolved oxygen reaching concentrations higher than 250 %Sat (Morillas-España et al., 2021a). The control of temperature and dissolved oxygen concentration in large-scale thin-layer reactors continues to be a challenge.

3.2. Recovery of nutrients

Concerning nutrients, microalgae are specialists in the removal of nutrients such as N and P, especially when available at low concentrations, due to the low saturation constants concerning these compounds (< 5 mgN/L and <1 mgP/L). During the experiments, the concentration of nutrients at the inlet varies from 88.2 mgN·L^{-1} and 7.4 mgP·L^{-1} in winter to 53.0 mgN·L^{-1} and 3.6 mgP·L^{-1} in summer (Figure 3). These values of nutrient concentration are low when compared with standard culture medium used for the production of microalgae containing up to 200 mgN·L^{-1} and 20 mgP·L^{-1}. Moreover, biomass productivity is expected to be higher in thin-layer photobioreactors compared to raceway reactors, which could lead to nutrient limitation conditions. Results show that in wintertime both N and P remain at the outlet at concentrations of 20 mgN·L^{-1} and 2 mgP·L^{-1}, whereas in summer the nitrogen was completely removed and the phosphorous concentration at the outlet was lower than 0.6 mgP·L^{-1}. This behavior confirms that a nitrogen limitation is present in the cultures, particularly during summer, which is responsible for the low biomass productivity observed. However, no differences were observed in the concentrations of nutrients due to the presence of pharmaceuticals. Thus, equal nutrient concentrations were measured at the outlet, confirming that the spiked compounds do not affect microalgal growth and nutrient assimilation at the concentrations studied.

The existence of nutrient limitation conditions determines the nutrients removal capacity, thus during summer, the nitrogen removal capacity ranged between 770 and 840 mg·m^{-2}·day^{-1}, resulting in the removal of 98% of the nitrogen in the inlet and outlet concentrations near zero. Similar results were previously obtained in pilot HRAPs (Gutiérrez et al., 2016). The removal of nitrogen during winter was lower (73-78%). This was attributed to (i) the total nitrogen inlet concentrations, which were higher, and (ii) the temperature and solar radiation values which were lower. A similar trend was observed for phosphorus, with higher inlet concentrations (7.4 mg·L^{-1}) in winter than in summer (3.6 mg·L^{-1}). This is due to the low inlet concentration and the summer conditions (higher solar radiation and temperature) enhancing microalgal activity and, consequently, nutrient removal. To maximise the productivity of the system, a recent study evaluated the potential use of membranes in raceway reactors to separate the cellular from the hydraulic retention time, achieving a 40% increase in the areal biomass productivity (Morillas-España et al., 2021b). One of the points to highlight is the ability of microalgae to recover nitrogen and phosphorus from water to transform it into biomass. Especially phosphorus, which is a limited resource on the planet.

The recovery of nitrogen and phosphorus, respectively, is represented in Figure 3C and 3D. As in the rest of the work, the comparison is also made in winter and summer. If we focus on nitrogen, it is observed that there are no significant differences between the seasons, the recovery being between 800 and 950 mg·m^{-2}·day^{-1}. In contrast, in the case of phosphorus (Figure 3D), although there is no distinction between the doped and undoped reactor, there are significant differences between winter and summer. On the one hand, the maximum recovery of phosphorus has been around 60 $mg \cdot m^{-2} \cdot day^{-1}$ in winter, being similar in the spiked and non-spiked reactor. On the other hand, the minimum recovery of phosphorus has been in summer in the spiked reactor (25 $mg \cdot m^{-2} \cdot day^{-1}$). In the non-spiked reactor in summer the recovery has exceeded 40 $mg \cdot m^{-2} \cdot day^{-1}$.

3.3. Removal of pharmaceuticals

Pharmaceuticals such as those studied in this work are gaining increased attention due to their potential risk to human health and the environment (Maryjoseph and Ketheesan, 2020). Five out of the six (sulfamethoxazole, trimethoprim, metronidazole, ofloxacin, and ciprofloxacin) are antibiotics whose occurrence in the environment promotes the production of resistance genes in pathogenic bacteria. On the other hand, diclofenac is on the current Watch List included in the Europen Union Water Framework (European Commission, Joint Research Centre, 2020), as it exerts inhibition effects on certain animal and human enzymatic pathways.

The concentration at the influent and effluent of the reactor in experiments performed using raw wastewater, without spiking it with these compounds ,was measured. Results show that the considered pharmaceuticals can be found in this type of effluent. Ofloxacin, diclofenac, and ciprofloxacin were detected both in winter and summer. In winter, sulfamethoxazole and trimethoprim were also detected. Finally, metronidazole was not detected in either season. The concentrations of these compounds ranged from maximal values of 420 $ng \cdot L^{-1}$ for diclofenac to 40 $ng \cdot L^{-1}$ for ciprofloxacin. Remark that the total concentration of pharmaceuticals at the inlet was much higher in winter, up to 1150 $ng \cdot L^{-1}$ than in summer, up to 840 $ng \cdot L^{-1}$. Although the presence of these compounds is significant, their measured concentrations are very low, which facilitates their removal. However, the removal process varies for each compound. Data shows that ciprofloxacin was the most easily removed, it being completely removed both in winter and summer.

Next, ofloxacin was removed by 63 and 75% in winter and summer respectively, whereas diclofenac was removed by 52 and 70% in winter and summer respectively. Sulfamethoxazole and trimethoprim were only detected in winter, where they were removed by 53% and 60%, respectively. Since most of these compounds were not completely removed, trace concentrations were still detected at the outlet. Thus, the effluent total concentration of CECs was 400 $ng \cdot L^{-1}$ in winter and 220 ng·L-1 in summer, corresponding to 65% and 74% removals, respectively**.** These results confirm that the removal capacity was higher in summer than in winter.

To better evaluate the capacity of microalgae culture to remove the selected pharmaceuticals, the inlet water was spiked by adding the selected compounds at concentrations up to 20 times higher.

As regards the experiments after spiking the influent of the reactor, metronidazole was removed by 85% and 99% in winter and summer, respectively, which agrees in general terms with the results of previous work. In that work, metronidazole was completely removed in microalgae cultivated in a culture medium. Sulfamethoxazole was also largely removed from the water, removal percentages were about 97% and 91% in winter and summer, respectively. This contrasts to previous works in which sulfamethoxazole was relatively persistent in microalgae degradation assays carried out using urban wastewater with removal rates lower than 47% (Gentili and Fick, 2017; Xiong et al., 2019). Similarly, the removal rates of diclofenac were high, 86% in winter and 98% in summer following other work that concluded that diclofenac, in concentrations of 25 $mg.L^{-1}$, could be removed by up to 98% in culture media (Sánchez-Sandoval et al., 2021). The removal of ofloxacin in this work was 75% and 86% in winter and summer, respectively. These percentages were higher than those previously published in a work using urban wastewater, with removal values in the range of 44-54% (Zhou et al., 2014). The removal of ciprofloxacin resulted to be markedly different in winter and summer, with removal rates of 56% and 99% for winter and summer respectively. Overall, the data in the literature are highly variable, with removal percentages from urban wastewater ranging from 48 to 97% in some works (Zhou et al., 2014; Hom-Diaz et al., 2017a,b) and a removal rate of ciprofloxacin 11% in other studies (Gentili and Fick, 2017). Trimethoprim was the most persistent antibiotic. The removal percentages in this experiment were 44% in winter and 50% in summer, which are somewhat higher than the values below 40% reported in studies focused on urban and industrial wastewaters or culture media (Zhou et al., 2014; Gentili and Fick, 2017; Gojkovic et al., 2019). In summer the removal percentage was similar but in winter the removal capacity was around 44% lower. In the same way, the removal rates for the other compounds were around 10-40% lower in comparison with those monitored in the spiking experiments. The content of sulfamethoxazole in summer decreased by 53%. The differences in the removal rates of ofloxacin concerning the spiking experiments were less marked, its removal percentages were 63% (winter) and 75 % (summer) varying only by 11% from one another. Trimethoprim, whose presence was only observed in winter, was not removed.

The concentration of these compounds found in the influent, effluent, and biomass grown in the reactors, and their removal percentages are resumed in Figure 4.

It is inferred from the data of the spiked and non-spiked experiments that the removal rates are generally higher in summer, which is understandable because in this season the solar radiation intensity and environmental temperature are higher, and they favour the metabolic processes of microalgae. Photochemical reactions can also promote their degradation .

The concentration of pharmaceuticals determined in the harvested biomass was relatively low. In the reactor spiked the concentrations, expressed as $ng \cdot g^{-1}$ of biomass (on a dry weight basis), resulted to be about 30, 200-250, 200-250, 300-400 and 5 $ng \cdot g^{-1}$ for sulfamethoxazole, trimethoprim, ofloxacin, ciprofloxacin, and metronidazole, respectively. There is no data for diclofenac. When the biomass of the non-spiked reactor, with lower concentrations in the influent, was analyzed, the concentrations were notably lower: about 20, 10-100, 10-60 and 70-80 $ng \cdot g^{-1}$ for sulfamethoxazole, trimethoprim, ofloxacin, and ciprofloxacin, respectively. Metronidazole was not detected in the biomass. Thus, the percentage of pharmaceutical incorporated into the biomass from the reactor influent was relatively high for trimethoprim (up to 6%), ciprofloxacin (up to 4%), and ofloxacin (up to 4%), and relatively low for sulfamethoxazole (below 0.1%). The concentrations determined in biomass, influent and effluent are all at trace levels. They are mostly lower than 1 $ng \cdot g^{-1}$ and the accuracy of their determination is scarce as a consequence of two factors: the uncertainty associated with the determination of traces and a possible inhomogeneity of the samples collected in the reactor. In any case, a mass balance has been done, considering for each pharmaceutical that the addition of the removal percentage in the effluent and the percentage of accumulation on biomass must be 100%. Only in five balances did the error exceed 40%, 4 out of them were observed in experiments at low concentrations in the winter season (sulfamethoxazole, trimethoprim, ofloxacin and diclofenac) and the fifth one involved the mass balance for trimethoprim in summer season after spiking the reactor with a high concentration.

As previously mentioned, the removal of pharmaceuticals is ascribed to a combination of mechanisms, whose predominance in the experimentation can be roughly estimated despite of the different findings published in the bibliography. Thus, the low quantities of pharmaceuticals found in biomass are coherent because the pharmaceutical adsorption on the biomass is not the most important mechanism for most of the assayed compounds (Hom-Diaz et al., 2017b; Reyman et al. , 2020; de Wilt et al., 2016). Also, our results show that metronidazole and sulfamethoxazole are the compound with lowest accumulation in biomass with agrees with previously published reports, which remark the scarce relevance of the adsorption mechanism in the removal of these pharmaceuticals (García-Galán et al., 2020; Reyman etr al., 2020; Zambrano et al., 2021). On the contrary, it has been stated that the adsorption is a relatively relevant mechanism for trimethoprim since an accumulation percentage of 7.6%, has been found in the biomass with respect the initial concentration (De Wilt et al., 2016). This percentage is of the same order that that determined in this work (6%). In the same way, it is commonly assumed that the removal by chemical reactions, mainly hydrolysis processes, is almost negligible (Hena et al., 2021; Leng et al., 2020).

The direct and indirect photodegradation, and the biodegradation by microalgae and their bacteria consortium are the predominant mechanisms to remove pharmaceuticals from wastewaters by using photobioreactors. Thus, published data suggest that ciprofloxacin (Bay and Acharya, 2017; Hom-Diaz et al., 2017b ; Zambrano et al., 2023) and metronidazole (Liu et al., 2021) are prone to notable degradation by photolysis. Diclofenac (de Wilt et al., 2016; Sánchez Sandoval et al., 2021) and sulfamethoxazole (Bay and Acharya, 2017) are removed by photodegradation, too. Moreover, it has been pointed out that the biodegradation of sulfamethoxazole (Gómez et al., 2013) and levofloxacin, optical isomer of ofloxacin (Xiong et al., 2017) are also significant. On the contrary, trimethoprim is a rather persistent compound, with degradation rates lower than 60% in the best of the cases (Bay and Acharya, 2016; Zeeman, 2016); in fact, trimethoprim was the lowest removed pharmaceutical confirming previous observations. The concentration of the rest of pharmaceuticals decreased by 56-99% in the assays carried out in the photobioreactor spiked at high concentration, and the photodegradation and biodegradation mechanisms are supposed to be the main contribution to the removal.

The effectiveness of the removal mechanisms can vary with the seasonal weather conditions. An increase of the solar radiation intensity and environmental temperature favor the biomass productivity, so that biodegradation mechanism should be enhanced. The biodegradation involves one or more reactions catalyzed by enzymes. On the other hand, an intense solar radiation entails a higher absorption of radiation by the pharmaceuticals, favoring the direct photodegradation (breakdown of its structure after electronic excitation) and the indirect photodegradation. In the latter case, the radiation hits the organic matter present in the medium, oxygen radical species are yielded and these species react with the pharmaceuticals. In the same way, an increase of temperature increases the rate of the photochemical and chemical reactions, although this circumstance is not enough for hydrolysis reactions in aqueous media to be relevant in the removal of these micropollutants. A possible drawback of a high temperature would be the lowest adsorption capacity of pharmaceuticals on the microalgae cell walls. In summary, assuming that the biodegradation and photodegradation mechanisms are the most relevant, removal rates are expected to be higher in summer in comparison with those obtained in winter. Except for sulfamethoxazole, our data are consistent with this assumption, the removal rates improved between 6 and 43% in summer. The removal of sulfamethoxazole in winter was a 6% higher

3.4. Use of the biomass as plant biostimulant

In the case of the root formation test (Figure 5a), all biomasses tested showed a statistically significant root induction with respect to the negative control. In addition, the biomasses harvested in winter showed a higher induction of root formation than the biomasses harvested in summer. On the other hand, the cucumber cotyledon expansion test showed no statistically significant differences between the different biomasses tested, nor with respect to the negative control. Finally, the chlorophyll retention in leaves was only statistically significant in the TL-Spiked 2 g/L treatment in summer.

The results obtained in the biostimulant activity (Figure 5) tests showed that the biomass from the spiked photobioreactor presented similar auxin results to the biomass from the non-spiked photobioreactor. Therefore, this biomass could be used in agriculture because its effect is maintained and is not affected by the presence of pharmaceuticals during the culture. These results agree with those obtained in the biomass analysis, which shows the ability of the microalgae to remove the pharmaceuticals present in the culture medium. Other studies have already proven the efficiency of treatments based on microalgae for the bioremediation of emerging micropollutants, such as Vassalle et al., (2020), who evaluated the efficiency of a semi-closed horizontal tubular photobioreactor (PBR) at demonstrative scale to remove a total of 35 target compounds, including benzotriazoles, benzophenones, antibiotics and different pharmaceuticals present in irrigation water in a peri-urban rural area, proving an efficient removal of pharmaceuticals and other compounds, with complete removal in most cases. On the other hand, most of the studies on the effects of these compounds in plants have been based on morphological variations and biochemical changes, but to fully understand the possible negative effects of pharmaceuticals, it will be necessary to extend these analyses to plant physiology and molecular biology. This will make it possible to detect physiological effects (both positive and negative) at lower concentrations of the toxicants than those necessary to cause biochemical or morphological alterations, thus increasing their predictive power (Mansilla et al., 2021).

4. Conclusions

The present study demonstrated the effectiveness of microalgae in removing several contaminants of concern during different seasons of the year. The results indicate that the biomass resulting from the removal process can be harnessed as a valuable plant biostimulant. These findings open new perspectives in the field of environmental biotechnology and propose the potential use of microalgae as a sustainable strategy to mitigate the presence of micro-contaminants in wastewater. However, further investigations are required to fully comprehend the mechanisms involved in the process. Considering the challenges in managing emerging compounds, this study provides a robust foundation for future research and the development of practical and effective solutions for safeguarding our environment and public health.

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6. Author contribution statement

A. Morillas-España: Formal analysis, Research, Writing – Original Draft; **R. López-Serna:** Formal analysis, Research, Writing – Review & Editing; **L.Y. Rodríguez Chikri:** Formal analysis, Research, Writing – Review & Editing; **J.J. Jiménez:** Supervision, Writing – Review & Editing; **T. Lafarga:** Supervision, Writing – Review & Editing, Funding acquisition; **E. Uggetti:** Research, Supervision, Writing – Review & Editing; **G. Acién:** Supervision, Funding acquisition, Writing – Review & Editing; and **C.V. González-López:** Supervision, Writing – Review & Editing, Funding acquisition.

7. Data availability statement

The data will be made available from the corresponding author upon reasonable request.

8. Conflicts of interest disclosure

The authors declare no competing financial interest.

LEGENDS OF FIGURE

Figure 1.- Environmental conditions, solar radiation and temperature, prevailing during the time on which the experiments were performed. Datas represent the mean values ± SD.

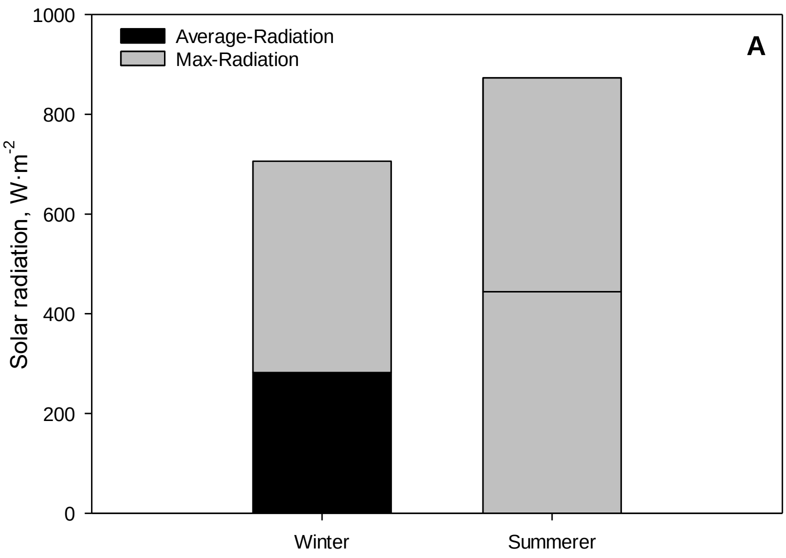
Figure 2.- Variation of biomass productivity, fluorescence of chlorophylls and culture conditions (dissolved oxygen, temperature) as a function of season and the addition of pharmaceuticals. Values represent the mean values ± SD. Different letters indicate significant differences ($p < 0.05$).

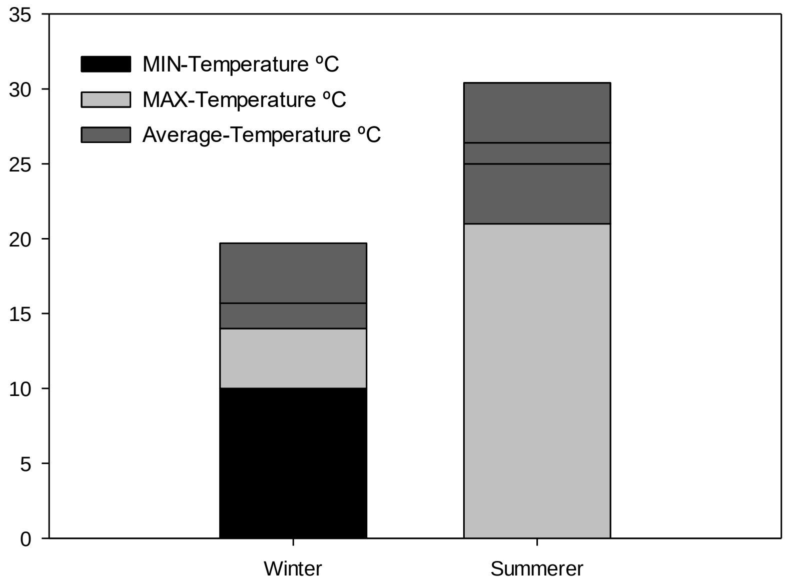
Figure 3.- Variation of nutrient (nitrogen and phosphorous) concentration, removal and recovery rate of as a function of season and the addition of pharmaceuticals. Values represent the mean values ± SD.

Figure 4.- Concentration of pharmaceuticals at the inlet/outlet and removal as a function of season in non-spiked and spiked cultures. Values represent the mean values ± SD.

Figure 5.- Biostimulant effects of the biomass produced in each season in spiked and non-spiked cultures. (A) Root development, (B) cotyledon expansion and (C) Chlorophyll retention. Values represent the mean values ± SD.

FIGURES





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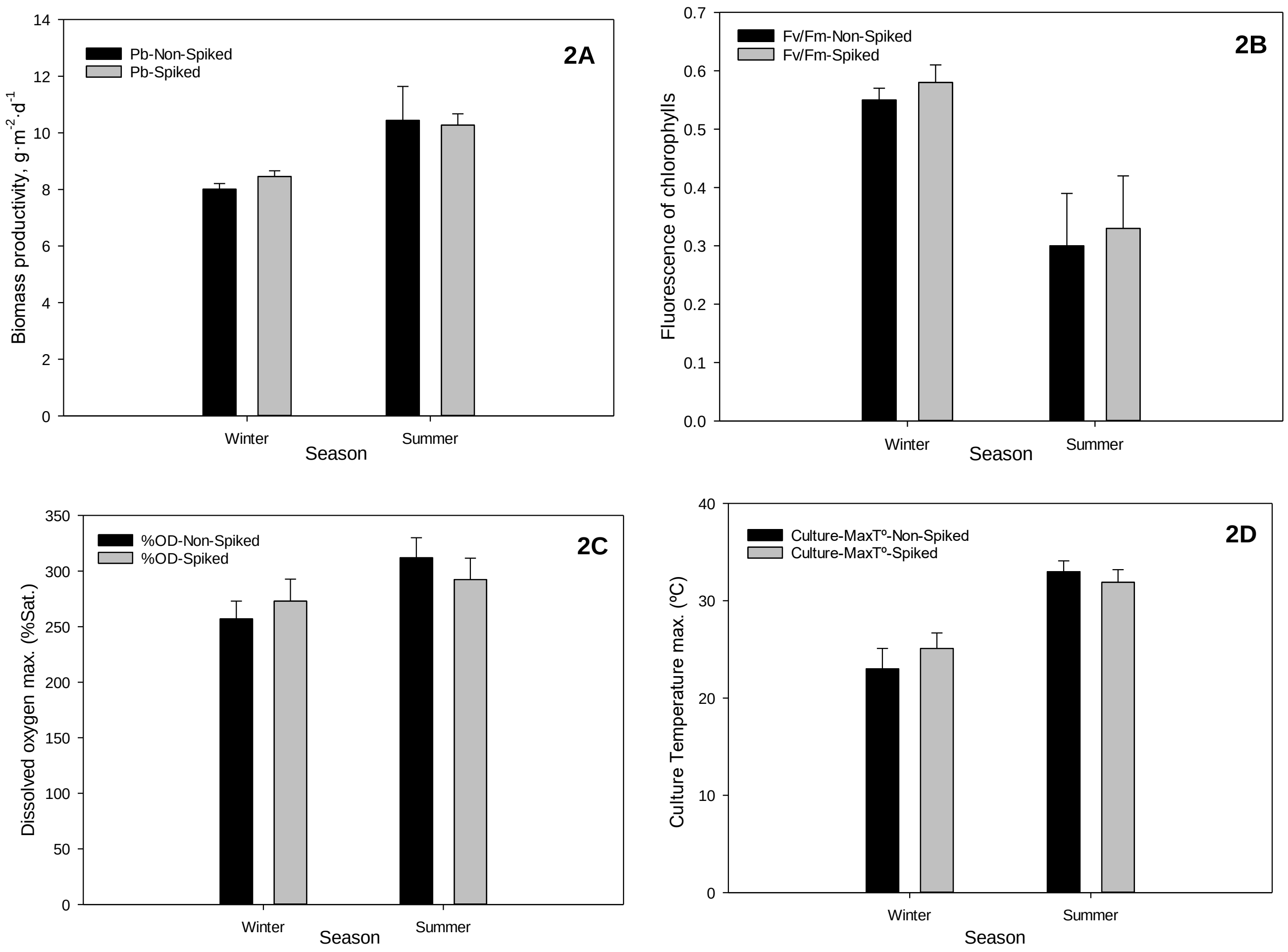

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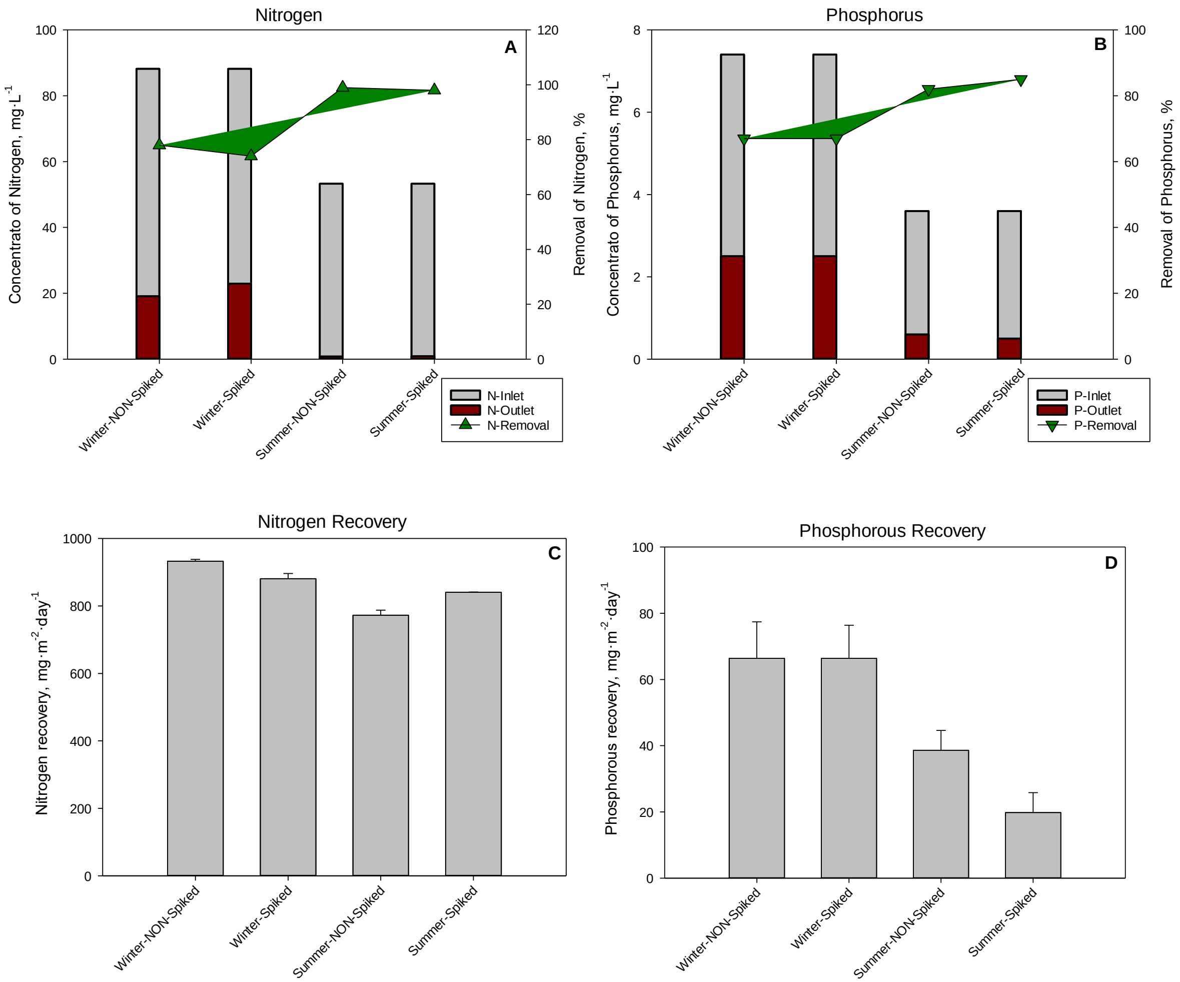
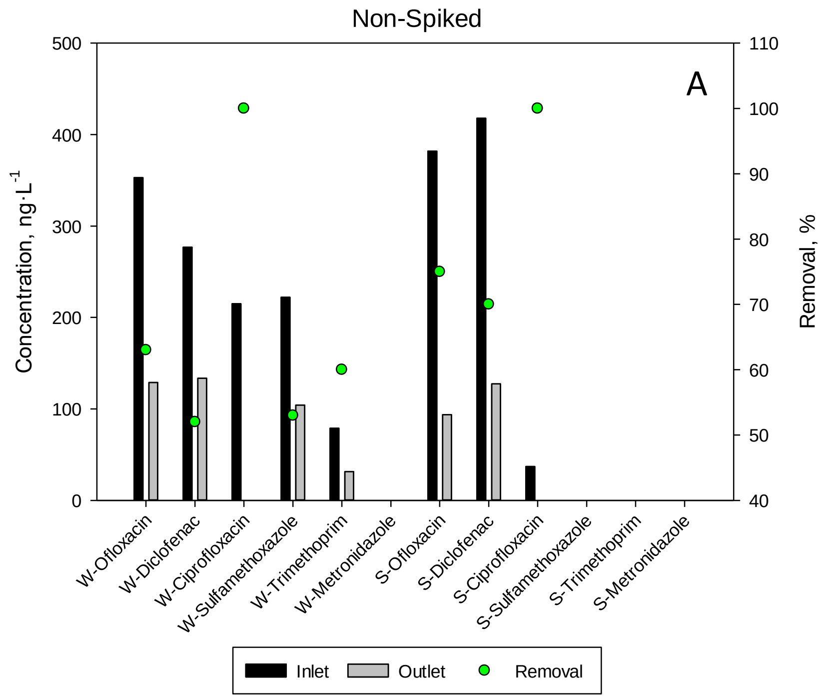
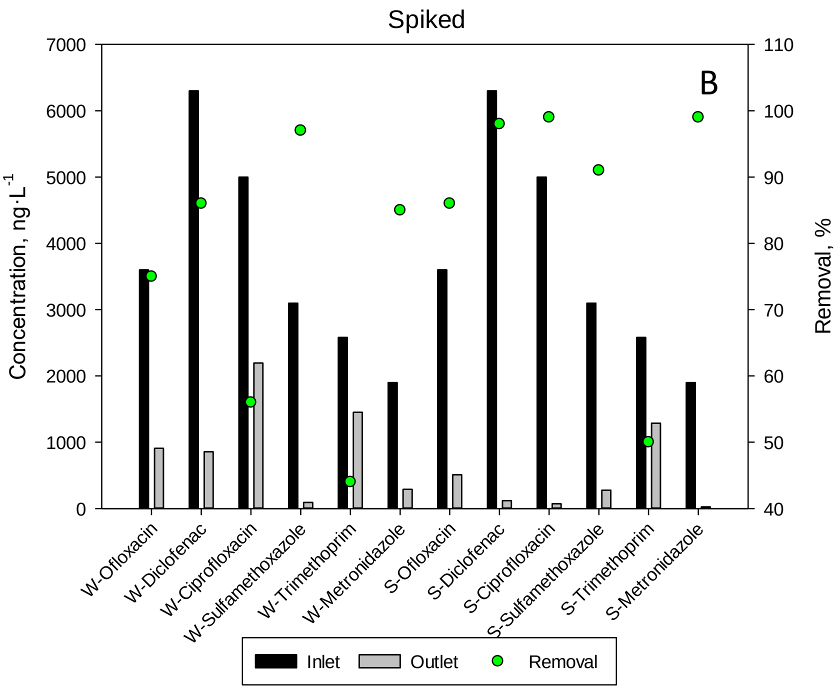


Figure 3.- Variation of nutrient (nitrogen and phosphorous) concentration, removal (A and B) and recovery rate of as a function of season and the addition of pharmaceuticals (C and D). Values represent the mean values ± SD.


Non-Spiked
A
Concentration, ng·L-1
Removal, %
500
400
300
200
100
0
110
100
90
80
70
60
50
40
W-Ofloxacin
W-Diclofenac
W-Ciprofloxacin
W-Sulfamethoxazole
W-Trimethoprim
W-Metronidazole
S-Ofloxacin
S-Diclofenac
S-Ciprofloxacin
S-Sulfamethoxazole
S-Trimethoprim
S-Metronidazole
Inlet
Outlet
Removal


Spiked
B
Concentration, ng·L-1
Removal, %
7000
6000
5000
4000
3000
2000
1000
0
110
100
90
80
70
60
50
40
W-Ofloxacin
W-Diclofenac
W-Ciprofloxacin
W-Sulfamethoxazole
W-Trimethoprim
W-Metronidazole
S-Ofloxacin
S-Diclofenac
S-Ciprofloxacin
S-Sulfamethoxazole
S-Trimethoprim
S-Metronidazole
Inlet
Outlet
Removal

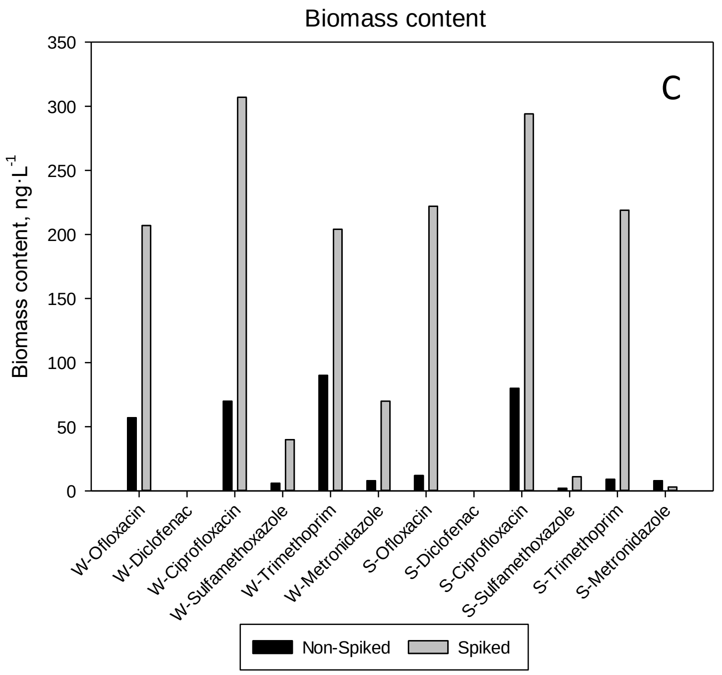
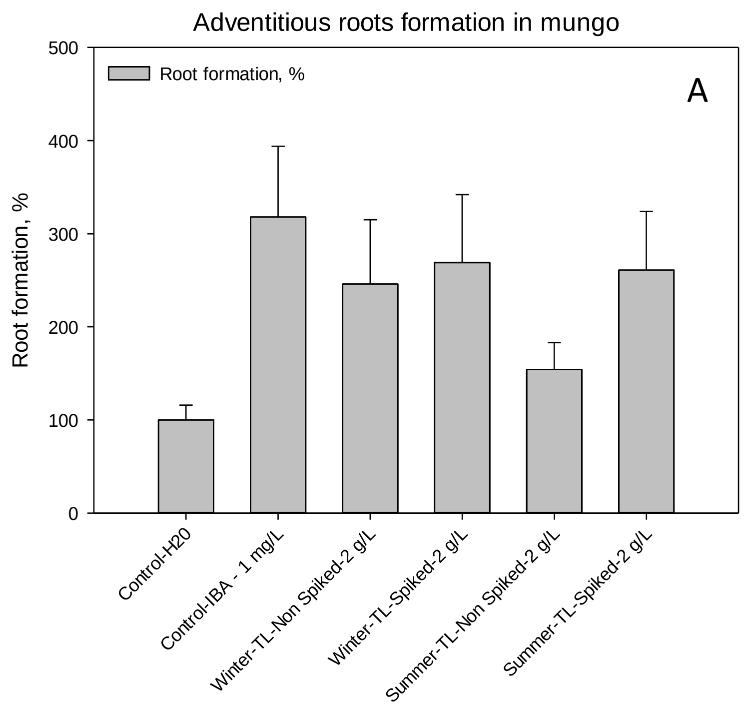
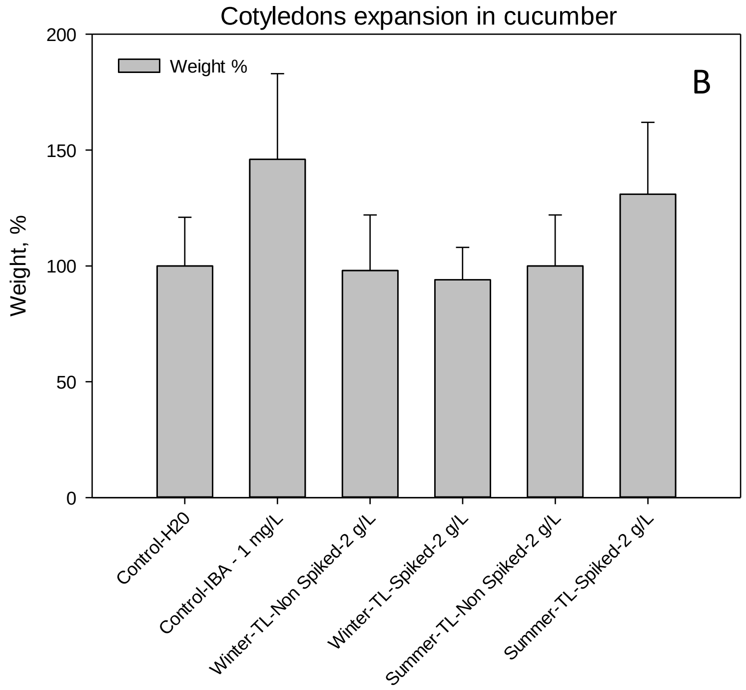


Figure 4.- Concentration of pharmaceuticals at the inlet/outlet and removal as a function of season in non-spiked (A) and spiked (B) cultures, and biomass content (C). Values represent the mean values ± SD.





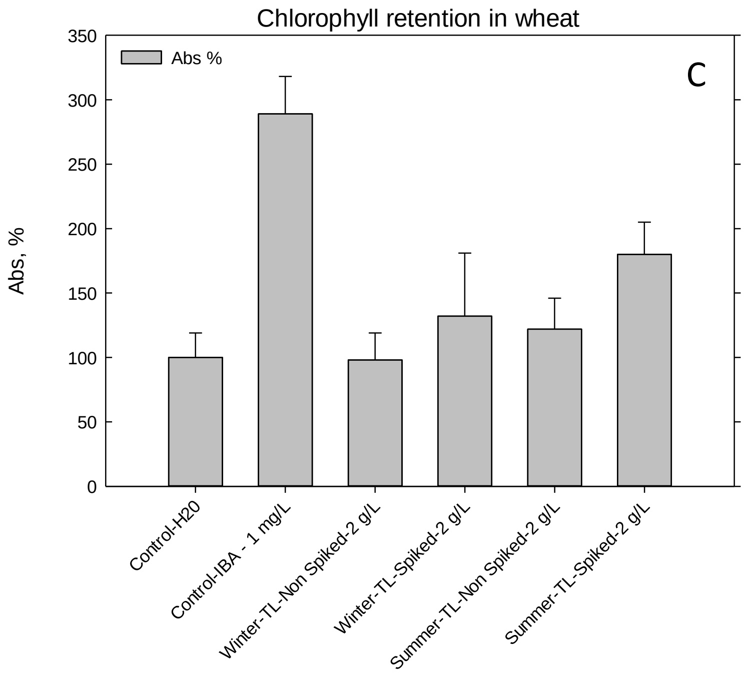


Figure 5.- Biostimulant effects of the biomass produced in each season in spiked and non-spiked cultures. (A) Root development, (B) cotyledon expansion and (C) Chlorophyll retention. Values represent the mean values ± SD.

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