Sequential use of Microalgae and Constructed Wetlands and its potential to remove organic load, toxicity and pharmaceuticals from urban wastewaters

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Abstract

The present study aimed at to evaluate the performance of integrated system composed of microalgae (MA) and constructed wetlands (CW). The MA production tank was set up as a raceway type and microalgae of the genus *Chlorella* were inoculated, while the CW was set up inspired by the first stage of the French model and vegetated with the macrophytes *Chrysopogon zizanioides*, *Typha domingensis* and *Dracaena trifaciata*. The Hydraulic Retention Time in each unit was of 7 days, totaling 14 days of treatment. Promising results were obtained regarding the reductions of dissolved organic carbon (67.2%) and Total N (68.6%). The treatment was also able to fully remove the acute ecotoxicity against *Dapnhia magna* and the genotoxicity in the *A. cepa* test assay. Chromatographic analysis showed the presence of 9 pharmaceuticals in the raw wastewaters, whereas after the CW unit only residuals of dipyrone (1%) were still detected. Future recommendations include the investigation of the main removal mechanisms of the pharmaceuticals and improvements of the separation process of the MA.

1. Introduction

The availability of adequate freshwater is a crucial factor for the survival of living organisms. Recent data of a UNICEF report show that half of the world's population could be living in areas facing water scarcity by as early as 2025 (UNICEF, 2023). So, wastewater treatment as a tool for sustainable water management is one of the global goals of the Sustainable Development Goals proposed by the United Nations (UN) (Scrucca et al., 2023). On the one hand, poorly managed wastewater can lead to risks to human health and the environment. But on the other hand, it might be considered as an opportunity for a circular bioeconomy (Satya et al., 2023).

In Brazil, only 53% of the population has access to wastewater treatment systems (SNIS, 2022). Furthermore, most of them consist of centralized units that make use of traditional treatment methods. The implementation of such treatment plants in large area countries such as Brazil is difficult, since due to low population density of some areas, the effluents has to be transported for long distances, often requiring pumping stations to reach the treatment area, raising considerable the costs associated with the treatment (Oliveira et al., 2021)

Over the last years, considerable attention has been paid to the presence of emerging contaminants (ECs) in water bodies. Despite the low environmental concentrations, authors have stated that this group of contaminants may pose threatens to living organisms due to their potential toxic effects (Shi et al., 2023). ECs cover a wide range of compounds, including pharmaceuticals and personal care products (PPCPs), microplastics (MPs) plasticizers, flame retardants, nanomaterials, endocrine disrupting compounds (EDCs), perfluorinated and polyfluoroalkyl substances (PFAS) (Kumar et al., 2023)

Traditional wastewater treatment plants (WWTP) are unable to fully remove from wastewaters compounds of high environmental relevance such as pharmaceutical active compounds (PACs) (Lutterbeck et al., 2016). These contaminants reach the natural environments with concentrations ranging from ng/L up to m/L and may cause several adverse effects both on aquatic biota and human beings (Ravikumar et al., 2022). Thus, there is a clear need to find treatment options that might combine reduced costs and efficiency to remove PACs from wastewaters. In this sense, Constructed Wetlands (CW) have been shown as a promising alternative for the decentralized treatment of effluents, since the costs associated with the implementation, operation and maintenance of the units are lower when compared to other traditional options (Colares et al., 2023). Moreover, several authors have reported good removal rates of PACs from wastewaters (AL Falahi et al., 2022; Salah et al., 2023; Shi et al., 2023).

Despite the advantages, the use of CW has some limitations, mainly related to higher land requirements that may difficult the implementation of this solution (Pereira da Silva et al., 2021). One strategy to overcome this drawback is the combined use of CW with another treatment technologies (Lutterbeck et al., 2022). A configuration that has received scientific attention over the last years is the sequential use of CW and Microalgae (MA) (Silveira et al., 2020). MA are classified as a group of tiny microscopic algae that have been used in the treatment of several kind of wastewaters, including domestic, industrial, dairy, food, brewing and pharmaceutical wastewaters (Song et al., 2022). According to Song et al. (2022) MA present several advantages over other traditional WWTP such as low energy demand, low cost-effectiveness, nutrient cycling, lower sludge formation, greenhouse gas suppression, recovery of nutrients in the form of useful biomass.

MA are also recognized as an efficient bioremediation solution to remove PACs from wastewaters. The study byXiong et al., (2018) approaches the main mechanisms of PACs removal by MA: bioadsorption, bioaccumulation, and intracellular and extracellular biodegradation. Moreover, the authors also highlight that the combined use of CW and MA may be considered an effective consortium for the removal of such group of micropollutants.

So, the present study aimed at to implement and investigate the performance of a combined system composed of sequential MA and CW with Vertical Flow-French type system (VFCW-FS), aiming at to remove nitrogen, organic load, ecotoxicity and PACs from wastewaters generated at a university campus.

2. Materials and Methods

2.1 Study site and set up of the integrated system

The research was carried out at the WWTP of the University of Santa Cruz do Sul (UNISC), with the following geographical coordinates: latitude 29°41'55.69"S and longitude 52°26'28.76"O. The climate in the region is considered subtropical, with warmer periods between November and May and cooler periods between June and September. An experimental unit consisting of an anaerobic reactor (AR), a microalgae production system (MA) and a constructed wetland (CW), configured as the first stage of the French model, was set up at the experimental site. The wastewaters generated at the University are drained from the urinals and toilets and conducted to the WWTP, which comprises seven treatment steps: grating; sand trap; equalizer tank; anaerobic upflow sludge blanket (UASB); aerated biofilter; decanter; and sludge drying bed.

The experimental unit was set up containing a cylindrical AR with a nominal volume of 1.1 m^3, measuring 1.1 m in diameter and 1.43 m in height, two oval-shaped water tanks of 1 m^3 each, 1.88 m long, 1.23 m wide and 0.68 m high, the first one used as a MA tank and the second one as the CW unit (Fig. 1).

Figure 1

The MA production system was set up as a raceway, i.e. a hydraulic pump connected to a Venturi-type air injector adds carbon dioxide to the effluent and at the same time circulates the water in the tank. A barrier was placed in the middle of the tank to create a path for the effluent to circulate.

The constructed wetland was set up inspired by the first stage of the French model, containing a lower drainage layer of 20 cm of gravel number 3 (25–50 mm), an intermediate transition layer of 10 cm of gravel number 1 (9,5 – 19 mm) and an upper layer of 30 cm filter medium, with gravel number 0 (4,8 mm – 9,5 mm), as indicated by Von Sperling & Sezerino (2018). The CW stage was vegetated with the following species: *Chrysopogon zizanioides* (6 units), *Typha domingensis* (4 units) and *Dracaena trifaciata* (4 units). The Vertical Flow-French type system was selected since it has presented satisfactory nitrogen removals rates, reducing the area for implementation and consequently lowering the operational costs (Silveira et al., 2015)

Weekly the system was fed with 250 L of university wastewaters from the equalization tank of the UNISC WWTP (raw effluent) into the AR. The wastewaters were then sent to the MA tank, where 10 L inoculum of microalgae of the *Chlorella* genus were initially added. As the integrated system operated in an open way, besides the presence of the predominant gender *Chlorella*, other MA genders (*Desmodesmus, Scenedesmus)* and bacteria also were found. After and hydraulic retention time (HRT) of seven days the wastewaters were transferred to the CW (first stage of the French system), using the same hydraulic pump responsible for injecting air into the MA unit and distributed throughout the tank, using a system with perforated PVC pipes that reached the entire surface bed of the CW. The adopted operational conditions were chosen based on previous experiments carried out at our workgroup.

Figure 2

2.2 Analytical characterization

Samples were collected at the outlets points of each unit. The following parameters were analyzed: electrical conductivity (EC), pH, total dissolved solids (TDS) and turbidity total organic carbon (TOC) and total nitrogen (TN) and dissolved oxygen (DO)

In order to assess the presence of PCs in the raw wastewaters and the removal capacity of these compounds by the integrated system, samples collected at tree points (raw wastewaters, MA and CW) were analyzed by ultra-high performance liquid chromatography coupled to tandem mass spectrometry

(UHPLC-MS/MS, Xevo TQ-XS, Waters), at the Pesticide Residue Analysis Laboratory (LARP), of the Federal University of Santa Maria (UFSM), in Santa Maria/RS. An ACQUITY UPLC HSS analytical column T3 (2.1 mm x 100 mm; 1.8 μm) was maintained at 45°C, with the mobile phase being: (A) water and (B) acetonitrile, both containing 0.1% (v/v) formic acid. The samples were centrifuged, diluted twice and filtered (0.22 μm).

The average historical temperatures (over the last thirty years), during the research period, i.e. February, March, April and May 2023, are 24, 23, 20 and 16.5°C, respectively (Climatempo, 2023).

Registration in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge was carried out for the microalgae *Chlorella vulgaris* and *Scenedesmus subspicatus* (A256CC3) and macrophytes *Chrysopogon zizanioides Typha domingensis*, *Dracaena trifaciata* (AA24A11).

2.3 Ecotoxicity assays

In order to evaluate the potential ecotoxicity of the raw and treated wastewaters, assays using the microcrustacean *Daphnia magna* were performed at the ecotoxicology laboratory of the University of Santa Cruz do Sul (UNISC). The experiments were carried out following the methodology described in ABNT NBR 12713 (2004). Untreated and treated samples were tested and prepared with volumetric precision at a geometric progression ratio of ½. Due to the variation in the composition of the raw effluents, 5 different concentrations were tested (100–6.25%).

The EC (I) 50 48 h, which corresponds to the Median Initial Effective Concentration - concentration of the sample at the beginning of the test that causes an acute effect on 50% of the organisms in 48h, under the test conditions, is calculated using the Trimmed Sperman-Karber statistical method Method (Hamilton et al., 1979)

2.4 *Allium cepa* Bioassays

The *Allium cepa* test system has been recognized as a versatile bioassay, allowing for the determination of different endpoints. Moreover, it presents several advantages over other assays, such as the facilities to store and handle plants, the good chromosomes conditions, the low costs and a good correlation with other test systems, e.g. mammals. (Lutterbeck et al., 2015)

The analyses were carried out using the methodology proposed by Fiskesjö (1985), taking into account the modifications made by Leme and Marin-Morales (2008). In the tests, phytotoxicity potential was assessed through the germination index (GI), cytotoxicity through the mitotic index (MI) and root length followed by genotoxicity. For genotoxicity, the frequency of nuclear anomalies (NA) was considered, which include both the presence of micronuclei (MN) and nuclear budding and lobulated nucleus, in addition to observing the frequency of total chromosomal aberrations (TCA) and its subtypes, aneugenic chromosomal aberrations (ACA) and clastogenic aberrations (CA) classified according to the criteria of Leme and Marin-Morales (2009). All tests were accompanied by a negative control (NC) consisting of

H_2O_d and the positive control (PC) made using $CuSO_4$ at a concentration of 1µg/mL. A more detailed description of the test procedures can be found in the Supplementary material (Text S1)

2.5 Statistical Analysis

Continuous variables were expressed as mean and standard deviation and median and interquartile range between 25 and 75%. The data were subjected to the Shapiro-Wilk normality test and as they did not present a normal distribution, it was decided to use non-parametric statistics. Thus, the effluents were purchased from each other and from the NC using the Kruskall-Wallis test followed by Dunn's post-test for multiple comparisons. The level of statistical significance was considered to be $p < 0.05$. All tests were accompanied by PC, which served to show that the tests presented significant differences in relation to NC when compared by the Mann-Whitney U test. Therefore, it was decided to compare only the NC and the treatments. All statistical analyzes were performed using the *GraphPad Prism* 10.0 statistical program.

3. Results and Discussion

3.1 Analytical Characterization

The results of the analytical characterization are depicted in Fig. 3. Regarding the organic load (Fig. 3A), a mean reduction of 67.2% was attained after the MA unit. Low differences were observed between the MA and CW, indicating that the removal of the DOC mainly occurred by the MA. Despite the good removal, no significant differences were registered among the treatment units. This might be attributed to the high standard deviation of the RW, since the studies were conducted with real samples and presented fluctuations of the organic load during the research period.de Souza Celente et al. (2019) investigated the combined used of algae turf scrubber and down-flow vertical constructed wetlands for the treatment of university treatments. The authors observed DOC reductions of 48. 2% after the treatments by MA, while at the end of the whole treatment these values reached 69.8%.Ferreira et al., (2021) carried out a research to evaluate the efficiency of MA in different photoperiod cycles, followed by CW. The outcomes showed total organic carbon (TOC) removals of 93.6% after the MA tank and absence of TOC after the treatment by the CW unit.Dell'Osbel et al. (2020) verified a DOC removal of 80% by using an integrated system composed of AR and 3 sequential CW when treating university wastewaters.

Figure 3

The Total N results are presented in Fig. 3B. As can be visualized, the MA tank removed 40.7% of the Total N (not significant), whereas after the CW stage, the mean values reached 68. 6%, indicating significant differences ($p < 0.05$) in comparison to the RW. On the one hand, it is necessary to point out that after the MA stage the TN load was yet high, indicating that, in order to obtain better removal results by this process, a more efficient separation of the MA from the rest of the liquid is necessary, and coagulation/flocculation methods can be used, followed by flotation or decantation. On the other hand, it must be pointed out that the whole treatment was efficient, with the effluent almost reaching emission standards present in stricter guidelines such as the UWTD 91/271/EEC (15 mg L^{-1} of Total N considering generation from 10,000–100,000 p.e.). de Souza Celente et al. (2019) verified similar TN decreases (68.4%) after the whole treatment by MA and CW. In the study by Ferreira et al., (2021) the authors observed deductions of 67.7% after the MA tank and of 85.7% after the CW. In an experimental demonstration plant that combined 3000 m^2 of MA, followed by 200 m^2 of CW and operated for one year, Saúco et al., (2021) verified removal values of 76.6% of total nitrogen (NT) in urban effluents. In addition, the authors mentioned that energy demand required in the hybrid system was five times lower than a conventional activated sludge.

Figure 3C present the values of the DO. The obtained results showed significant increases after the MA ($p < 0.01$) and CW ($p < 0.05$) units. The raises in these values show an improvement in the conditions of the effluents, since low DO values are a limiting factor for the survival of several aquatic species (Li et al., 2020).

Concerning the turbidity analysis (Fig. 3D), significant reductions were achieved after the MA (86,5%) ($p < 0.001$) and CW (78,8%) ($p < 0.001$) stages. High turbidity is responsible for the increased oxygen consumption usually associated with high sediment loads (Liu et al., 2023).

The presence of TDS increased by 20% from the RW until the MA. After the CW, the mean values were reduced by 27.4%, in comparison with the RW (Fig. 3E). This initial increase might be possible attributed to the growth of the MA themselves, which increases the concentration of organic material. Nevertheless, none of the obtained results presented significant changes. Total dissolved solids (TDS) consist of inorganic salts such as chlorides, calcium, magnesium, sodium, bicarbonate, sulphates and organic matter in water, are harmful to human health and can be toxic to aquatic life such as fish, insects and amphibians. For example, TDS levels above 250 mg L^{-1} affect some fish and an increase in TDS from 270 to 1170 mg L^{-1} can kill almost all their eggs (Herawati et al., 2023).

A similar tendency was observed for the Electric Conductivity (EC). As can be observed in Fig. 3F, after an initial raise of 25,8% at the MA tank, the final values decreased 25,6% at the outlet of the CW. Increased conductivity can interfere with the growth of MA and bacteria, reducing therefore their activity (Amadu et al., 2023). It is also well-known that high conductivity values in the effluent alter the transport of chemical compounds between the environment and the interior of the microbial cell, causing changes in metabolism and inhibitory effects, contributing to a reduction in the efficiency of the biological treatment of the effluent (Cui et al., 2023). Furthermore, it can also be used to analyze the stability of CW substrate materials, as well as their adsorption (Wang et al., 2021). In this sense, a lower EC in CW may indicate the stability of the materials used as a substrate.

Finally, the pH did not suffer great alterations and remained at a neutral range, with values of 7,1, 6,9 and 7,0 at the RW, MA and CW, respectively, indicating that the raw effluent is not toxic to MA growth and that both during the microalgae production process and after passing through the CW, the pH remains stabilized. The pH values in an effluent can identify various characteristics of the effluent with regard to its treatment, especially related to the growth conditions of microorganisms and actions such as coagulation/flocculation when chemical agents are used for this purpose. In a study carried out in Qatar, during the cultivation of *Scenedesmus sp.*, the pH of the culture, cultivated in sanitary effluent, remained in the range of 7.2–7.5 (Cassini et al., 2017; Das et al., 2016).

3.2 Chromatographic Analysis

Chromatographic analysis of the raw wastewaters showed the presence of nine PCs at different concentrations, as shown in Table 1. From them, dipyrone (DIP), caffeine (CAF) and ampicillin (AMP) presented the highest concentrations.

Table 1
Average concentrations of drugs found in the raw effluent and in the stages of the treatment system in μg L^{-1}. n.d.: not detected.

Pharmaceuticals	RW (μg L^{-1})	MA (μg L^{-1})	CW (μg L^{-1})
Acetaminophen	6.11	n.d	n.d
Mefenamic acid	3.34	n.d	n.d
Ampicillin	16.79	n.d	n.d
Caffeine	21.88	0.93	n.d
Ketoprofen	0.29	n.d	n.d
Diclofenac	0.12	n.d	n.d
Dipyrone	29.52	1.42	0.33
Losartan	0.43	0.18	n.d
Naproxen	0.35	n.d	n.d

Table 1

As can be observed, after the treatment by the MA unit, only three compounds were detected yet (Dipyrone, Caffeine and Losartan) and at the end of the whole treatment, only a small fraction of DIP (1%) remained in the effluent.

The presence of CAF has been reported by several authors in wastewaters and surface waters, with concentrations ranging from ng L^{-1} to μg L^{-1}(de Souza et al., 2021; Matamoros et al., 2015; Hijosa-Valsero, Matamoros, et al., 2011). Caffeine is a stimulant drug, that is very polar (logKow = − 0.1) and highly stable at neutral pH range. The study carried out byGojkovic et al., (2019) tested the ability of different microalgal species to remove 19 PACs from a culture medium. The results showed that CAF was among the most persistent compounds, remaining relatively stable at the end of 12 days of the experiment. Conversely,Matamoros et al., (2015) reported CAF removal rates of > 90% from urban wastewaters by high-rate algal ponds (HRAPs) at pilot scale in Barcelona, Spain. According to the authors, the removal of PACs by MA is influenced not only by the population density, but also by the specific ability of each algal species to adsorb, absorb, and degrade specific compounds.Hijosa-Valsero et al., (2011) investigated the removal of CAF by CW with different configurations and plant species. The authors reported good removal rates, with values up to 99%. Our results showed a reduction in the CAF concentration of 96% at the outlet of the MA unit.

AMP is a β-Lactam antibiotic that is used in therapeutic treatments and, due to both high consumption and excretion rate (30%), has been frequently detected in natural environments (Papageorgiou et al., 2016). Molecules with a β-lactam ring are relatively stable at neutral pH-values, which is the case of raw wastewaters of our study.Hijosa-Valsero, et al., (2011) measured lower Ampicilin concentrations at the (0. 69 μg L^{-1}) inlet wastewaters of the WWTP of Léon, Spain. They also investigated the removal of AMP by different mesocosm-scale CW and found that all the configurations were not effective. To the best of our knowledge, to the present there are no data about the removal of AMP by MA. So, our study showed promising results, since AMP was not detected anymore after the MA stage.

Dipyrone (metamizole sodium) is a widely used anti-inflammatory drug that may cause adverse effects on aquatic fauna and on human beings (Valadez-Renteria et al., 2024). Because of its large consumption it has been frequently found at different water matrices (Feldmann et al., 2008). DIP has also been detected at the outlets of WWTP, which indicates that this compound is recalcitrant to traditional treatment methods (Feldman et al., 2008). In our research, the results showed elimination rates of 95% and 99% after the MA and CW units, respectively. We also did not find literature data about the removal of DIP by MA and CW.

The degradation of PACs may occur by different mechanisms, namely. bioadsorption, bioaccumulation, and intracellular and extracellular biodegradation According to Xiong et al., (2018), biodegradation is the most effective way by which MA remove organic contaminants from an aqueous phase. The studies performed by Gojkovic et al. (2018) and Matamoros et al., (2016) support this assumption for CAF.

3.3 Ecotoxicity assays

Based on the relative toxicity scale (Table 2) proposed by Lobo et al. (2006), and recently endorsed by de Souza Celente et al., (2020), the results of the raw samples presented an EC (I)50 48h value = 74.30%, corresponding to a “moderately toxic” sample. After the treatment by the integrated system both samples (MA and CW) presented an EC (I)50 48h value = 100%, indicating absence of acute ecotoxicity for this test organism. Silveira et al., (2020) also investigated the treatment of university wastewaters by the sequential use of MA and CW. The obtained results showed the raw wastewaters classified as “slightly toxic” whereas the treated wastewaters did not present acute toxic effects against *D. magna*.

Table 2
Relative toxicity scale for 48 h EC_{50} determinations with *D. magna*

Percentile	**EC_{50}**	**Toxicity**
25%	< 25%	Extremely toxic
50%	25–50%	Highly toxic
75%	50–75%	Moderately toxic
	> 75%	Slightly toxic

Table 2

3.4 *A. cepa* Bioassays

The results of the analyzes referring to the phytotoxicity, cytotoxicity and genotoxicity parameters of the *A. cepa* assay are summarized in Table 3.

Table 3
– Results of phytotoxicity, cytotoxicity and genotoxicity analysis

	NC	RW	AR	MA	CW
GI (%)	**55(55–70)**	**75(65–75)**	**60(52.5–67.5)**	**60(55-72.5)**	**65(55-72.5)**
	61.0 ± 8.2	71.0 ± 8.9	60.0 ± 9.4	63.0 ± 9.7	64.0 ± 9.6
Radicle	0.6(0.4–0.9)	0.7(0.5-1)	0.7(0.5-1)	0.7(0.5–0.9)	0.6(0.5-0.875)
(cm)	0.64 ± 0.29	0.73 ± 0.32	0.74 ± 0.33	0.69 ± 0.32	0.66 ± 0.31
MI (%)	9.9(7.9–15.6)	10.1(4.0-15.5)	12.6(4.8–17.2)	12.2(6.6–17.4)	7.7(5.8–13.5)
	11.6 ± 5.9	10.2 ± 5.7	11.6 ± 5.7	12.6 ± 7.1	8.8 ± 3.6
TCA (%)	0.0(0.0–15.0)	27.0(18.5–40.0)	22.0(0.0-47.8)	41.0(20.0-47.5)	15.5(6.0–24.0)
	7.0 ± 9.7	29.0 ± 14.5	24.4 ± 23.1	35.5 ± 18.5	15.0 ± 10.1
ACA (%)	0.0(0.0–14.0)	14.5(10.5–40.0)	14.5(0.0-42.8)	33.5(20.0-37.3)	14.0(6.0–18.0)
	5.9 ± 7.8	21.9 ± 18.8	21.1 ± 22.6	29.3 ± 13.7	12.6 ± 8.9
CCA (%)	0.0(0.0-2.5)	14.0(7.8–18.8)	11.5(0.0-22.3)	11.0(3.8–17.5)	8.0(0.0-14.3)
	3.1 ± 7.0	13.4 ± 6.0	11.9 ± 11.7	11.5 ± 8.9	7.7 ± 7.3
NA (‰)	0.0(0.0–0.0)	3.5(0.0-7.3)	1.5(0.0–4.0)	0.0(0.0-3.3)	0.0(0.0–4.0)
	0.0 ± 0.0	3.8 ± 3.3	2.4 ± 3.2	1.4 ± 1.8	2.4 ± 3.9

NC: negative control; **RW**: raw wastewaters; **AR**: anaerobic reactor; **MA**: microalgae; **CW**: constructed wetland; **MI**: mitotic index; **TCA**: total chromosomal aberrations; **ACA**: aneugenic chromosomal aberrations; **CCA**: clastogenic chromosomal aberrations; **NA**: nuclear anomalies. The results are expressed as median (25–75%) and mean ± standard deviation.

Table 3

Regarding phytotoxicological analysis, based on the percentage of germination index (GI), no statistical difference was found between the samples and the negative control (Fig. 4A). These results suggest that the samples do not present phytotoxicity.

Concerning cytotoxicity assessments, which correspond to radicle length and mitotic index (MI), it was possible to observe that there was no statistical difference between the negative control (NC) and the analyzed samples (Figs. 4B and C).

According to Frota et al. (2019), the MI evaluates the cytotoxicity of many substances. Both the increase and decrease of this parameter suggest cytotoxicity of the analyzed sample. However, as in the present work no statistical significant differences between the samples and the NC were observed, it is suggested that there is no cytotoxicity of the RW and the samples treated by the integrated system.

Figure 4

As can be visualized in Figs. 4D-G, in relation to genotoxicity, the RW presents a significantly increased frequency of chromosomal aberrations and nuclear anomalies when compared to CN, thus demonstrating genotoxic potential.

On the one hand, for nuclear anomalies, it can be seen that the frequency was already reduced in the AR wastewaters and remained low throughout the other stages of the integrated system (Fig. 4D). On the other hand, in relation to total chromosomal aberrations (TCA), it was possible to verify statistical significant increases until the MA stage (Fig. 4E). It is likely that this increase is mainly occasioned by aneugenic chromosomal aberrations (Fig. 4F) which are also significantly increased in the MA stage.

Aneugenic aberrations are classified into: wandering chromosome, spindle abnormalities, duplications or polyploidies, abnormal metaphase, C-metaphase, Stickiness (metaphases with adhesions), chromosome lost in anaphase and chromosome delayed in telophase (Leme & Marin-Morales, 2008). Given these parameters, the treatment using MA was the one that demonstrated most anomalies throughout the mitosis process with 30% of aneugenic aberrations i.e., even higher than the RW. The percentage of aneugenic aberrations found in prophase, metaphase, anaphase and telophase was significant higher in the samples collected after the MA stage, indicating a pattern of abnormalities that occur in all stages of cell division (Fig. 4).

The values of clastogenic chromosomal aberrations are evident as being characteristic of the RW. An important analysis is that throughout the treatments this type of aberration decreases considerably, being close to 10% in the CW step. It is also noteworthy to mention that, in the case of aneugenic aberrations, the value obtained after the treatment by the CW unit is very similar to the amount of aberrations of the same type found in the NC. This again highlights the efficiency and importance of this type of treatment. It is interesting to note that the wastewaters collected after the CW stage no longer present any genotoxicity. This suggests that the integrated treatment system was efficient in removing compounds with genotoxic potential present in RW.

When it comes to nuclear anomalies, it can be seen that this is a predominant characteristic of the RW in relation to the NC. The treatment using MA showed a greater number of clastogenic and aneugenic chromosomal aberrations (Fig. 5). This may be due to the recirculation used at this stage of the system and that brought back a product or drug, causing an increase in toxicity in this sample. Another possibility might be related to the partial degradation of some compounds present in the wastewaters by the MA, leading to the formation of byproducts that can be more recalcitrant and/or toxic than the parent compounds (Lutterbeck et al., 2015)

On the other hand, the CW unit was able to eliminate the toxicity from the whole system, so that the treated wastewaters presented similar values to the NC regarding total chromosomal aberrations (TCA), which configure aneugenic, clastogenic chromosomal aberrations and nuclear aberrations. This demonstrates that the treatment was efficient and that the treated wastewaters can be returned to the environment.

Previous studies have also evidenced the efficiency of MA and CW for reducing the toxic potential of different types of effluents. Melo et al., 2018 verified that the treatment using the MA *C. vulgaris* was able to fully remove corn steep liquor toxic power and minimized the vinasse genotoxic potential by reducing nuclear abnormalities in the *A. cepa* test system. The study by Silveira et al 2019 investigated the potential of combined CW and MA to remove toxicity from urban wastewaters. The results obtained by the authors showed that the system was completely removed the genotoxicity and mutagenicity and significantly reduced the cytotoxicity of the investigated wastewaters.

In summary, the results brought in these both toxicity sections demonstrated that the combined use of MA and CW may constitute an interesting alternative to reduce the toxicity potential of different wastewaters.

4. Conclusions

The present study aimed at to treat the wastewaters generated at a university campus by an integrated system combining MA and CW. Significant total N was attained, indicating the efficiency of the system to reduce the eutrophying potential of the wastewaters. Furthermore, the treatment also achieved good removals of organic matter and was able to remove PACs from the effluents. The toxicity assays showed interesting findings, since the system was capable to fully remove the acute toxicity against *D. magna* and the genotoxic potential of the investigated wastewaters in the *A. cepa* assay.

Despite the promising results, some improvements to the system should be considered in order to improve even more the obtained results, such as better separation of the MA, using, for example, coagulation and flocculation, combined with decantation or flotation processes. Finally, future studies should also investigate the removal mechanisms of PACs by MA and CWs.

Declarations

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CRediT authorship contribution statement

Fábio Rodrigo de Oliveira: Methodology - development of methodology, Investigation - conducting research, Writing - original draft.

Maurício Kersting: Investigation - performing experiments.

Bruna Allebrandt: performing experiments and data collection.

Alexandre Rieger Conceptualization - Ideas - provision of resources. Supervision

Tuanne Carvalho Nunes performing experiments and data collection.

Guilherme Lemões Iepsen performing experiments and data collection.

João Vitor Frantz performing experiments and data collection.

Julia Antunes de Oliveira: performing experiments and data collection.

Carlos Alexandre Lutterbeck: Investigation - Writing - reviewing and editing. Supervision

Carine Baggiotto: Writing - reviewing and editing.

Rosana de Cassia de Souza Schneider: Conceptualization - Ideas - provision of resources. Supervision

Renato Zanella: Conceptualization- Ideas - research goal and aims.

Eduardo Alcayaga Lobo: Provision of resources – ecotoxicity analysis.

Adriane Lawisch Rodriguez: Conception of the treatment system, Provision of resources - reagents - materials.

Enio Leandro Machado: Conception of the treatment system, Writing - review and editing. Supervision

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Informed consent was obtained from all individual participants included in the study.

“Consent for publication”

The authors consent for publication of the manuscript in Environmental Science and Pollution Research.

Competing Interests

The authors declare they have no competing interests

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Figures

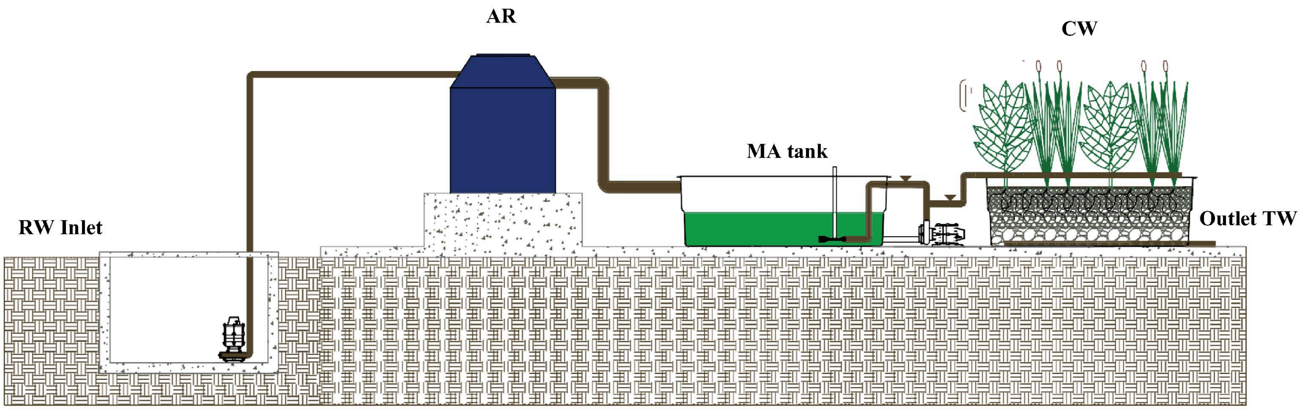

Fig. 1. Schematic representation of the Integrated Treatment System. Abbreviations: RW: Raw Wastewater; AR: Anaerobic Reactor; MA: Microalgae CW: Constructed Wetland; TW: Treated Wastewaters

Figure 1

See image above for figure legend.

Figure 2. Upper view of the Integrated Treatment System. A) Microalgae Unit B) Constructed Wetland.

Figure 2

See image above for figure legend.

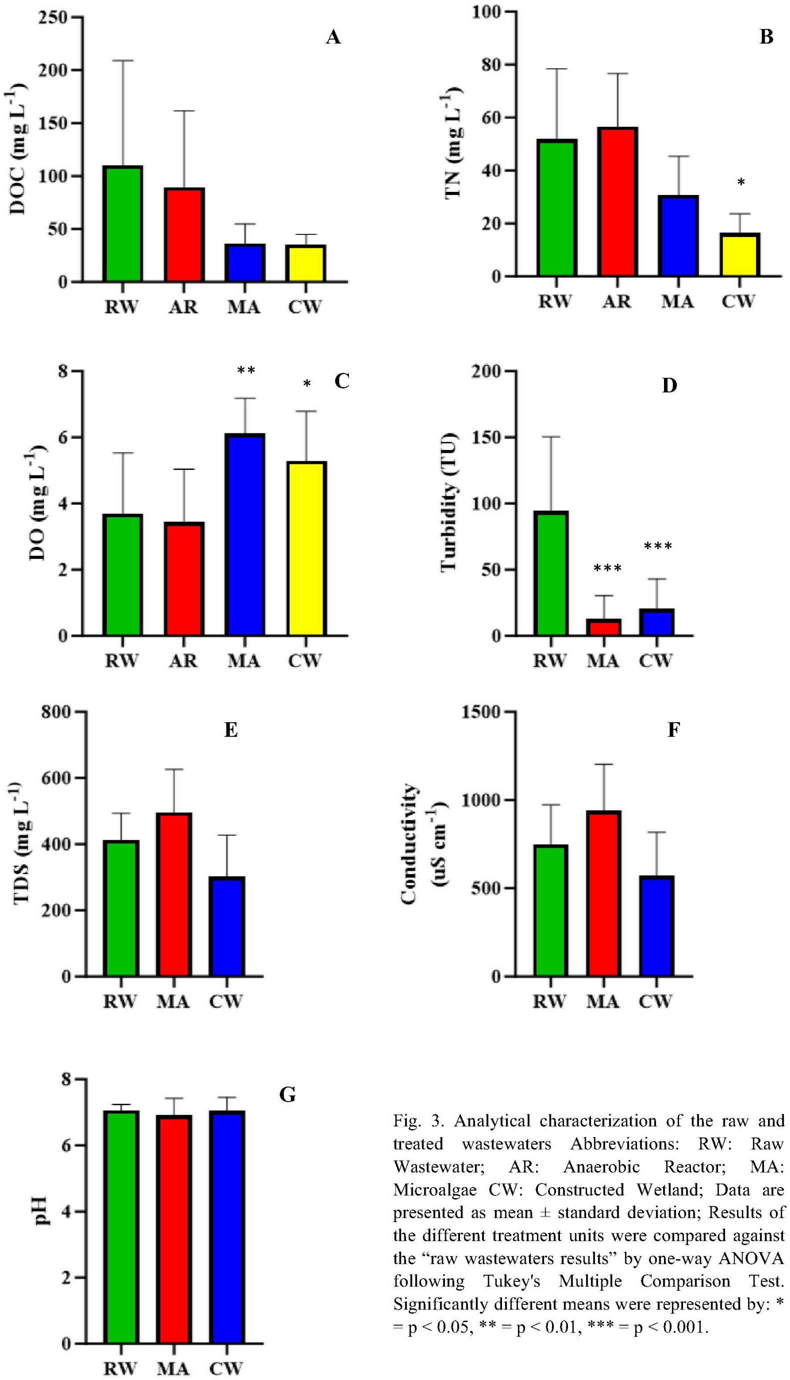

Fig. 3. Analytical characterization of the raw and treated wastewaters Abbreviations: RW: Raw Wastewater; AR: Anaerobic Reactor; MA: Microalgae CW: Constructed Wetland; Data are presented as mean ± standard deviation; Results of the different treatment units were compared against the "raw wastewaters results" by one-way ANOVA following Tukey's Multiple Comparison Test. Significantly different means were represented by: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Figure 3

See image above for figure legend.

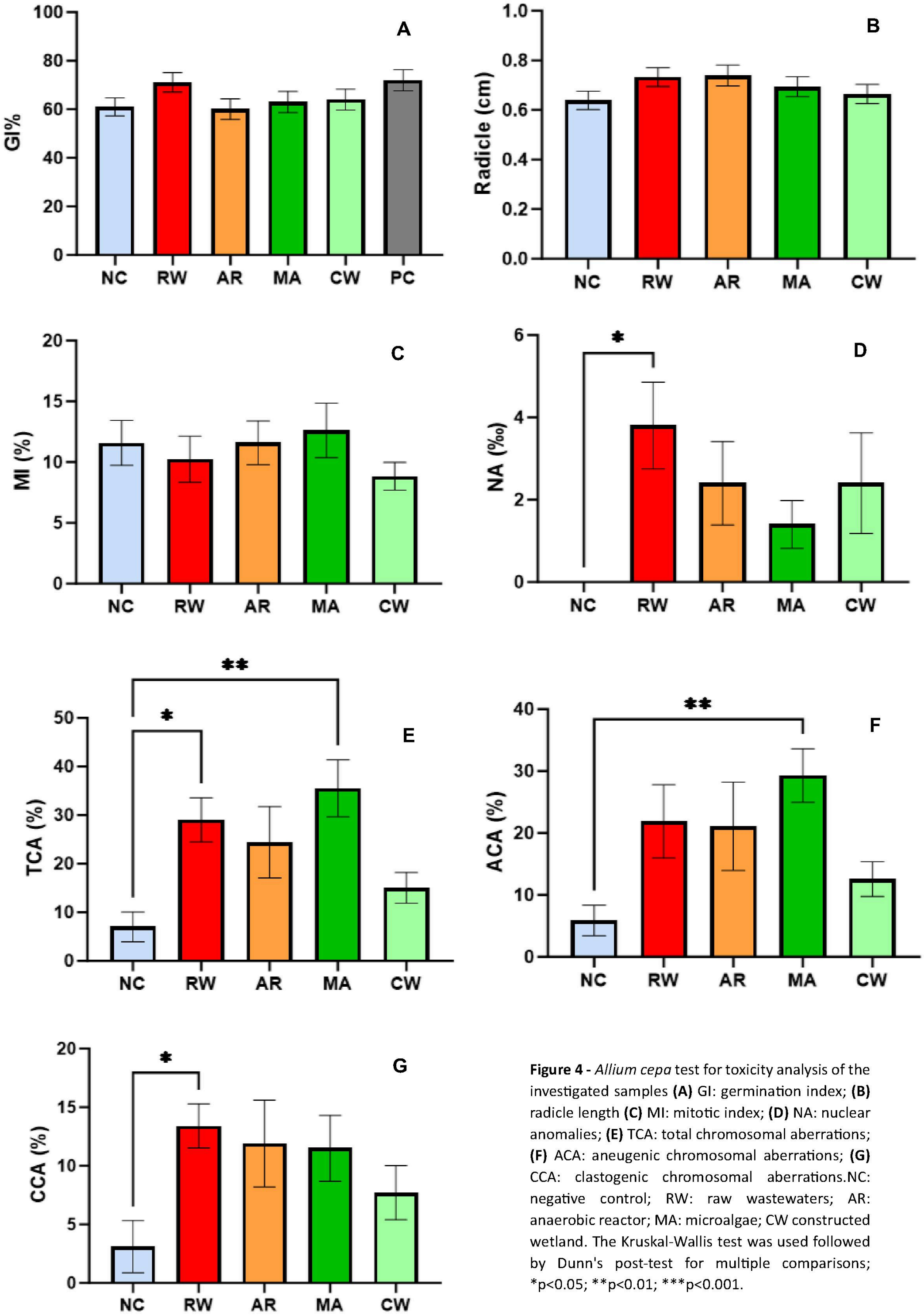

Figure 4 - *Allium cepa* test for toxicity analysis of the investigated samples **(A)** GI: germination index; **(B)** radicle length **(C)** MI: mitotic index; **(D)** NA: nuclear anomalies; **(E)** TCA: total chromosomal aberrations; **(F)** ACA: aneugenic chromosomal aberrations; **(G)** CCA: clastogenic chromosomal aberrations.NC: negative control; RW: raw wastewaters; AR: anaerobic reactor; MA: microalgae; CW constructed wetland. The Kruskal-Wallis test was used followed by Dunn's post-test for multiple comparisons; *p<0.05; **p<0.01; ***p<0.001.

Figure 4

See image above for figure legend.

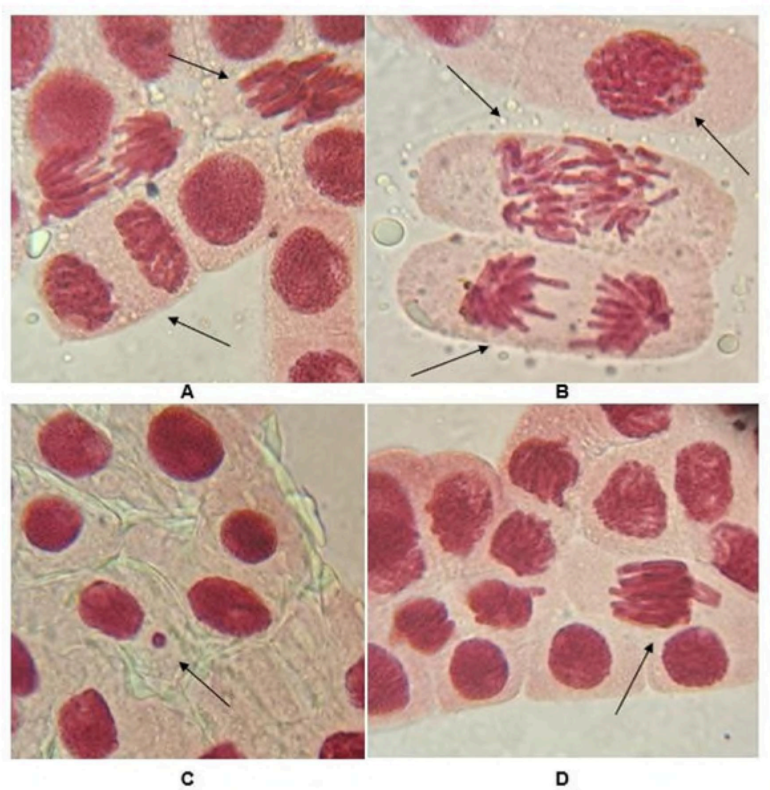

Fig. 5 - Schematic representation of some meristematic cells of *A. cepa*. **A**: Telophase, Anaphase **B**: Prophase, Anaphase with wandering chromosome and polyploidy; **C**: Micronucleus; **D**: Spindle abnormality.

Figure 5

See image above for figure legend.

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