Chapter

Evaluation of the Use of Advanced Ozone Oxidative Process in Reducing the Danger of Environmental Toxicity by Endocrine Interferences of Magistral Pharmacy

Thais Francinne, Suellen Zucco Bez, Julia Carolina Soares, Sabrina Martins da Rosa, Aline Mirian Paszuck, Luciana Ferreira Karsten and Luciano Henrique Pinto

Abstract

The presence of emerging pollutants in the waters has been observed worldwide, resulting from improper domestic disposal, non-recommended veterinarian use, and product waste from pharmaceutical industries and magistral pharmacies. The contamination provoked, besides causing damage to the environment, remains in potable water even after passing through the treatment plants. The objective of this work was to verify the existence of environmental toxicity of raw effluents from gross pharmacy laboratories, as well as the same effluent treated with POA via ozone in the time of 1 hour, having as a risk parameter the changes that they cause in *Euglena gracilis* algae. Photosynthetic efficiency tests were conducted via PAM, and chlorophyll concentration and behavioral evaluation were checked via NGTOX. The results demonstrate that the hormone laboratory was considered the most impacted effluent treated, with lower production and significant chlorophyll reduction. It presented reduction in photosynthetic post-ozonation activity, due to the hormone decomposition, oxidative potential and ethylene formation. Effluents from psychotropic and solid laboratories presented different production demand, but similar follow-up, with impact on the behavior and algae's photosynthetic activity, due to the presence of active substances on cellular action potentials. The treated effluent from dermocosmetics laboratory influenced the chlorophyll concentration, as well as the general speed and velocity of surface ascent. The behavioral differences between the laboratories and the pre and post-ozonation conditions demonstrate that the effluent treatment should be distinguished, according to the characteristics of the manipulated substances in each laboratory.

Keywords: *Euglena gracilis*, oxidative process, ozonation, biomonitoring

1. Introduction

All over the world, the presence of medicines and cleaning and health product waste has been identified in the waters, and nowadays they are classified as emergent pollutants. This contamination is the result of many factors, like improper domestic disposal; non-recommended veterinarian use, which is exacerbated, making the excretion of medicine active metabolites reaches the groundwater in higher-than-expected quantities; and product waste from pharmaceutical industries and magistral pharmacies (also called manipulation pharmacy), that dispose their compounds to the effluents. Although the wastewater treatment plants treat this water, many medicines still remain in the drinking water [1].

In general, it has been seen that traditional wastewater treatment processes are not very efficient in removing this kind of emergent pollutants. In biological processes, for example, the degradation efficiency is highly influenced by the presence of other macrocompounds, what makes the drug degradation, besides rarely, only partially [2]. Systems based on absorption processes have been recently proposed, which use standard (active carbon) and modern (pre-absorbed micelles in montmorillonite) sorbents. However, their efficacy is questionable [3].

In this perspective, investigations indicating the environmental risk of these pollutants and the methods of removing these contaminants are increasingly more needed, since neither the treatment approaches nor the awareness of this issue are enough. The legislation can be cited here. It must be updated when it comes to emergent pollutants.

The Brazilian Water Resources Management Policy aims to assure the proper water availability to human consumption [4]. The Order no. 2.914/11, of the Ministry of Health, defines the potability patterns to water consumption. In this document, the drugs with potential risk to human health are not mentioned [5]. This condition makes the compounds neither be identified nor even treated on the wastewater treatment plants [6].

Differently from the Brazilian reality, organizations like European Union, the United States Environmental Protection Agency and the World Health Organization have already published guidelines and rules that warn about the risks of the presence of medicines in water and require studies that lead to their removal, in order to stablish acceptable limits for drinking water [7].

In this scenario, there are the magistral pharmacies, which, in the last decade, manipulated 8% of all the prescriptions in Brazil [8]. Nowadays, there are more than 7000 facilities like them over the country, and they are responsible for the small-scale and personalized medicine production, besides all the precautions required by the current legislation. In Joinvilly city specifically, the study site, at present 25 magistral pharmacies are registered with the Pharmacy Board and Sanitary Surveillance – which also attend the traditional segment –, dealing with drug and cosmetic demands, beyound the specialized pharmacies, that work with veterinarian products and hormone manipulation.

When reaching the environment, the hormones are then called endocrine disruptors (ED). These ED are defined as natural or synthetic exogenous chemical substances that, when in the environment, are capable of modifying the endocrine system, since they simulate the actions of natural hormones. These compounds might cause disorders that affect human and animal health [6], provoking, for example: breast and uterine cancer, increase in the incidence of polycystic ovarian, reduction in male fertility and prostatic neoplasm [9]. Considering the disorders the ED may cause in health and the environment, their chemical removal has been largely studied.

A technique used to remove ED is based on the development of advanced oxidation processes (POA), that correspond to a type of water treatment. POA promotes the composition of highly reactive and little selective hydroxyl radicals, being able to act on chemical oxidation of a wide range of organic substances, like medicines, converting them in substances that do not present, *a priori*, the same biological interactions than the original molecule does. In ED situation, there is the estradiol, whose oxidant action leads to the decomposition of pharmacophores and cessation of estrogenic activity [10].

A way to obtain the advanced oxidation is through the ozone, which is highly used along with other oxidizing agents, like hydrogen peroxide, titanium dioxide and ultraviolet. This process has been showing efficiency in emerging environmental decontamination [11].

The use of POA in this case is justified by the previously presented points related to the need of reducing the risks. However, it is important to say that compounds originated from degradation (COD) will be formed, and their evaluation will be relevant as well. The evaluation of the environmental impact provoked by these COD and the toxicity hazards in different trophic levels may clarify the use of this decontamination procedure and the results on the suppression of a certain environmental risk.

In this investigation, the results found out by Pinto et al. [8] with *Euglena gracilis* algae will be taken into account, in relation to raw effluents from a hormone manipulation laboratory after the ozone/ultraviolet POA, being analyzed the alterations and solutions COD may cause, comparing these results to other ones observed in other (psychotropics, dermo-cosmetic and solids) laboratories.

2. Materials and methods

2.1 Study design

This experimental study was performed in the Laboratory of Photochemistry and Photobiology and in the Environmental Laboratory, at the University of the Region of Joinville. It involved the *Euglena gracilis* KLEBS algae from the University of Göttingen's collection, Germany. Behavioral changes, photosynthetic activity and chlorophyll level alterations were analyzed, when the algae were submitted to chlorinated water from the pharmacy, as well as the raw effluents and the post-ozone/UV-POA effluents.

2.2 Sample collection

Three types of samples were analyzed:

- Water from access: chlorinated, to be used as control;
- Raw effluent, collected from the pharmacy;
- Post-POA-treated effluent.

All of them were collected from the four pharmacy laboratories.

In order to conduct the study, there was the collaboration of a magistral pharmacy from Joinville city, northeast of Santa Catarina, the same facility that was part of Pinto et al.'s investigation [8]. The pharmacy made possible the samples collection from the four production environments of the pharmacy:

- psychotropics laboratory, responsible for the manipulation of controlled-sale medicine prescriptions, accordingly to the Order 344/98;

- hormone laboratory, responsible for the manipulation of strictly hormonal prescriptions;
- solids laboratory, responsible for tablets and other solids formulation production;
- dermo-cosmetic laboratory, responsible for the solids and semisolids formulation production of dermatological properties.

The samples considered as water of access were collected directly from the faucets of the washing sinks in each laboratory, through previously sterilized borosilicate glass jars. They were used here as control samples (water from the wastewater treatment plant). The raw effluents samples were collected from the syphons connected to the washing sinks using a peristaltic pump, and here sterilized borosilicate glass jars were also used. Samples were taken up to 12 L^{-1} from each laboratory. Afterwards, the samples were kept in polystyrene boxes with ice and away from light up to their packaging in the freezer.

2.3 Production estimate of the magistral pharmacy laboratories

In order to conduct the study, there was the collaboration of a magistral pharmacy from Joinville city, northeast of Santa Catarina, that made possible the samples collection from the four production environments of the pharmacy.

An important factor to be considered here is the quantity of actives and other substances disposed through the sinks and that compound the laboratories' raw effluent. For this purpose, the pharmacy's average monthly production of six months was taken into account, and the monthly average production was calculated, in order to verify the laboratories' activity average, accordingly Eq. (1):

$$MP = \left(\frac{\sum Monthlyquantityofproducts}{30} \right) x \left(\frac{\sum mgofrequestsmade}{quantityofrequests} \right) \quad (1)$$

2.4 Fractions tests preparation

2.4.1 Removal process

Removal reaction occurred in a 500-mL^{-1} reactor, which contained the raw effluent samples from all the studied laboratories. The other samples removals occurred after 1-hour ozonation, through a Trump TCB ozone generator, that injected the ozone in a 10-mg L^{-1} flow.

The total time was of 2 hours, in accordance with Ferreira [9]. The volume removed was up to 10% of the total volume (50 mL), following the recommendation, to avoid interferences related to a larger oxidizing agents' exposure to a smaller contaminant volume [12]. Afterwards, the samples were kept in freezer to be later analyzed.

2.5 Tests for environmental toxicity risks

For the purpose of tests for environmental toxicity risks, four sample categories were investigated:

- Algae pure culture;
- Water of access;
- Raw effluent;
- Treated-post-POA effluents.

2.5.1 Tests with Euglena gracilis algae

From each one of the four sample categories, a 5-mL aliquot was removed, and it was added in a 40-mL *Euglena gracilis* algae culture. The collections were performed for photosynthetic efficiency tests, chlorophyll concentration and behavioral evaluation via NGTOX after a period of at least 48 hours, according to Ekelund [13].

2.5.1.1 Algae photosynthetic efficiency test via PAM

For testing the photosynthetic efficiency via PAM, the photosynthetic parameters were measured through a modulated pulse-width PAM 2000 fluorimeter (Walz, Effeltrich, Germany). The PAM measurement principle is based on changes on the chlorophyll fluorescence level, after the application of saturated light pulses. The photosynthesis performance (Yeld) was, then, calculated accordingly Eq. (2), on Yeld photosynthetic efficiency.

$$Yeld = \frac{fm - f0}{f0} \quad (2)$$

Approximately 5 mL^{-1} of the tested cultures were taken and transferred to the cuvette of the PAM equipment. They were then submitted to saturating light pulse emission, for photosynthetic activity evaluation. The saturating light pulse emission made possible the detection of the maximum fluorescence Fm, indicating total reduction of the electrons FSII receptor. The light-curve response was determined for all the treated samples. The algae were exposed to an increasing luminous intensity (generated by an internal halogen bulb) in 10 steps, from 0 to 3111 molm-2/s. After 10 s of each luminous step, a saturating pulse was applied, and the photosynthetic performance and the electron transport rate were measured automatically.

Afterwards, the average performance on test situation was calculated, considering all the values obtained on the saturation process. The global photosynthetic efficiency was measured according with Eq. (2):

$$EFG = \frac{\sum Yeld\ during\ saturation}{Quantity\ of\ submitted\ irradiating\ pulses} x\,100 \quad (3)$$

This way, it was intended to analyze the interference the compound of raw effluents and the waste will promote in the algae culture, when compared to the control one.

2.5.1.2 Evaluation of algae behavioral changes through biomonitoring via NGTOX

The behavioral tests with *Euglena gracilis* and the samples from the laboratories were conducted using a real-time biomonitoring tool called NGTOX, developed and homologated by Ecobabitonga Tecnologia Ltda. The instrument has monitored, through the analysis of real-time images, the algae behavior, considering different movement parameters of the photosynthetic single-celled flagellate [12].

The equipment is a system of connections that involve four silicon tubes responsible for the suction of *Euglena gracilis* cells culture, water samples with hormones for the test, water for sample dilution and the analyzed material disposal.

Three pumps activated by peristaltic motors transported the cells, the diluent and the sample up to a glass cuvette of 22 mm of internal diameter and 0.2 mm of thickness. The trial bodies in contact with the control were blended and transferred to an observation cuvette, connected to a microscope, which captured the images of the cells in movement. The images were recorded by a charged coupled device camera and digitalized by a board connected to a microcomputer, in which they were presented in a monitor. Then, the software calculated the movement parameters, the movement speed, the ascent rate, the average cell size, etc. After that, the samples were added separately, and the analyses were performed by the software one more time 10 minutes later. Any alteration on the movements, average speeds, ascent rates and cell size were calculated and compared with the previous results [14].

2.5.1.3 Test for alterations on concentration of chlorophyll present in algae: chlorophyll removal from the algae and UV analysis (160 SHIMADZU)

This test had the objective of verifying if the parameters previously analyzed affected the chlorophyll concentration. After the time of exposure to contaminants, 5-mL of the culture media submitted to the presence of the test samples and the control were taken. These aliquots were treated according with the procedures conducted by [15]. Aliquots were vacuum-filtered through Whatman® 47-mm filter paper.

The papers containing the filtered (precipitated cells) were transferred to a Falcon tube, received 5 mL of ethanol and were afterwards kept at 4°C for 60 minutes, for the pigments extraction. Then, the mixtures were centrifugated at 6000 g for 10 min at 4°C in order to aggregate on the waste cells.

The absorption spectrum of the supernatant was measured accordingly Lorenz's equation for the calculation of chlorophyll concentration.

2.6 Data statistical analysis

The data were evaluated through ANOVA, a univariate technique which deals with the quantitative data relating them to three-level independent category variables.

For the groups' analysis (tests and control), comparing all the effects, the used technique was an ANOVA extension, called ANOVA for repeated measures, that consists on a better developed approach for paired data. Then, comparisons of results and averages based on the samples' quantitative items were performed.

Following, the other variables were described, since formally there is not a statistical hypothesis test, although it works on confirming or not the a-priori expectations about the results.

The statistical analysis on algae behavior evaluated via NGOTX were conducted by ImagingTox®, a software especially developed and written for Microsoft platform, with multilingual Net 64-bit and MS SQL Server database. It has seven threads (the main one, three for video 1 and three for video 2), two functions (one for controlling the PC and NGTOX connection and the other one for database connection and validation), making possible the storage of bioassays performed for forensic analysis and real-time results exhibition screen. ImagingTox® conducted the 5-PL integrated statistical analysis.

3. Results and discussion

The environmental toxicity related to emergent pollutants has been increasingly causing concern among the scientific community, especially because a lot of studies have been pointed out to clear health and environmental risks [6]. Recent investigations have found essential results for other researches, as well as for the ones which study the development of decontamination processes of these products that are emergent and dangerous to the environment. Then, nowadays there is a scenery that leads to the revision and reorientation of conducts and legislations that involve environmental issues, both in national and international context [2].

In this perspective, magistral pharmacies are potent candidates to generate emergent pollutions potentially harmful to health and to the environment. Studies performed by the National Health Surveillance Agency (ANVISA) have showed that in Brazil around 120 thousand tons of garbage is generated every day, and between 1 and 3% of this total is produced by health facilities, comprising the magistral pharmacies (manipulation of magistral formula). From 10 to 25% of health waste represents risks to the environment and population's health, including the medicines [16].

Among the magistral pharmacy aspects that worked as effluent collection, an important factor to be considered was the quantity of actives and other substances disposed though the sinks and which compound the raw effluents from the laboratories. In the present investigation, the idea was not only to know the characteristics of hormone laboratory, but also the other ones of the other laboratories, in order to compare the found results and to evaluate the LMH risk before the production and other points. To know how many items the pharmacy averagely produced, the average monthly production of six months was examined, accordingly Eq. (1), previously presented.

The found results were collected and placed in a worksheet (**Table 1**):

On the results, it was seen a more prominent production of solids formulae, which include orally used tablets. They represent more than half of the formulae produced in the verified period. On second place, there are the dermato-cosmetic formulae, and among them there are creams in which a multiple of actives are incorporated to. In smaller quantity, there are the Order 344/98 formulae, that, in accordance with the current legislation, require an extra laboratory. The same occurs with the hormones – main study object here. Regarding the manipulation of hormones, almost all the formulae (99%) corresponded to the manipulation of estradiol valerate, with a similar structure of the 17 β estradiol used for many clinical conditions.

As the purpose of this investigation was to evaluate the impact of ED in the effluents and of the formed COD, a CGMS analysis of the LMH sample was

Laboratory	Quantity of formula	Representation (%)
Psychotropics	3150	18.6
Hormones	728	4.3
Solids	9388	55.5
Dermato-cosmetics	3638	21.5
Total	16,904	99.9

Source: pharmacy's registers.

Table 1.
Quantity of formulae produced between January and July of 2014.

conducted, in order to identify the presence of estradiol valerate ED, and another analysis was performed after ozone-based POA and UV for 120 minutes. The acquired chromatograms are showed in **Figure 1**.

3.1 Influence of COD in raw effluents on the behavior and chlorophyll concentration in algae

The presence of hormones in the effluent has significantly affected the velocity of surface ascent rate, since it was inhibited (**Figure 2**). The same was observed in the algae's r-value. The phenomenon was similar to the one observed by Pinto et al [8].

However, it is important to say the COD, unlike the original ED, presents a certain potential of chlorophyll degradation.

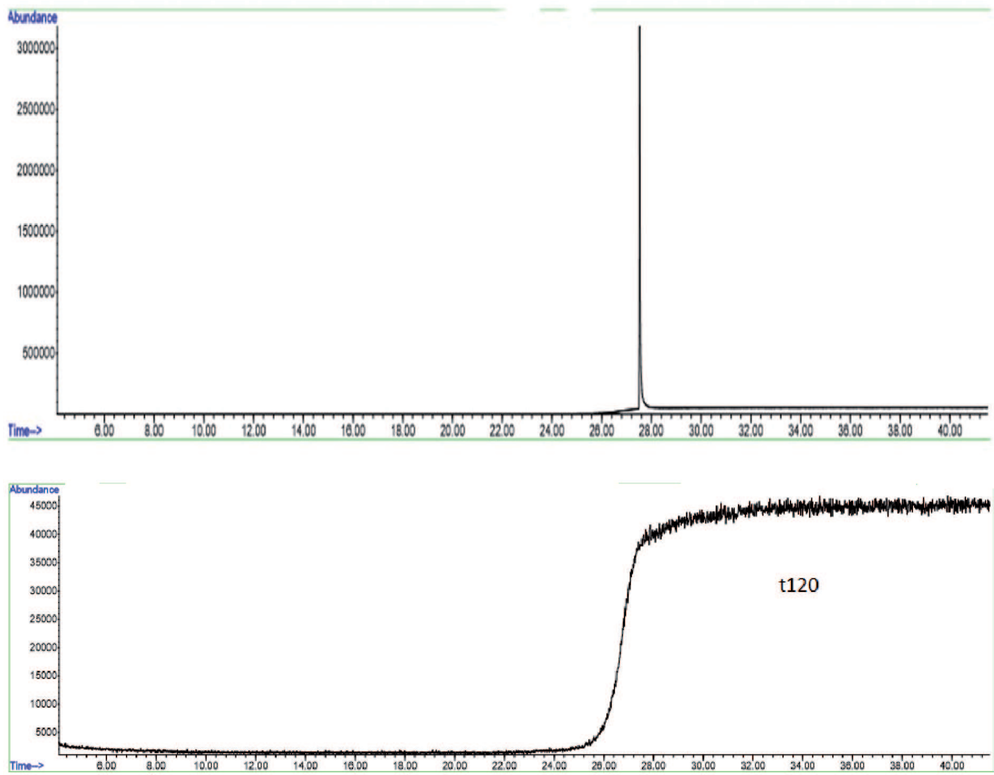

Figure 1.
CGMS chromatogram showing ED absence after POA/UA for 120 minutes.

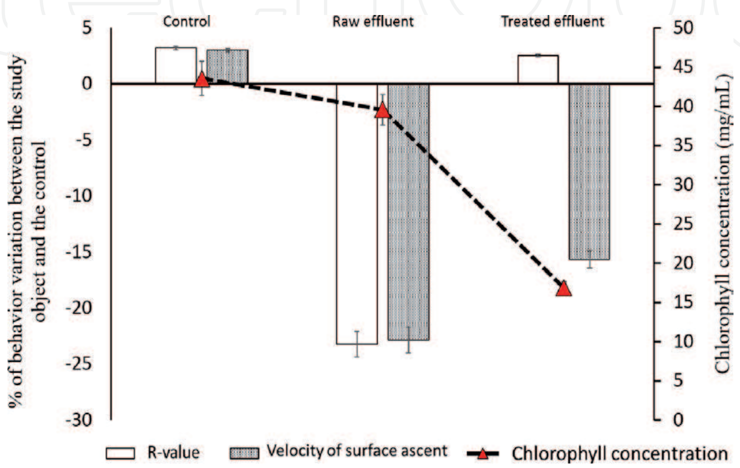

Figure 2.
Changes in behavioral parameters of Euglenas gracilis exposed to post-ozonating effluents at the Laboratory of Hormones, compared to the raw effluent. Pearson correlation test: There are significant connections between the pairs of variables (P > 0,050).

To better comprehend this effect on chlorophyll degradation by COD, a comparison between the concentration before and after the ozonating process was performed (**Figure 3**). Considering the influence over the chlorophyll concentration, it was seen a clear difference between the laboratories and the pre- and post-ozonating, as it is possible to seen in **Figure 3**.

In this comparison, it was seen that in the solids and psychotropics laboratories there was reduction in *a*-chlorophyll in the presence of raw effluent. The result was very similar when compared with the same waste treated using ozonating. However, in the dermato-cosmetics laboratory, as well as in the LMH, it was difficult to see influence of raw effluent, but is was observed influence of the treated effluent, which now has a concentration similar to the ones from the other laboratories treated with ozone/UV. It is important to emphasize the ozone in excess was not present, because in reaction with potassium permanganate the result was negative for the ozone. The justification for the LHM was the antioxidating activity, while a probable explanation for the dermato-cosmetics was the small quantity of highly lipophilic products manipulated there, besides the little influence in the algae physiology [17].

Another relevant variable is the quantity of actives and substances disposed through the sinks and that compound the raw effluent of the laboratories.

For this purpose, the pharmacy's average monthly production of six months was taken into account, and the monthly average production was calculated, in order to verify the laboratories' pre-ozonating activity average. The results obtained accordingly Eq. (1) are presented in **Figure 4**.

The most impacting treated effluent that has COD, in an independent way, on *a*-chlorophyll concentration came from the laboratory of hormones, since it has the lowest production and significant reduction of *a*-chlorophyll concentration. At the same time, in the presence of raw effluent, its concentration was similar to the control one.

Then, it is clear that the removal process via ozone/UV, in the total estrogenic activity removal, interferes on *a*-chlorophyll degradation with COD. Both the raw effluent and the treated one influence on *a*-chlorophyll concentration, reducing it, except at LMH. The reduction of chlorophyll concentration might affect the algae's photosynthetic efficiency and cause ecotoxicity hazards, when there is not compensatory mechanism to assure the algae's survival. Anyway, the condition does not dispense the monitoring of effluents [18].

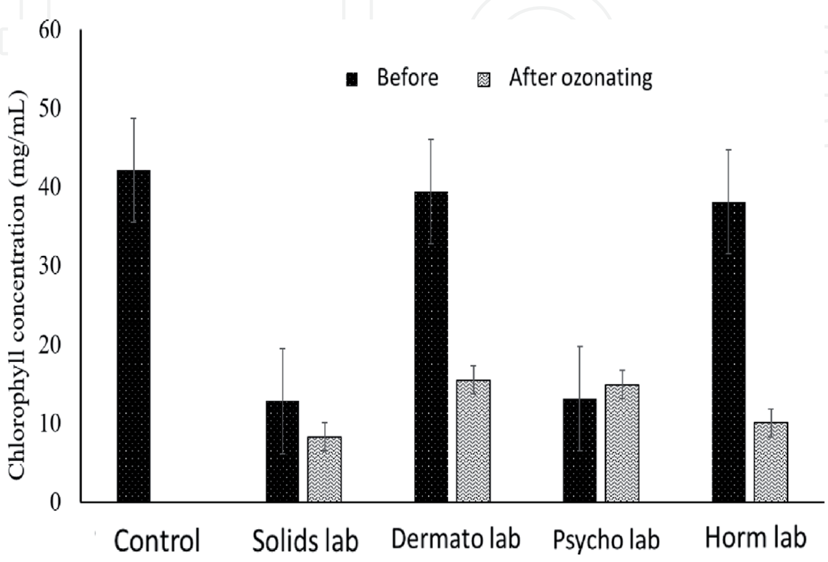

Figure 3.
Influence on chlorophyll concentration. Variance analysis on Kruskal-Wallis variables. H = 7.812 with 2 liberty degrees. P (est.) = 0.020 P (exact) = 0.011. The difference among the average value between the treatment groups are greater than the expected. There is a statistically significant difference (P = 0.011).

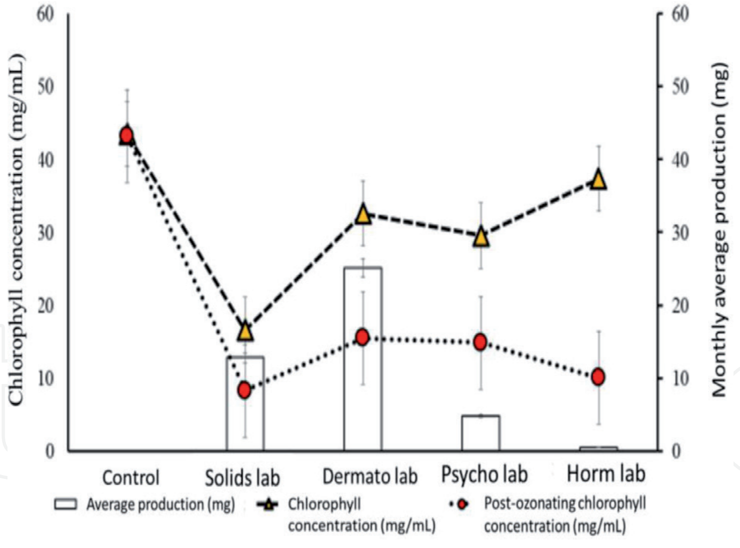

Figure 4.
Influence of the produced quantity versus pre-ozonating chlorophyll concentration. Pearson correlation test: Alteration on chlorophyll concentration at the laboratory (P > 0,050).

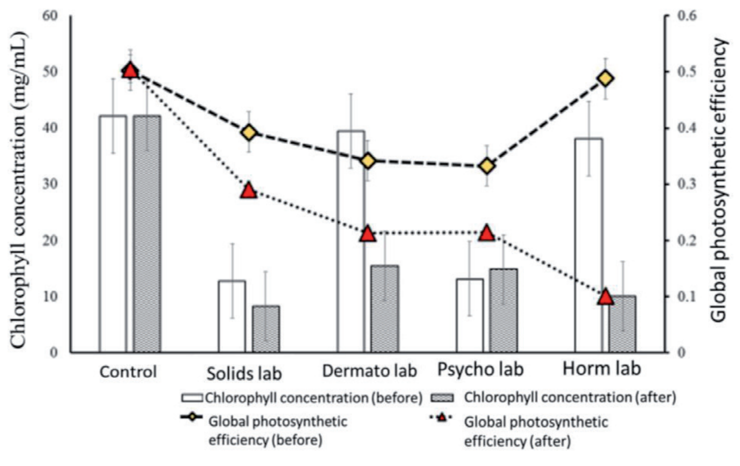

Figure 5.
Comparison between chlorophyll concentration and global photosynthetic efficiency. Variance analysis on Kruskal-Wallis variables. H = 7.812 with 2 liberty degrees. P (est.) = 0.020 P (exact) = 0.011. The difference among the average value between the treatment groups are greater than the expected. There is a statistically significant difference (P = 0.011).

Thus, considering the reduction of *a*-chlorophyll concentration was strongly influenced by the COD, it was also pursued if these reductions would interfere on the global photosynthetic efficiencies of the post-ozonating samples, in order to confirm if the chlorophyll concentration influence anyhow this physiologic parameter, denoting one more risk/environmental hazards (**Figure 5**).

The global photosynthetic efficiency has presented variations among the raw effluent samples, revealing a direct correlation between the chlorophyll concentration and the global photosynthetic efficiency in all the laboratories, except in the hormones one, in which the probable antioxidating action attributed to the steroidal hormone structure helps the electrons transference and contributes to the global photosynthetic efficiency improvement [8, 19].

The global photosynthetic efficiency was influenced by the COD in all laboratories, and the laboratory of hormones has showed the lowest value in the presence of COD and the highest difference between the efficiencies found in the exposure of raw effluents and the treated ones. The hormones' antioxidating activity has assured a good global photosynthetic efficiency performance. The reduction of global photosynthetic efficiency after the ozonation occurred because the hormone decomposition and its oxidizing potential, as well as the possible ethylene formation, which affects the chlorophyll activity [20].

Regarding the solids and psychotropics laboratories, the global photosynthetic efficiency reduction was due to the presence of substances that act on action potentials, listed in the production of both laboratories. These changes may influence the algae's movement ability, being an extra factor on the global photosynthetic efficiency, or the reduction is only attributed to the decline on chlorophyll concentration.

Maybe, the potential cellular action attributed to the manipulated medicines in these laboratories prevent these mechanisms, which affect the algae's flagellar mobility [21].

4. Conclusions

The present investigation made possible an analysis on how the ozone-based oxidative processes influence the reduction of the ecotoxicity risk caused by emergent pollutants. The obtained results have showed algae's behavioral changes among the four examined laboratories–hormones, solids, dermo-cosmetics and psychotropics–, comparing raw effluent samples, treated effluent samples and control.

Significant alterations on *Euglena gracillis* behavior were observed for the effluents from the laboratories of hormones and dermo-cosmetics, with modifications in general speed and velocity of surface ascent. The psychotropics and solids laboratories have not presented significant statistically difference over the algae's behavior. However, complementary studies are appropriate, in order to confirm the long-term toxicity, since the algae find compensatory mechanisms to fit the adversities.

The variations on algae's behavior before the exposure to different pollutants have suggested it is important to distinguish the effluent treatment, according with the characteristics of the substances manipulated in each laboratory, to reduce the environmental toxicity risks.

Therefore, the found biomonitoring data were relevant for better knowing and to be more aware of the issue, indicating the environmental toxicity caused by the effluents from magistral pharmacies may provoke great impact to the environment if revisions of actions and legislation are not performed in a way that in long term the environmental points related to this type of emergent pollutants are reduced.

For future studies, the evaluation of fish's behavior, before similar conditions, may point out better comprehension over the influence of pharmaceutical ecosystem risks.

Acknowledgements

The researchers thank Univille and the Research Support Funds, that make possible the Environmental Impacts Integrated Project (ECOSAM) development.

Author details

Thais Francinne[1], Suellen Zucco Bez[1], Julia Carolina Soares[1],
Sabrina Martins da Rosa[2], Aline Mirian Paszuck[2], Luciana Ferreira Karsten[3]
and Luciano Henrique Pinto[4*]

1 Pharmacy Course, Health and Environmental Research Laboratory, University of the Region of Joinville (UNIVILLE), Joinville, SC, Brazil

2 Nursing Course, Health and Environmental Research Laboratory, University of the Region of Joinville (UNIVILLE), Joinville, SC, Brazil

3 Department of Nursing, University of the Region of Joinville (UNIVILLE), Joinville, SC, Brazil

4 Department of Pharmacy/Nursing/Medicine, Health and Environmental Research Laboratory, University of the Region of Joinville (UNIVILLE), Joinville, SC, Brazil

*Address all correspondence to: lucianoefar@gmail.com

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