Impact of venlafaxine in the growth of the microalga *Pseudokirchneriella subcapitata*

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Abstract It has been observed an increase in the consumption of antidepressants, and venlafaxine appears among the most consumed. Its presence in the environment, together with other antidepressants, has been reported all over the world. Nevertheless, the knowledge in its possible ecotoxic effects is still limited. Therefore, it is imperative to evaluate their impact in the aquatic ecosystems. In this context, the effect of venlafaxine in the growth of the microalga *Pseudokirchneriella subcapitata* was evaluated. The experiments were carried out in agreement to the inhibition test for algae (EC Regulation 440/2008, which was based on the OCDE Guideline 201). *P. subcapitata* was chosen, because it is highly sensitive to contamination from aquatic environment and it is also recommended as a standard organism for ecotoxicity tests. The effect of different concentrations of venlafaxine in the growth of the microalgae was evaluated by determining the content of chlorophyll *in vivo* by fluorescence and comparing to a control culture. Venlafaxine showed to have toxic effects to *P.subcapitata*, with EC_{10} and EC_{50} of 0.9 and 11.0 mg/L, respectively. According to the "Globally harmonized system of classification and labelling of chemicals (GHS)" (United Nations), venlafaxine can be classified as toxic to the aquatic organisms.

Keywords: Ecotoxicity, growth inhibition, *Pseudokirchneriella subcapitata*, venlafaxine

1. Introduction

The advances of analytical techniques, especially in the detection methods, allowed the reduction of the detection limit values thus trace levels of pharmaceuticals have been detected in the environment. In this way, the scientific community became more aware and concerned about this problematic, since the presence of residual quantities of pharmaceuticals in the environment may represent a risk not only for aquatic organisms but, ultimately, for human health (Santos *et al.*, 2010).

Most of the pharmaceuticals used by the population has as final destination the wastewater. When used, these compounds are either excreted in their original form or as metabolites, and follow the normal course of wastewater, being conducted to wastewater treatment plants (WWTPs), which discharge their treated effluents into surface water. If pharmaceuticals are not efficiently removed at the WWTPs the environment will be contaminated. There are evidences that conventional WWTPs do not efficiently remove most of these compounds (Mackuľak *et al.*, 2015). Besides wastewaters, household waste of unused medicinal products (for example when out of date) can also contribute to the environmental contamination by pharmaceuticals.

Hundreds of pharmaceuticals have been identified in surface and wastewaters. Among the most commons are the anti-inflammatories diclofenac and ibuprofen, the psychiatric drugs carbamazepine and diazepam, as well as other classes of pharmaceuticals: antibiotics, beta-blockers, steroids and hormone regulators, lipid regulators and even antineoplastics (Nikolaou, Meric e Fatta, 2007).

Today's lifestyle causes worry, stress, and depression to a large extent of the population. To help in the combat at these unwell conditions, the pharmaceutical industry has developed pharmaceuticals that act on the central nervous system, which act as antidepressants or mood stabilizers. In the last years it has been observed an increase in the consumption of antidepressants in the countries of the European Union as is evidenced by the Organization for Economic Co-operation and Development (OECD, 2015) between the years 2000 and 2013. The average consumption in these countries has increased to almost twice over the period analyzed. Values range from 32 to 58 daily defined doses (DDD) per 1000 people per day. By 2013, consumption almost tripled, exceeding the average consumption indicators and reaching a value of 88 DDD per 1000 people per day (OECD, 2015).

Due to the increasing consumption of antidepressants, their presence in different environmental compartments has been reported. For instance, a maximum concentration of 59 ng/L of venlafaxine was detected in the Llobregat River (Spain) (Huerta-Fontela, Galceran e Ventura, 2011) while in the ST. Lawrence river (Montreal, Canada) venlafaxine reached a mean concentration of 195.7 ng/L (Lajeunesse, Cagnon e Sauvé, 2008). Similar levels of venlafaxine were also detected in the Lis river (Portugal) (Paíga *et al.*, 2016).

The evaluation of the ecotoxicity of a psychoactive pharmaceutical, venlafaxine, which acts as a selective serotonin and noradrenaline reuptake inhibitor (SSRI), for the microalga *Pseudokirchneriella subcapitata* carried out in this work intends to contribute to the analysis of the environmental risks involved in the presence of pharmaceuticals in domestic wastewater.

Pseudokirchneriella subcapitata is a chlorophyte, highly sensitive to the contamination of aquatic environments being recommended as a preferred organism in the ecotoxicity tests by EPA (United States Environmental Protection Agency), OECD and Commission Regulation (EC) 440/2008. Standardized toxicity tests for each species are a way of projecting the hazards to which a non-target organism may be subjected to a particular substance or group of substances. In conjunction with the assessment of the presence of a particular compound, these tests form the basis for the establishment of water quality criteria with the objective of protecting aquatic life fromanthropogenic activity (Brooks *et al.*, 2003).

2. Materials and methods

The tests were carried out according to the algae inhibition test described in Commission Regulation EC 440/2008 of 30 May 2008, which was based in the OECD guideline nº 201 for testing of chemicals Freshwater Alga and Cyanobacteria Growth Inhibition Test (OECD, 2011) and the Short-term Methods for

Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms (EPA, 2002).

A stock solution of venlafaxine (50 mg/L) was prepared by dissolving the venlafaxine (powder) directly in culture medium, which was used in the preparation of the solutions with different concentrations used in the test, so that the culture medium has the same concentration in all test cultures. The culture of *P. subcapitata*, strain 278/4, was originally obtained by Culture Centre of Algae and Protozoa (United Kingdom).

The temperature was controlled to 22 ± 2 °C). The required light intensity was provided by four universal white light fluorescent lamps (Osram, L36W/865) at a distance of 0.35 m from algae cultures. All substances used in the preparation of the culture medium were of a high purity grade, at least 95%.

Deionised water (conductivity bellow than 5 μScm^{-1}) used in the preparation of these solutions and it was obtained in a deionizer Millipore; model Helix 3 (France).

The assays were prepared and carried out under aseptic conditions using an autoclave (AJC, model uniclave 88, Portugal) and a laminar flow chamber (Faster, model two 30, Italy). The test substance was introduced into 250 mL flasks and dissolved in culture medium so as to form a geometric series of concentrations with an approximate ratio of 2 to not more than 2.2 and finally, an inoculum of algae was added, yielding an approximate cell density of 10^5 cells/mL and a final volume of 50 mL in all flaks. All the experiments were made in triplicate and controls, i.e. without test substance, and blanks were also included. The flasks were incubated for 72 h. The shaking of the flasks was performed manually, twice a day (EPA, 2002).

The quality and validation criteria foreseen in the method are the pH variation and the test substance concentration. A test with a reference substance, potassium dichromate, confirmed the validation of the test conditions and the sensitivity of the alga.

The growth of the biomass of the cultures was evaluated by the determination of the variation of the fluorescence.

2.1. Analytical control

The pH and the concentration of venlafaxine were controlled at the begin and the end of the assay

The pH was determined using a combined glass electrode, connected to the potentiometer Crison, micropH 2002 (Spain).

Control of the venlafaxine concentration was done by chromatographic analysis by high performance liquid chromatography coupled with fluorescence detection

(HPLC-FLD) using a HPLC Prominance Shimadzu LC (Shimadzu Corporation, Japan) equipped with a 20AB LC pump, a DGU-20A5 degasser, a SIL 20A automatic injector, a CTO-20AC column oven and a RF-10AXL fluorescence detector. LCsolution software (Shimadzu Corporation, Japan) was used to control the chromatographic system and to acquire and treat the chromatographic data. Chromatographic analysis was performed using the following conditions: Luna C18 chromatographic column (4.6 × 150 mm, 5 μm particle size) (Phenomenex, USA), column temperature of 35 ° C, flow rate of 1.0 mL/min and Injection volume of 20 μL. As mobile phase, ultra-pure water, acidified with 0.1% formic acid as eluent A, and acetonitrile as eluent B was used. The run time was 14 min, gradient eluting initially with 10% eluent B. Reaching 80% at 7 min, returning to baseline conditions up to 10 min and rebalancing the column in the initial conditions up to 14 min. Fluorescence detection was performed at the excitation wavelength of 274 nm and at the emission wavelength of 610 nm, which is the optimal emission / excitation pair for venlafaxine

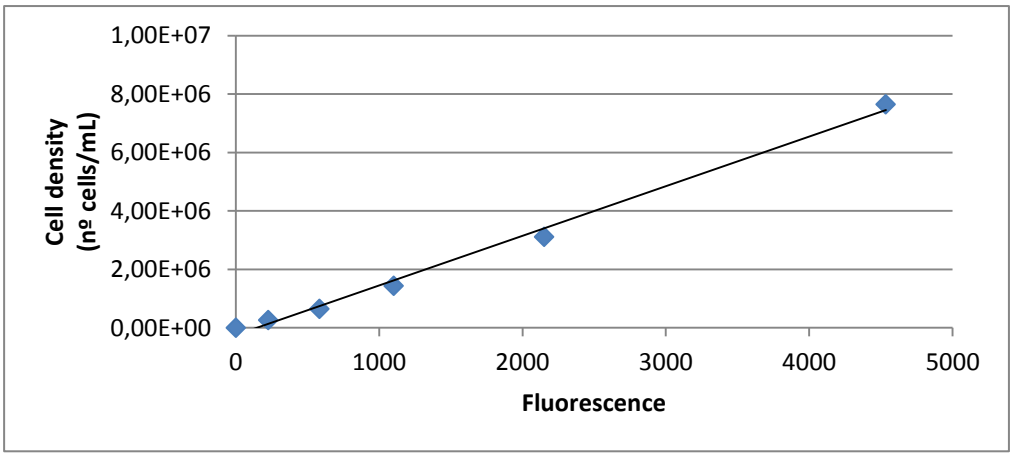

Figure 1: Calibration curve cell density x fluorescence

The determination of biomass growth as recommended by EC Regulation 440/2008 would be the cell density, number of cells/mL, by direct cell counting in a microscope. However, as an alternative of quantification, in a more agile determination, the measurement of the fluorescence *in vivo* was used, provided that its correlation was demonstrated (EPA, 2002; OECD, 2011). Calibration curves were made relating cell density to chlorophyll fluorescence *in vivo*. (cell density = 1.700E+3*fluorescence – 2.533E+5, R^2 =0.994) (Fig. 1). Direct counting of cells was done on a Leica DM500 optical microscope (Germany), with the aid of a counting chamber model Neubauer improved, Blaubrand (Germany). Determination of the *in vivo* chlorophyll content by fluorescence is a measure recommended by EPA (2002) for its sensitivity. Fluorescence measurements were performed in a Biotek Synergy HT microplate reader with Gen5 software version 2.0.18 (USA), using an excitation wavelength of 485 nm and an emission wavelength of 645 nm. Fluorescence determinations were performed with a suspension volume of 0.25 mL, after 10 seconds of shaking to suspend the microalgae. All the measurements were made in triplicate.

3. Results and Discussion

The growth of the cultures was determined from the chlorophyll fluorescence variation between the beginning and the end of the assay. The validity criterion of pH variation less than 1.5 was met. The results of venlafaxine analysis indicated that there was no variation of concentration during the test, meeting another validity criterion, which indicates that the substance should remain at more than 80% of the initial concentration value, meaning that there was no adsorption by the flask, nor evaporation neither degradation of venlafaxine. Comparing with the analysis of the blank tests it is confirmed the stability of venlafaxine and that there was no adsorption by the algae (Table 1).

For each concentration tested, growth inhibition was calculated from the mean growth of replicates at that concentration (□Fi), compared to the mean growth of replicates of the control (□Fc), as shown in the equation below:

$$\text{Inhibition \%} = \frac{\Delta Fc - \Delta Fi}{\Delta Fc} \text{ x } 100$$

The results of the experimentally determined concentrations (C), log (C), fluorescence variation (ΔF) and growth inhibition (I %) obtained in the tests performed with venlafaxine are presented in Table 2 and Fig. 2.

Table 1: Variation of venlafaxine concentration in replicates and controls

Concentration (mg/L)					
Replicates		variation	Control		variation
Inicial	Final	%	Inicial	Final	%
0,234	0,225	3,83	0,234	0,229	2,14
0,435	0,429	1,67	0,428	0,430	-0,47
0,813	0,800	1,60	0,811	0,827	-1,97
1,647	1,623	1,48	1,627	1,649	-1,35
3,313	3,278	1,06	3,297	3,325	-0,85
6,946	7,003	-0,78	6,458	6,539	-1,25
9,566	9,581	-0,20	9,911	9,952	-0,41
12,844	13,281	-3,40	12,924	13,533	-4,71
48,446	49,668	-2,53	49,431	41,806	15,43

Following the procedure suggested by OECD Standard 201 (2011) the ECs were estimated at 50, 20 and 10% inhibition from the regression line of the plot of inhibition of growth versus log of the concentration tested.

In order to better represent the data two linear regressions were used: one for the low inhibition values and the other for the higher values. The results of the ecotoxicity tests led to the calculation of the effective concentrations EC_{50} (growth inhibition = 127.56*log C–82.96, R^2 = 0.9155) EC_{20} and EC_{10} (growth inhibition = 15.642*logC+10.891, R^2 = 0.9243).

Figure 2, growth inhibition x log C chart, shows the linear regression equations for the calculation of the EC estimates. Two ranges of values were chosen, one for the

calculation of the EC estimates one for EC_{50} and another for EC_{20} and EC_{10}, in order to obtain better adjustment.

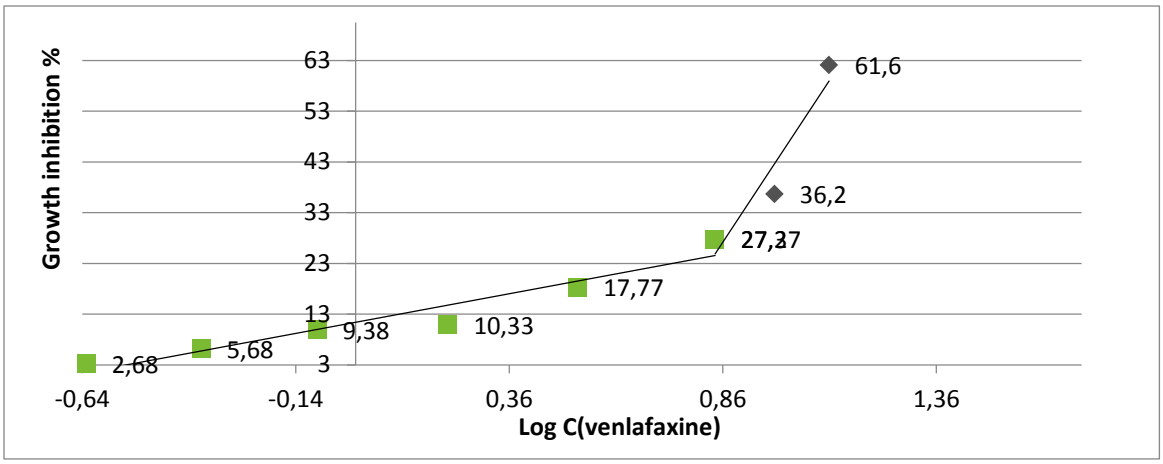

Figure 2: Growth inhibition x log C (venlafaxine)

Venlafaxine showed an EC_{50} of 11.0 mg/L, an EC_{20} of 3.8 mg/L and an EC_{10} of 0.9 mg/L. Considering this study as a first approach and following the logical decision algorithm proposed in the GHS (UN, 2015), this pharmaceutical should be classified in category 2: toxic to the aquatic environment. It has been shown an EC_{50} slightly above the limit of category 2 (11.0 mg/L), however, within the limits of category 2 for EC_{10}, 0.9 mg/L. The recommendation is to use the more restrictive situation, so the classification given should be of a toxic substance to the aquatic environment. So far the highest value found in the aquatic environment is from St. Lawrence river (Montreal, Canada) where venlafaxine concentration reached a mean of 195.7 ng/L (Lajeunesse, Cagnon e Sauvé, 2008), which is much lower than the EC_{10}.

Table 2: Results obtained in the venlafaxine tests

C (mg/L)	log C	ΔF	I%
0		1452	0
0.23	-0.631	1525	2.7
0.44	-0.362	1478	5.7
0.81	-0.090	1420	9.4
1.65	0.217	1302	10.3
3.31	0.520	1194	17.8
6.95	0.842	1056	27.3
9.57	0.981	927	36.2
12.84	1.109	558	61.6
48.45	1.685	652	68.9

Considering the classification proposed by the GHS, (UN, 2015) and the type of test, chronic toxicity, the substances harmful to the environment can be divided into four classes.

The classification is made according to parameters EC_{50} and NOEC (comparable to EC_{10}) and a degree of toxicity is assigned to each category. According to these data, it is recommended that they be labeled on the marketing packaging or in the product technical sheet, containing the environmental risk statements: Category 1 product data sheet, the classification is highly toxic to the aquatic environment; category 2, toxic to aquatic environment; category 3, which is harmful to the environment and category 4, possible harmful effects on aquatic organisms (UN, 2015).

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On the other hand, a high EC_{50} (141.24 mg/L) was obtained for *Daphnia magna* in an acute toxicity test , immobilization test, 48 h exposure (Minguez *et al.*, 2014).

For the concentrations that showed the highest growth inhibitions, a microscope observation (Nikon Eclipse Ti) was performed, with a 100-fold increase. The photographs were obtained with the software NIS - Elements F 4.30.01. Figure 3 shows a control culture and Figure 4 shows the test culture of venlafaxine at concentration corresponding to the maximal growth inhibition found, 48.45 mg/L. Changes in cell shape can be observed in the culture

exposed to venlafaxine.

4. Conclusion

This study aimed to evaluate the toxicity of venlafaxine, a commonly used psychoactive pharmaceutical, to the alga *P. subcapitata*. Venlafaxine showed an EC_{50} of 11.0 mg/L and EC_{10} of 0.9 mg/L. According to the "Globally harmonized system of classification and labelling of chemicals", venlafaxine can be classified as toxic to the aquatic organisms. In order to be aware of the real situation monitoring and ecotoxicity studies focused in the commonly used pharmaceuticals are needed together with research in tertiary treatments that guarantee high removal efficiencies at moderate costs.

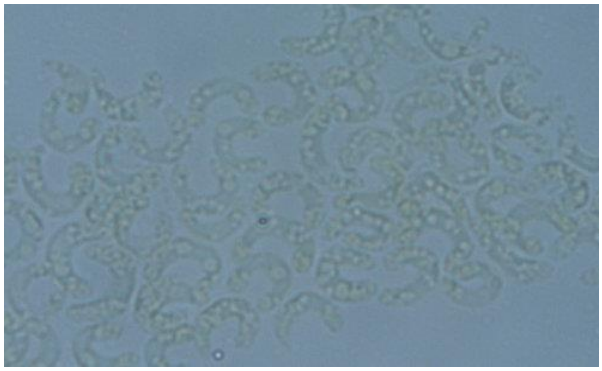

Figure 4: Control culture

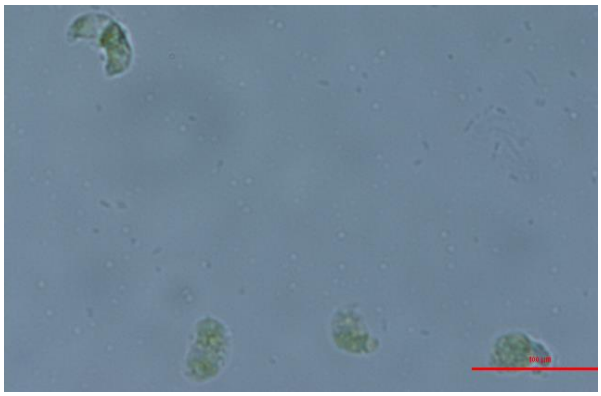

Figure 4: Culture Exposed to Venlafaxine

Acknowledgments

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